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Tese

Investigação de alterações bioquímicas, moleculares e comportamentais relacionadas à patogênese da hipermetioninemia: estudos *in vitro* e *in vivo*

Mayara Sandrielly Pereira Soares

Pelotas, 2019

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Bioquímica e Bioprospecção da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutora em Ciências (Bioquímica e Bioprospecção).

Orientadora: Profª. Drª. Roselia Maria Spanevello

Co-orientadora: Profª. Drª. Francieli Moro Stefanello

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Tese apresentada, como requisito parcial, para obtenção do grau de Doutora em Bioquímica e Bioprospecção, Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas.

Data da Defesa: 13/06/2019 às 14 h

Banca examinadora:

Roselia Maria Spanevello

Profª Drª Roselia Maria Spanevello (Orientadora) – Doutora em Ciências Biológicas - Bioquímica pela Universidade Federal do Rio Grande do Sul.

César J. Bruning

Prof. Dr. César Augusto Bruning – Doutor em Ciências Biológicas - Bioquímica Toxicológica pela Universidade Federal de Santa Maria.

Adriano M. de Assis

Prof. Dr. Adriano Martimbianco de Assis - Doutor em Ciências Biológicas - Bioquímica pela Universidade Federal do Rio Grande do Sul

Prof. Dr. Augusto Schneider – Doutor em Biotecnologia pela Universidade Federal de Pelotas

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos”.

(Isaac Newton)

Resumo

SOARES, Mayara Sandrielly Pereira. **Investigação de alterações bioquímicas, moleculares e comportamentais relacionadas à patogênese da hipermetioninemia: estudos *in vitro* e *in vivo*.** 2019. 172. Tese (Doutorado) - Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas, 2019.

A hipermetioninemia é uma desordem metabólica hereditária caracterizada por elevados níveis de metionina (Met) e de seus metabólitos como a metionina sulfóxido (MetO). A sintomatologia e os sinais clínicos ainda permanecem pouco compreendidos, além de serem variados e inespecíficos, dificultando o diagnóstico e a escolha da terapia mais adequada. Sendo assim, este estudo teve como objetivo investigar o efeito de elevadas concentrações de Met e MetO sobre alterações comportamentais, celulares e moleculares em modelos experimentais *in vitro* e *in vivo*. Nos estudos i*n vitro* foram avaliados os efeitos citotóxicos da exposição de astrócitos corticais a Met (1 e 2 mM), MetO (0,5 mM) e a associação de Met (1 e 2 mM) e MetO (0,5 mM). Para o modelo *in vivo*, utilizou-se um protocolo agudo e um crônico nos quais os ratos *Wistar* foram divididos em quatro grupos: I (controle); II (Met 0,2 – 0,4 g/kg); III (MetO 0,05 – 0,1 g/kg) e IV (associação de Met+MetO). Após 1 h, 3 h e/ou 21 dias foram avaliados parâmetros comportamentais, bioquímicos séricos, neuroquímicos e tromboregulatórios em cérebro, fígado, rim, sangue e plaquetas. Primeiramente foi observado que a administração crônica de Met e/ou MetO alterou parâmetros bioquímicos séricos como níveis de glicose, triglicerídeos, colesterol total e ureia, além de aumentar a atividade da enzima ALA-D e induzir estresse oxidativo em fígado e rim. Nos protocolos agudo e crônico a Met e/ou MetO reduziram a atividade das enzimas NTPDase, 5'-nucleotidase e adenosina deaminase em plaquetas e soro. Ainda, observou-se um aumento da produção de espécies reativas de oxigênio e peroxidação lipídica, diminuição dos níveis de compostos antioxidantes não enzimáticos, redução da atividade das enzimas superóxido dismutase, catalase e aumento na atividade da glutationa S-transferase em plaquetas e soro de ratos submetidos aos protocolos agudo e crônico. Em córtex cerebral, no protocolo agudo, a Met e MetO induziram estresse oxidativo, diminuíram a viabilidade celular, causaram dano ao DNA e morte celular por apoptose, aumentaram a atividade das caspases 3 e 9 e reduziram o potencial de membrana. No protocolo crônico, Met e/ou MetO induziram prejuízo na memória espacial e de curto prazo sem alterar a locomoção do animais. No córtex cerebral, hipocampo e estriado Met e/ou MetO induziram estresse oxidativo e aumentaram a atividade da acetilcolinesterase (AChE). Além disso, no hipocampo houve redução da atividade da Na^+,K^+-ATPase, dos níveis do fator neurotrófico derivado do cérebro e do número de neurônios na região CA3 e giro denteado. Nos astrócitos, observou-se que a Met e/ou MetO não alteraram a viabilidade e proliferação celular, entretando alteraram a morfologia dessas células, causaram estresse oxidativo, aumentaram a atividade da AChE e reduziram a atividade da Na^+,K^+-ATPase. Além disso, nos astrócitos tratados com Met e/ou MetO houve uma redução na atividade da NTPDase usando ATP como substrato e da 5'-nucleotidase. Por outro lado, houve um aumento na atividade da NTPDase utilizando ADP como subtrato. Os achados do presente trabalho demonstraram que a hipermetioninemia é capaz de alterar diversos mecanismos celulares e moleculares induzindo importantes alterações na homeostase de diferentes tecidos e células, prejudicando aspectos comportamentais

e fisiológicos. Portanto, essas descobertas podem ser bastante importantes para auxiliar no diagnóstico e futuramente em estudos de possíveis alvos terapêuticos, como compostos antioxidantes, para pacientes portadores dessa patologia.

Palavras chave: metionina; metionina sulfóxido; astrócitos; plaquetas; encéfalo; fígado; rim

Abstract

SOARES, Mayara Sandrielly Pereira. **Investigation of biochemical, molecular and behavioral changes related to the pathogenesis of hypermethioninemia: *in vitro* and *in vivo* studies.** 2019. 172. Thesis (Doctorate)-Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas, 2019.

Hypermethioninemia is an inherited metabolic disorder characterized by high levels of methionine (Met) and its metabolites such as methionine sulfoxide (MetO). The symptoms and clinical signs remain poorly understood, besides being varied and nonspecific, making it difficult to diagnose and select the most appropriate therapy. Thus, this study aimed to investigate the effect of high concentrations of Met and MetO on behavioral, cellular and molecular changes in experimental models *in vitro* and *in vivo*. In the *in vitro* protocol, the cytotoxic effects of exposure of cortical astrocytes to Met (1 and 2 mM), MetO (0.5 mM) and the combination of Met (1 and 2 mM) and MetO (0.5 mM) . For the *in vivo* model, an acute and a chronic protocol were used in which the Wistar rats were divided into four groups: I (control); II (Met 0.2-0.4 g/kg); III (MetO 0.05 - 0.1 g/kg) and IV (association of Met + MetO). After 1 h, 3 h and/or 21 days, behavioral, serum biochemical, neurochemical and thromboregulatory parameters were evaluated in brain, liver, kidney, blood and platelets. First, it was observed that chronic administration of Met and/or MetO altered serum biochemical parameters such as glucose, triglycerides, total cholesterol and urea levels, besides increased ALA-D enzyme activity and induced oxidative stress in liver and kidney. In the acute and chronic protocols Met and/or MetO reduced the activity of the enzymes NTPDase, 5'-nucleotidase and adenosine deaminase in platelets and serum. In addition, there was an increase in the production of reactive oxygen species and lipid peroxidation, reduction of the levels of non-enzymatic antioxidant compounds, reduction of superoxide dismutase activity, catalase and increase in glutathione S-transferase activity in platelets and serum of rats submitted to acute and chronic protocols. In the cerebral cortex, acute administration of Met and/or MetO induced oxidative stress, decreased cell viability, caused DNA damage and cell death by apoptosis, increased the activity of caspases 3 and 9 and reduced membrane potential. In the chronic protocol, Met and/or MetO induced impairment in spatial and short-term memory without altering the animals' locomotion. In the cerebral cortex, hippocampus and striatum Met and/or MetO induced oxidative stress and increased acetylcholinesterase (AChE) activity. Additionally, in the hippocampus there was a reduction of Na^+, K^+-ATPase activity, levels of neurotrophic factor derived from the brain and number of neurons in the CA3 region and dentate gyrus. In the astrocytes, it was observed that Met and/or MetO did not change cell viability and proliferation, but altered the morphology of these cells, induced oxidative stress, increased AChE activity and reduced Na^+, K^+-ATPase activity. In addition, in the astrocytes treated with Met and/or MetO there was a reduction in NTPDase activity using ATP as substrate and 5'-nucleotidase. On the other hand, there was an increase in NTPDase activity using ADP as substrate. The findings of the present study demonstrated that hypermethioninemia is able to alter several cellular and molecular mechanisms inducing important alterations in the homeostasis of different tissues and cells, impairing behavioral and physiological aspects. Therefore, these findings may be very important to aid in the diagnosis and future studies of possible therapeutic targets, as antioxidants, for patients with this pathology.

Keywords: methionine; methionine sulfoxide; astrocytes; platelets; brain; liver; kidney

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Lista de abreviaturas e siglas

ACh - Acetilcolina

AChE - Acetilcolinesterase

ALAD - Ácido Delta Aminolivilínico Desidratase

ATP - Trifosfato de Adenosina

ADP - Difosfato de Adenosina

ADA - Adenosina Deaminase

ADO - Adenosina

AMP - Monofosfato de Adenosina

BDNF - Fator Neurotrófico Derivado do Cérebro

BuChE - Butirilcolinesterase

CAT - Catalase

ChAT- Colina Acetiltransferase

CHT1 - Transportador de Colina de Alta Afinidade

EIM - Erros Inatos do Metabolismo

ERN - Espécies Reativas de Nitrogênio

ERO - Espécies Reativas de Oxigênio

GFAP - Proteína Fibrilar Glial

GPx - Glutationa Peroxidase

GST - Glutationa S-transferase

GSH - Glutationa

H_2O_2 - Peróxido de Hidrogênio

IL-6 - Interleucina 6

IL-10 - Interleucina 10

MAT - Metionina Adenosiltranferase

Met - Metionina

MetO - Metionina Sulfóxido

NPPs - Ecto-Nucleosideo Pirofosfatase / Fosfodiesterases

NTPDase - Ecto-Nucleosideo Trifosfato Difosfohidrolases

SAM - S-Adenosilmetionina

SNC - Sistema Nervoso Central

SOD - Superóxido Dismutase

TNF α - Fator de Necrose Tumoral α

VAChT - Transportador Vesicular de Acetilcolina

Sumário

1. INTRODUÇÃO

Os erros inatos do metabolismo (EIM) são alterações genéticas as quais podem se manifestar através da síntese anormal de enzimas (AHRENS-NICKLAS et al., 2015; EL-HATTAB, 2015; VERNON, 2015). Dentre os EIM mais prevalentes, estão as aminoacidopatias, como a hipermetioninemia, a qual é caracterizada pelo acúmulo tecidual e plasmático do aminoácido metionina (Met) e de seus metabólitos como a metionina sulfóxido (MetO) (MUDD, 2011; MUDD et al., 2001). A hipermetioninemia pode ter origem genética, como na deficiência da enzima metionina adenosiltransferase (MAT) ou origem não genética, como na prematuridade e/ou baixo peso ao nascer. Nessa condição, os pacientes podem apresentar sintomas hepáticos, neurológicos, deficiência de ferro e zinco e alterações de pele e capilar. Entretanto a fisiopatologia envolvida nesses sintomas ainda permanece pouco compreendida (MUDD, 2011; MUDD et al., 2001).

Diante disso, estudos têm demonstrado que elevadas concentrações de Met e/ou MetO, como as encontradas na hipermetioninemia, podem ser tóxicas, além disso, o estresse oxidativo e o processo inflamatório parecem estar envolvidos em alterações neuronais e não-neuronais observadas em modelos experimentais *in vitro* e *in vivo* dessa patologia (SOARES et al., 2018; DOS SANTOS et al., 2017; COSTA et al., 2013).

Em encéfalo, já foi relatado que elevadas concentrações de Met podem alterar a atividade de enzimas antioxidantes, da acetilcolinesterase (AChE), Na^+,K^+-ATPase, induzir peroxidação lipídica e reduzir o conteúdo de importantes lipídios de membrana (gangliosídios, fosfolipídios e colesterol), além de causar déficit de memória em ratos (STEFANELLO et al., 2007b; STEFANELLO et al., 2007c). Em fígado, estudos prévios de nosso grupo de pesquisa demonstraram que a exposição *in vitro* e *in vivo* à Met e/ou MetO modifica a homeostase hepática por alterar o estado redox celular, além de promover alterações histológicas em fígado de ratos jovens (COSTA et al., 2013; STEFANELLO et al., 2009).

Recentemente, foi demonstrado que a hipermetioninemia *in vitro* e *in vivo* promove um ambiente pró-inflamatório, através do aumento de interleucina 6 (IL-6), do fator de necrose tumoral alfa (TNF-α), da proteína C reativa e diminuição dos níveis de interleucina 10 (IL-10) (SOARES et al., 2018, DOS SANTOS et al., 2017). Além disso, elevadas concentrações desses aminoácidos são capazes de modular a

atividade de enzimas do sistema purinérgico em linfócitos e macrófagos, bem como os níveis dos nucleotídeos e nucleosídeos de adenina em soro, os quais são importantes moduladores dos processos imunes, inflamatórios e tromboregulatórios (SOARES et al., 2018, DOS SANTOS et al., 2017). Também alterações na atividades das colinesterases, AChE em linfócitos e butirilcolinesterase (BuChE) em soro já foram relatadas associadas a presença de um ambiente pró-inflamatório em modelos pré-clínicos de hipermetioninemia (SOARES et al., 2018).

Neste contexto, considerando que muitos mecanismos ainda não são compreendidos em relação à fisiopatologia da hipermetioninemia e a fim de dar continuidade aos estudos do nosso grupo de pesquisa, este trabalho teve por objetivo avaliar os efeitos da Met e/ou MetO em parâmetros comportamentais, neuroquímicos, renais, hepáticos e tromboregulatórios em modelos experimentais *in vitro* e *in vivo*.

2. OBJETIVOS

2.1 Objetivo geral

Investigar os efeitos de Met e/ou MetO em parâmetros comportamentais, neuroquímicos, renais, hepáticos e tromboregulatórios em modelos experimentais *in vitro* e *in vivo*.

2.2 Objetivos específicos

a) Investigar o efeito *in vitro* do tratamento com Met e/ou MetO em cultura primária de astrócitos quanto a viabilidade, proliferação e morfologia celular, *status* redox, bem como atividade das ectonucleotidases, acetilcolinesterase e Na^+,K^+-ATPase.

b) Avaliar o efeito do tratamento agudo (1 e 3 h) e crônico (21 dias) com Met e MetO em córtex cerebral, hipocampo e estriado de ratos jovens quanto a:

- Viabilidade celular, dano ao DNA, tipo de morte celular, atividade das caspases 3 e 9 e o potencial eletroquímico mitocondrial.
- Parâmetros de estresse oxidativo.
- Atividade locomotora e memória espacial, atividades das enzimas acetilcolinesterase e Na^+, K^+- ATPase, bem como os níveis do fator neurotrófico derivado do cérebro.
- Alterações na morfologia e no número de neurônios hipocampais.

c) Avaliar o efeito do tratamento crônico (21 dias) com Met e MetO em fígado e rim de ratos jovens quanto aos seguintes parâmetros:

- Níveis de glicose, colesterol total, ureia e triglicerídeos em soro.
- Parâmetros de estresse oxidativo.

d) Avaliar o efeito do tratamento agudo (1h e 3h) e crônico (21 dias) com Met MetO em plaquetas e soro de ratos jovens quanto:

- Parâmetros de estresse oxidativo.
- Atividade das enzimas do sistema purinérgico em soro e plaquetas de ratos.

3. REVISÃO DA LITERATURA

3.1 Erros Inatos do Metabolismo (EIM)

Os erros inatos do metabolismo (EIM) são doenças genéticas que envolvem anormalidades em processos celulares bioquímicos. Majoritariamente, essas doenças são causadas por defeitos enzimáticos que resultam na conversão insuficiente ou ausente de substratos em produtos. Diante disso, podem surgir problemas devido à acumulação de substâncias tóxicas, aos efeitos dos compostos essenciais reduzidos e/ou ao metabolismo alternativo anormal do substrato (AHRENS-NICKLAS et al., 2015; EL-HATTAB, 2015; VERNON, 2015).

O grande número de transtornos e a complexidade bioquímica de cada um dos mais de 500 EIM já descritos são pontos desafiadores para o diagnóstico clínico (VERNON, 2015). Nesse sentido, propostas de classificação desse grupo de desordens foram desenvolvidas para auxiliar a categorização e o entendimento dos EIM, com consequente benefício para a prática clínica (VERNON 2015). Fisiopatologicamente, os EIM podem ser classificados de acordo com a área do metabolismo afetado, subdividindo-se em metabolismo de aminoácidos, carboidratos, lipídios, glicoproteínas, ácidos orgânicos, lipoproteínas, dentre outros (EL-HATTAB, 2015; SCRIVER et al., 2001).

Embora os EIM sejam individualmente raros, coletivamente atingem 1:1000 nascidos, sendo que 25% deles apresentam manifestações ainda no período neonatal. Na maioria dos casos, os recém nascidos são saudáveis mas podem começar a desenvolver sinais típicos dessas desordens em horas a dias após o nascimento (AHRENS-NICKLAS et al., 2015; EL-HATTAB, 2015). Os sinais geralmente são inespecíficos e podem incluir má alimentação, dificuldade respiratória, letargia ou convulsões, sendo esses muitos comuns a várias outras condições neonatais como sepse e disfunção cardiopulmonar (AHRENS-NICKLAS et al., 2015; EL-HATTAB, 2015). Dentre os EIM, os mais frequentes são as aminoacidopatias que envolvem alterações no metabolismo dos aminoácidos. Nesse grupo, destacam-se os EIM do metabolismo dos aminoácidos sulfurados, como a homocistinúria e a hipermetioninemia (SAUDUBRAY et al., 1990).

3.2 Hipermetioninemia

A hipermetioninemia foi primeiramente relatada associada à deficiência da enzima metionina adenosiltransferase (MAT), a qual é responsável por catalisar a reação de metabolização do aminoácido metionina (Met) até o seu produto S-adenosilmetionina (SAM) (MUDD, 2011; MUDD et al., 2001). Embora essa seja a principal causa de hipermetioninemia isolada e persistente, atualmente outras cinco alterações genéticas já foram descritas como sendo fatores determinantes para casos de hipermetioninemia como: deficiência da enzima MAT I/III; homocistinúria devido à deficiência da cistationina beta-sintase; deficiência da glicina N-metiltransferase; deficiência de S-adenosil-homocisteína hidrolase; deficiência de citrina; deficiência de fumarilacetoacetato hidrolase - tirosemia tipo I. Além disso, disfunções não genéticas como baixo peso ao nascer, prematuridade e dieta rica em Met também estão relacionadas a essa condição (SCHWEINBERGER e WYSE 2016; MUDD, 2011; MUDD et al., 2003). Dados epidemiológicos mundiais da hipermetioninemia ainda não foram relatados até o momento, entretanto estudos prévios têm demonstrado que a deficiência da MAT I/III é de um em cada 27.000 recém-nascidos na Península Ibérica (MARCÃO et al., 2014).

O gene que codifica a enzima MAT 1A está localizado no braço longo do cromossomo 10, e é expresso somente em células hepáticas maduras. As mutações nesse gene podem levar a ausência ou atividade enzimática residual, resultando em hipermetioninemia plasmática variando de elevação leve até grave, podendo atingir até 2.500 µmol/L de Met no sangue, sendo que os níveis normais ficam em torno de 30 µmol/L (MUDD, 2011; CHAMBERLIN et al., 1996). Ademais, outros achados bioquímicos têm sido encontrados na deficiência da MAT e incluem baixos níveis de SAM, elevação de homocisteína, além de aumento da concentração de metabólitos secundários da Met, como o metanotiol, sulfeto de hidrogênio e a metionina sulfóxido (MetO) (SCHWEINBERGER e WYSE 2016; MUDD, 2011; CHAMBERLIN et al., 1996).

Clinicamente, embora a maioria dos pacientes sejam assintomáticos, alguns podem apresentar sintomas neurológicos e/ou hepáticos (LU et al., 2008, 2001; AVILA et al., 2000). Nesse sentido, tendo em vista que a Met é principalmente metabolizada no fígado, os sintomas mais relatados em pacientes hipermetioninêmicos têm sido associados com alterações nesse órgão, como

esteatose e cirrose (MUDD, 2011). Além disso, é importante ressaltar que a hipermetioninemia isolada e persistente é capaz causar danos neurológicos como edema, déficit cognitivo, retardo no desenvolvimento psicomotor e desmielinização cerebral (NASHABAT et al., 2018; MUDD, 2011). Ainda, esses pacientes podem apresentar sinais clínicos como odor incomum na respiração, suor, hálito e urina possivelmente devido ao metabolismo alternativo da Met. Esses sintomas e sinais clínicos podem ser observados desde a infância, ainda no período neonatal (AVILA et al., 2000; LU et al., 2008, 2001). Entretanto, não há consenso, em relação ao plano de manejo recomendado na literatura para pacientes hipermetioninêmicos (NASHABAT et al., 2018; MUDD, 2011).

O diagnóstico da hipermetioninemia pode ser dificultado devido à variação de sinais e sintomas os quais são bastante inespecíficos, no entanto essa patologia pode ser descoberta ainda nos primeiros dias de vida. Nesse caso, para a confirmação do diagnóstico, muitos dados devem ser considerados como, nível plasmático de Met e envolvimento do sistema nervoso central (SNC), antes de iniciar o tratamento. Quando os níveis de Met atingem 48 mol/L, pode ser sugestivo para deficiência da MAT, sendo necessário a determinação da atividade dessa enzima no fígado. Também, alterações nos níveis de SAM e de homocisteína (acima de 59 µmol/L) podem amparar o diagnóstico (CHIEN et al., 2005; COUCE et al., 2008; SUAREZ et al., 2010).

As modalidades de tratamento utilizadas são a dieta com restrição de Met e a suplementação com SAM (S-adenosilmetionina dissulfato tosilato) em doses de 400 a 800 mg, duas vezes ao dia (NASHABAT et al., 2018; CHIEN et al., 2005; MUDD, 2011). A suplementação com SAM tem como objetivo melhorar ou prevenir os sintomas neurológicos associados ao processo de desmielinização no SNC. No caso de pacientes assintomáticos, não é necessário abordagem terapêutica, mas o monitoramento constante da doença é essencial para prevenção de aparecimento dos sinais e sintomas (CHIEN et al., 2005; COUCE et al., 2013, 2008).

Cabe salientar que embora importantes avanços na identificação dos sintomas associados à hipermetioninemia tenham sido identificados nos últimos anos, esses continuam pouco compreendidos. Além disso, essa é uma doença que não tem cura e cuja abordagem terapêutica é bastante limitada, principalmente devido ao pouco conhecimento quanto a sua patogênese. Portanto, estudos cujos objetivos são identificar e entender possíveis alterações e seus mecanismos na

hipermetioninemia se tornam extremamente relevantes. Nesse sentido, modelos experimentais utilizando animais são de extrema importância, uma vez que podem auxiliar de maneira significativa no entendimento dos mecanismos fisiopatológicos frente a elevadas concentrações de Met e/ou MetO como as encontradas na hipermetioninemia.

Estudos envolvendo elevadas concentrações plasmáticas da Met já foram realizados e demonstraram que esse aumento pode levar a alterações prejudiciais em alguns órgãos como encéfalo e fígado, bem como favorecer um ambiente pró-inflamatório. Em cérebro de ratos submetidos a modelos agudos e crônicos de hipermetioninemia também já foi relatado a presença de estresse oxidativo, caracterizado por aumento da peroxidação lípidica e alteração na atividade de enzimas antioxidantes tanto em córtex cerebral quanto em hipocampo, alterações na atividade de enzimas acetilcolinesterase (AChE) e Na^+,K^+-ATPase, redução do conteúdo de gangliosídeos, fosfolipídeos e colesterol e diminuição do número de neurônios (SCHWEINBERGER et al., 2018; STEFANELLO et al., 2007a, 2007b, 2007c, 2005). Também já foram descritas alterações em marcadores de dano oxidativo e histológicos em fígado de ratos jovens submetidos ao modelo de hipermetioninemia (COSTA et al., 2013; STEFANELLO et al., 2009).

Além das alterações cerebrais e hepáticas, recentemente, também foi demonstrado que a Met e MetO tanto *in vitro* quanto *in vivo* são capazes de induzir um ambiente pró-inflamatório através do aumento de interleucina-6 (IL-6), do fator de necrose tumoral alfa (TNF-α), de proteína C-reativa e redução da interleucina-10 (IL-10), bem como alterações em enzimas envolvidas na modulação do sistema imune e inflamatório como AChE em linfócitos e butirilcolinesterase (BuChE) em soro, além de alterações em enzimas envolvidas na sinalização purinérgica em macrófagos e linfócitos (SOARES et al., 2018; DOS SANTOS et al., 2017).

Levando-se em consideração o exposto pode-se destacar que: (1) a hipermetioninemia é uma condição com sintomas variados e inespecíficos cuja fisiopatologia ainda é pouco compreeendida o que dificulta o diagnóstico precoce e a instituição de terapia apropriada, (2) que essa é uma doença rara e que não tem cura, (3) que pode afetar de maneira significativa a qualidade e a expectativa de vida, (4) que a principal característica bioquímica dessa patologia é o acúmulo da Met e de seus metabólitos como a MetO, (5) e que estudos prévios do nosso grupo já demonstraram que elevadas concentrações plasmáticas de Met e MetO causam

várias alterações em diferentes órgãos e células. Todos esses aspectos constituem argumentos que embasam a justificativa de realizar estudos que visem investigar possíveis mecanismos relacionados com a patogênese da hipermetioninemia. Ademais, considerando que essa é uma doença neurometabólica são necessários estudos que abordem o impacto dessa em componentes mais específicos do SNC, a fim de auxiliar na detecção precoce e também de novas terapias específicas objetivando melhorar a qualidade de vida dos portadores desse EIM.

3.3 Hipermetioninemia e Estresse Oxidativo

A Met é um aminoácido essencial para o crescimento e desenvolvimento saudáveis de mamíferos (PRUDOVA et al., 2005). Por ser um aminácido sulfurado, a Met é especialmente sensível a processos oxidativos mediados por espécies reativas de oxigênio (ERO), sendo o principal metabólito formado a partir desta reação de oxidação, a MetO (Figura 1), a qual também pode sofrer oxidação dando origem a metabólitos com potencial tóxico, como a metionina sulfona e o ácido homocisteico (MARTÍNEZ et al., 2017; LIANG et al., 2012; ZHAO et al., 2012; MUDD, 2011; LEVINE et al., 2000).

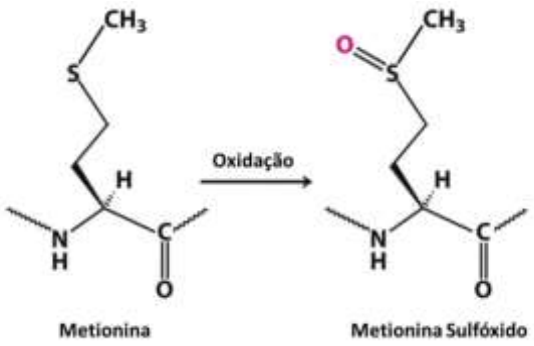

Figura 1: Oxidação da Metionina a Metionina Sulfóxido (Adaptado BERG et al., 2012).

A presença de elevadas concentrações de MetO tem sido associada a doenças neurodegenerativas e com a perda da atividade biológica de proteínas (SUZUKI et al., 2016; MOSKOVITZ, 2014). Além disso, o aumento nas concentrações de MetO pode acarretar na sua oxidação irreversível a metionina sulfona, desbalanceando o ciclo oxidação/redução da Met que constitui um sistema endógeno com capacidade local antioxidativa (MOSKOVITZ, 2014; KOC e

GLADYSHEV, 2007; STADTMAN, 2004). Nesse sentido, considerando a intrínseca relação da Met e MetO com a manutenção do balanço redox (Figura 2), a investigação do mesmo frente a elevadas concentrações desses aminoácidos se torna interessante.

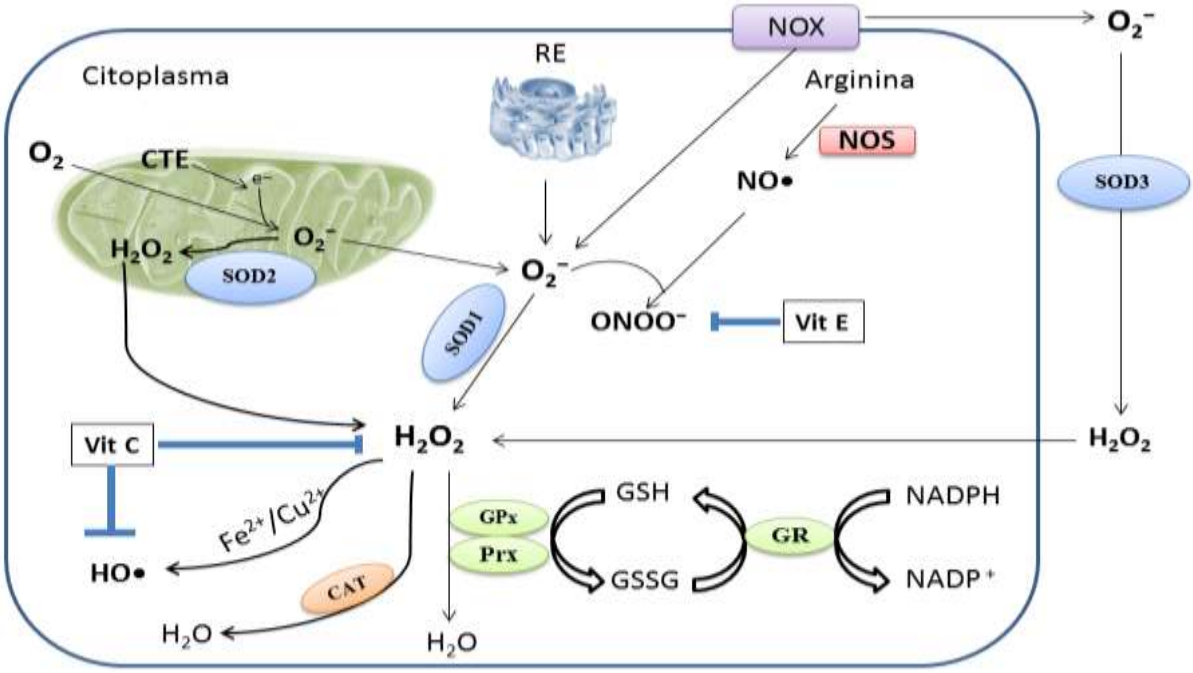

Figure 2: Ilustração esquemática da manutenção celular da homeostase redox. A cadeia de transporte de elétrons das mitocôndrias (CTE), o complexo NADPH oxidase (NOX) ligado à membrana e o retículo endoplasmático (ER) são as três principais fontes intracelulares de espécies reativas de oxigênio (ERO). O ânion superóxido ($O_2^{\cdot-}$) é a principal forma de ERO e pode ser rapidamente convertido em peróxido de hidrogênio (H_2O_2) pelas superóxido dismutases (SODs) ou, alternativamente, formar peroxinitrito ($ONOO^-$) através da reação com o óxido nítrico (NO•). O H_2O_2 pode ser catalisado para radical hidroxila ($HO^{\cdot}$) na presença de íons Fe^{2+} ou Cu^{2+} ou ser convertido em H_2O e O_2 pela catalase (CAT), glutationa peroxidase (GPx) ou peroxirredoxinas (Prx). Vitamina C (Vit C); Vitamina E (Vita E); NOS, óxido nítrico sintase; GR, glutationa redutase; GSH, glutationa reduzida; GSSG, glutationa oxidada.

Cabe salientar que o desbalanço da homeostase redox é denominado de estresse oxidativo, o qual é caracterizado por um desequilíbrio entre compostos pró-oxidantes e antioxidantes podendo assim causar danos irreversíveis às biomoléculas e levar à disfunção celular e tecidual (LJUBISAVLJEVIC, 2016; GONSETTE, 2008). Em contrapartida, o organismo possui um mecanismo antioxidante composto por sistemas não enzimáticos como as vitaminas e a glutationa e enzimas endógenas, como a superóxido dismutase (SOD), que catalisa a dismutação de radicais superóxido em peróxido de hidrogênio (H_2O_2), que pode ser degradado pela catalase (CAT) e glutationa peroxidase (GPx) (BÓ et al., 2015; LOWE 2014).

Considerando que a Met e a MetO são aminoácidos sulfurados sensíveis a oxidação, vários estudos têm associado o estresse oxidativo com diversas alterações encontradas em modelos experimentais de hipermetioninemia. Além disso, tem sido demonstrado também que Met e MetO são capazes de alterar tanto a produção de espécies oxidativas como a atividade de enzimas antioxidantes (STEFANELLO et al., 2011, 2009, 2007; COSTA et al., 2013; DOS SANTOS et al., 2017) (Tabela 1).

Tabela 1: Parâmetros de estresse oxidativo avaliados em diferentes modelos experimentais de hipermetioninemia.

Autor/ano	Modelo	Tecido	Resultado
Lynch e Strain 1989	Dieta rica em Met	Fígado	Aumenta peroxidação lipídica Reduz atividade da SOD Aumenta a atividade da CAT e GPx
Mori e Hirayama 2000	Dieta rica em Met	Fígado	Aumenta peroxidação lipídica e GSH Aumenta a atividade da GPx e da CAT
Lu et al., 2001	Deficiência da MAT	Fígado	Redução de GSH e de GSSG
Martínez-Chantar et al., 2002	Deficiência da MAT	Fígado	Aumenta peroxidação lipídica Reduz níveis de GSH
Stefanello et al., 2009	Hipermetioninemia crônica *in vivo*	Fígado	Aumenta peroxidação lípidica e carbonilação proteica Reduz atividade da CAT e GPx
Yalçinkaya et al., 2009	Dieta rica em Met	Fígado	Aumenta peroxidação lipídica Reduz níveis de GSH Reduz atividade da GST e da GPx
Costa et al., 2013	Hipermetioninemia *in vitro* e *in vivo* agudo	Fígado	*In vitro* reduz ERO e aumenta a atividade da SOD e da CAT *In vivo* reduz ERO e peroxidação lipídica e altera níveis de tios totais *In vivo* reduz a atividade da CAT e aumenta atividade da SOD
Stefanello et al., 2005	Hipermetioninemia *in vitro*	Hipocampo	Aumenta peroxidação lipídica Reduz capacidade antioxidante não enzimática
Stefanello et al., 2007	Hipermetioninemia aguda e crônica *in vivo*	Hipocampo	Aumento da peroxidação lipídica
Schweinberger et al., 2014	Hipermetioninemia gestacional *in vivo*	Cérebro	Redução do conteúdo tiólico total Diminuição na atividade de CAT
Schweinberger et al., 2015	Hipermetioninemia gestacional *in vivo*	Músculo esquelético	Aumento de ERO e de peroxidação lipídica Redução de tios totais e de nitritos Redução da atividade de SOD e CAT
Dos Santos et al., 2017	Hipermetioninemia *in vitro*	Macrofágos	Redução de ERO Redução da atividade da SOD e CAT

Nesse sentido, considerando que o estresse oxidativo está envolvido na fisiopatologia de diversas doenças tanto neuronais como não neuronais, em conjunto com a intrínseca relação da metabolização da Met e MetO com a manutenção do estado redox, além dos diversos achados já relatados da presença do estresse oxidativo com a hipermetioninemia, a investigação do envolvimento dos parâmetros envolvidos nessa condição é de extrema importância para auxiliar no entendimento das alterações clínicas encontradas em pacientes hipermetioninêmicos.

3.4 Sinalização Purinérgica

Além das alterações de estresse oxidativo observadas em modelos experimentais, recentemente, também foi demonstrado que a Met e MetO, tanto *in vitro* quanto *in vivo,* são capazes de induzir um ambiente pró-inflamatório através do aumento de IL-6, do TNF-α, de proteína C-reativa e redução da IL-10, bem como alterações em enzimas e moléculas envolvidas na modulação do sistema imune e na sinalização purinérgica em macrófagos e linfócitos (SOARES et al., 2018; DOS SANTOS et al., 2017).

O sistema purinérgico é constituído por nucleotídeos e nucleosídeos como trifosfato de adenosina (ATP), difosfato de adenosina (ADP), monofosfato de adenosina (AMP) e adenosina (ADO) que são as moléculas que medeiam a sinalização purinérgica, os transportadores dessas moléculas, os receptores pelos quais esses nucleotídeos e nucleotídeos exercem seus efeitos e as ectonucleotidases que são as enzimas que modulam a concentração dessas moléculas no meio extracelular (YEGUTKIN, 2008) (Figura 4).

É bem estabelecido na literatura que o ATP, ADP e ADO são importantes moléculas sinalizadoras em vários tecidos e em diversos processos biológicos (YEGUTKIN, 2008). O ATP é um neurotransmissor excitatório nas sinapses nervosas purinérgicas, podendo ser também coliberado juntamente com outros neurotransmissores como a acetilcolina (ACh) e a noradrenalina (PUCHAŁOWICZ et al., 2015; BURNSTOCK, 2006; GIBB e HALLIDAY, 1996), enquanto que a ADO age na neuromodulação regulando a liberação de vários neurotransmissores (PUCHAŁOWICZ et al., 2015; DUNWIDDIE e MASINO, 2001). Ademais, tem sido demonstrado que estas moléculas também estão envolvidas na sinaptogênese, na

plasticidade neuronal e na proliferação de células gliais (PUCHAŁOWICZ et al., 2015; RATHONE et al., 1999). Além disso, o ATP também possui funções pró-inflamatórias como a estimulação e a proliferação de linfócitos, sendo essencial para a liberação de citocinas pró-inflamatórias (DI VIRGILIO e VUERICH, 2015; BURNSTOCK e BOEYNAEMS, 2014; BOURS et al., 2006). Por outro lado, a ADO tem potentes atividades anti-inflamatórias e imunossupressoras por inibir a proliferação de células T e a liberação de citocinas pró-inflamatórias (GESSI et al., 2007). O ADP é um importante agonista estimulador de plaquetas, e por isso alterações na sinalização induzida por esta molécula tem sido associada com a patogênese de doenças tromboregulatórias (BURNSTOCK e RALEVIC 2013; BURNSTOCK, 2015).

A sinalização induzida por essas moléculas é regulada pela ação de enzimas como NTPDases (ecto-nucleosideo trifosfato difosfohidrolases), NPPs (ecto-nucleosideo pirofosfatase/fosfodiesterases), ecto-5’-nucleotidase e adenosina deaminase (ADA). A NTPDase é uma ectoenzima capaz de hidrolisar nucleosídeos tri- e difosfatados, como o ATP e o ADP, até seu respectivo nucleosídeo monofosfatado AMP (ZIMMERMANN, 2001). O AMP formado é posteriormente hidrolisado pela ação da 5´-nucleotidase até ADO, a qual é degradada pela ação da ADA em inosina (ROBSON et al., 2006; YEGUTKIN, 2008; PHILLIS, 1991). Sendo assim, a atividade dessas enzimas é essencial para a manutenção dos níveis normais dessas moléculas no meio extracelular.

Nos últimos anos, o papel dessas enzimas tem sido avaliado em várias doenças demonstrando que elas podem ser importantes alvos terapêuticos em muitas situações patológicas. Ademais, estudos preliminares demonstraram que Met e MetO induzem um ambiente pró-inflamatório em macrófagos *in vitro* e em linfócitos *in vivo* e que essa condição correlacionou-se positivamente com alterações na atividade das ectonucleotidases seguido de um desbalanço dos níveis dos nucleotídeos e nucleosídeos de adenina (SOARES et al., 2018; DOS SANTOS et al., 2017). Esses dados sugerem que alterações na sinalização purinérgica podem contribuir para complicações presentes na hipermetioninemia.

3.4.1 Plaquetas e Sistema Purinérgico

As plaquetas são importantes células anucleadas com formato discoide provenientes dos megacariócitos e que desempenham funções fisiológicas cruciais para a manutenção da hemostasia (HOLINSTAT, 2017; YUN et al., 2016). Além disso, participam centralmente da coagulação sanguínea juntamente com os vasos sanguíneos, fatores de coagulação e o sistema fibrinolítico. Ademais, essas células também atuam nos processos inflamatórios e de cicatrização (JENNE e KUBES, 2015). As plaquetas são metabolicamente ativas com numerosas organelas funcionais, uma ampla gama de receptores, moléculas de adesão, enzimas de membranas e numerosos grânulos (GREMMEL et al., 2016). A função hemostática dessas células inicia-se com a rápida ligação aos vasos sanguíneos danificados, agregando-se em seguida para formar trombos e prevenir o sangramento excessivo. Por outro lado, as plaquetas ativadas também podem se agregar no local da ruptura da placa aterosclerótica ou erosão das células endoteliais, estimulando a formação de trombos e promovendo a aterosclerose (HOLINSTAT 2017; GREMMEL et al., 2016; YUN et al., 2016; JENNE e KUBES 2015).

Um dos principais sistemas envolvidos na ativação e/ou redução da agregação plaquetária é a sinalização purinérgica. Os nucleotídeos e nucleosídeos de adenina podem ser liberados para o meio extracelular em diversas condições patológicas. Além disso, o ATP e ADP são liberados de grânulos densos durante a ativação plaquetária e propagam interações entre as plaquetas. A ADO, por sua vez, não é conhecida por estar presente nas plaquetas, mas é gerada no espaço extracelular a partir de ATP e ADP por duas ectonucleotidases, NTPDase 1 (CD39) e 5´-nucleotidase (CD73), as quais estão presentes tanto nas superfícies endotelial quanto plaquetária (KOUPENOVA e RAVID, 2018; BURNSTOCK, 2017; YUN et al., 2016; JONES et al., 2014; KUNAPULI et al.,2003).

Além disso, ATP, ADP e ADO desempenham as suas funções através das duas classes de purinoreceptores, o receptores do tipo P1 para ADO, e P2 para ATP e ADP. Os receptores P1 incluem quatro receptores acoplados a proteína G, sendo que dois deles (A2A e A2B) ativam a adenilil ciclase e geram AMP cíclico (AMPc), e os outros dois (A1A e A3) inibem essa enzima impedindo a formação de AMPc (KOUPENOVA e RAVID, 2018; BURNSTOCK, 2017; YUN et al., 2016; JONES et al.,

2014; KUNAPULI et al., 2003). Nas plaquetas, o principal receptor P2 presente é o A2A, o qual quando ativado pela ADO, medeia a função plaquetária através da ativação da adenilil ciclase que leva a um aumento dos níveis de AMPc que por sua vez leva a uma redução da agregação plaquetária. Também, a ativação do A2A, via adenilil ciclase, inibe o aumento mediado pela trombina nos níveis de cálcio intracelular, levando a redução da função plaquetária (KOUPENOVA e RAVID, 2018; BURNSTOCK, 2017; JONES et al., 2014; KUNAPULI et al., 2003).

Quanto aos receptores P2, estes são subdivididos em dois grupos, P2X e P2Y, e nas plaquetas são representados pelo receptor iônico ativado por ATP (P2X1) e os receptores acoplados a proteína G ativados por ADP (P2Y1 e P2Y12). A ativação do receptor P2X1 pelo ATP não medeia a agregação plaquetária; no entanto, ele pode amplificar a agregação mediada pelo ADP através do receptor plaquetário P2Y1 (KOUPENOVA e RAVID, 2018; BURNSTOCK, 2017; JONES et al., 2014; KUNAPULI et al., 2003). O P2Y12 é o receptor mais importante para a agregação plaquetária, e quando ativado por ADP, leva à inibição da adenilil ciclase. Um dos mecanismos pelos quais o AMPc reduz a agregação plaquetária envolve a ativação da proteína cinase A (PKA) que leva à fosforilação e inibição do aumento do cálcio mediado pelo receptor do inositol 1,4,5-trifosfato (IP3) do sistema tubular denso. Níveis reduzidos de AMPc têm o efeito oposto ao IP3 e consequentemente aumentam o cálcio intracelular (KOUPENOVA e RAVID 2018; BURNSTOCK 2017; JONES et al., 2014; KUNAPULI et al., 2003).

Por fim, o segundo nível de regulação da propagação da sinalização purinérgica nas plaquetas é mediado pelas ectonucleotidases. As plaquetas possuem toda a cascata enzimática responsável pela hidrólise dos nucleotídeos e nucleosídeos de adenina demonstrando, dessa forma, que essas possuem papel essencial para a manutenção, propagação ou terminação da sinalização induzida por essas moléculas (KOUPENOVA e RAVID, 2018; BURNSTOCK, 2017; JONES et al., 2014; KUNAPULI et al., 2003). Além das ectonucleotidases presentes na membranas das plaquetas, essas enzimas nas suas formas solúveis encontradas no soro, bem como as presentes nas células endoteliais dos vasos sanguíneos também podem contribuir para regulação da sinalização purinérgica (BURNSTOCK, 2017) (Figura 3). Muitos estudos têm relatado a importância da atividade dessas enzimas na fisiopatologia de diversas doenças, principalmente às relacionadas com processo inflamatórios e distúrbios na função plaquetária. No entanto, a função das plaquetas

bem como o papel das ectonucleotidases na função plaquetária ainda não tinha sido relatado na hipermetioninemia.

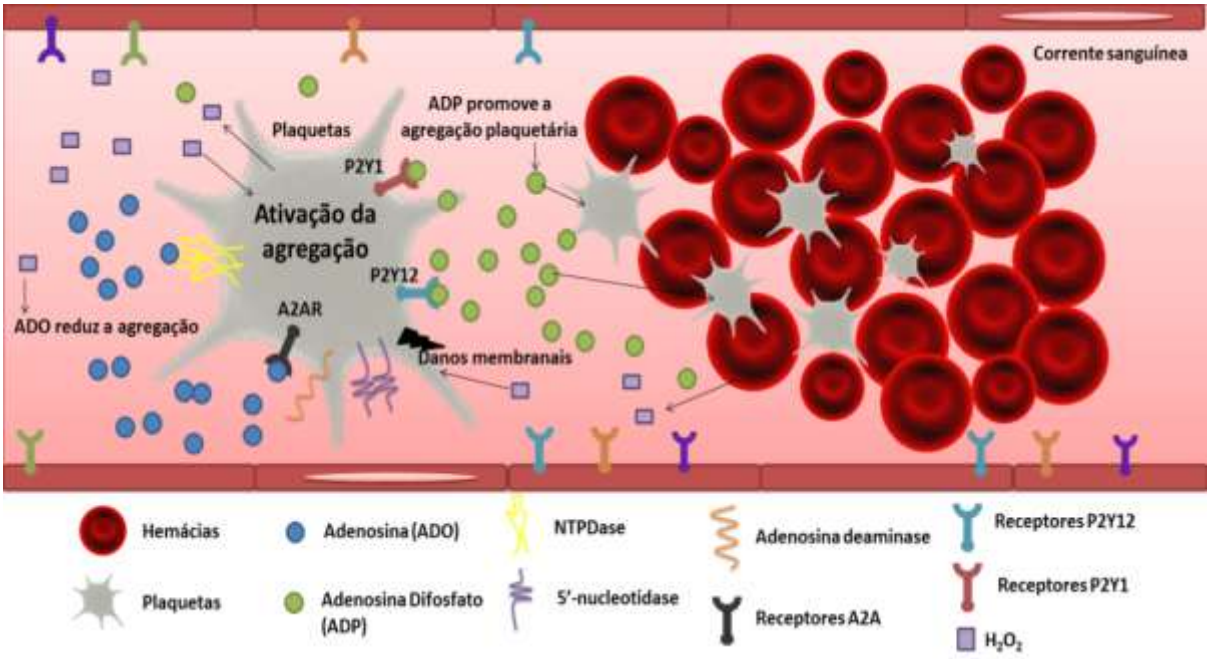

Figura 3: Participação dos componentes da sinalização purinérgica na ativação ou inibição da agregação plaquetária. As plaquetas são estimuladas por uma variedade de agonistas, incluindo adenosina difosfato (ADP). Trifosfato de adenosina (ATP) e adenosina (ADO) no meio extracelular também são capazes de modular vários efeitos no sistema vascular interagindo com receptores específicos em plaquetas. A membrana plaquetária expressa uma cascata de ectoenzimas composta de trifosfato de nucleosídeo fosfohidrolase (NTPDases), 5′-nucleotidase e adenosina deaminase (ADA) as quais são responsáveis pela hidrólise dos nucleotídeos e nucleosídeos de adenina. O peróxido de hidrogênio pode interagir no processo de ativação das plaquetas através de possíveis danos oxidativos na membrana dessas células.

3.4.2 Astrócitos e Sistema Purinérgico

Por muitos anos estendeu-se a ideia de que a principal função dos astrócitos era dar suporte estrutural passivo aos neurônios. Entretanto, desde a descoberta inicial da ampla gama de funções astrogliais, os astrócitos cimentaram a sua posição como determinantes e cruciais para o funcionamento neuronal adequado (VASILE et al., 2017; SICA et al., 2016; SOFRONIEW e VINTERS, 2010). Os astrócitos são células especializadas da glia, e são as células gliais de maior tamanho e número no SNC. Morfologicamente são células estreladas com núcleo grande, ovoide ou ligeiramente irregular com cromatina frouxa e nucléolo central. No citoplasma está presente a proteína fibrilar glial (GFAP) que são filamentos exclusivos dessas

células. Os astrócitos comunicam-se uns com os outros por junções gap (VASILE et al., 2017; SICA et al., 2016; SOFRONIEW e VINTERS, 2010).

Atualmente já é bem estabelecido que os astrócitos são participantes diretos dos circuitos e processamento cerebrais, e exibem um amplo espectro de funções em nível celular, tais como formação, maturação e eliminação de sinapses, homeostase iônica, eliminação de neurotransmissores, regulação do volume do espaço extracelular e modulação da atividade e plasticidade sináptica. Além disso, foi demonstrado que estão envolvidos na geração de ritmo e padrões de rede neuronal (VASILE et al., 2017; SICA et al., 2016; SOFRONIEW e VINTERS, 2010). A incrível variedade de processos nos quais os astrócitos estão envolvidos sugere que uma mudança em suas características irá alterar sua contribuição para as funções neuronais.

Além disso, os astrócitos respondem a todas as formas de danos e doenças do SNC com uma variedade de potenciais alterações na expressão gênica, estrutura celular e funcional. Tais respostas são comumente referidas como astrogliose (LIDDELOW e BARRES, 2017). As modificações sofridas pelos astrócitos reativos variam com a gravidade do insulto ao longo de um gradativo contínuo de alterações progressivas na expressão molecular, hipertrofia celular e, em casos graves, proliferação e formação de cicatriz (SOFRONIEW, 2015; PEKNY e PEKNA, 2014). As alterações da astrogliose reativa são reguladas de maneira específica por moléculas sinalizadoras inter e intracelulares. Também têm o potencial de alterar as atividades dos astrócitos tanto pelo ganho quanto pela perda de funções que podem afetar tanto benéfica quanto prejudicialmente as células neurais e não neurais adjacentes (COLOMBO e FARINA, 2016; SOFRONIEW, 2015; PEKNY e PEKNA, 2014).

Sabe-se que danos agudos e crônicos no SNC liberam grandes quantidades de ATP, podendo atingir níveis na faixa de milimolar, o qual pode se ligar nos receptores P2X e P2Y localizados nos astrócitos. A ativação dos receptores P2X7 e PY1 e 2 nos astrócitos estão intrinsicamente relacionados com o inicio da astrogliose (FRANKE e ILLES, 2014; BURNSTOCK, 2013, 2007). O ATP liberado pode atuar em poucos minutos como uma molécula excitotóxica; em uma escala de tempo maior, em poucos dias, causar neuroinflamação (CISNEROS-MEJORADO 2015; FRANKE e ILLES, 2014) (Figura 4).

Por outro lado, embora geralmente a ADO seja produzida pela quebra ecto-enzimática do ATP, podem haver subpopulações de neurônios e/ou astrócitos que liberam ADO diretamente no meio extracelular através de proteínas de transporte de nucleosideos de equilíbrio 1 e/ou 2 (ENT1, ENT2) (ILLES e VERKHRATSKY, 2016; CISNEROS-MEJORADO, 2015; CHU et al., 2014; FRANKE e ILLES, 2014; BURNSTOCK, 2013, 2007). A ADO exerce efeito contrário ao ATP, agindo como anti-inflamatória e imunossupresora através da sua interação com os receptores P1, que desempenham um papel importante na neuromodulação pré-sináptica. Ambos os receptores P1 e P2 participam nas interações neuro-gliais. A sinalização purinérgica está surgindo como um dos principais meios de integrar a atividade funcional entre os neurônios, células gliais e vasculares no SNC (CISNEROS-MEJORADO 2015; FRANKE e ILLES, 2014; BURNSTOCK, 2013, 2007) (Figura 4).

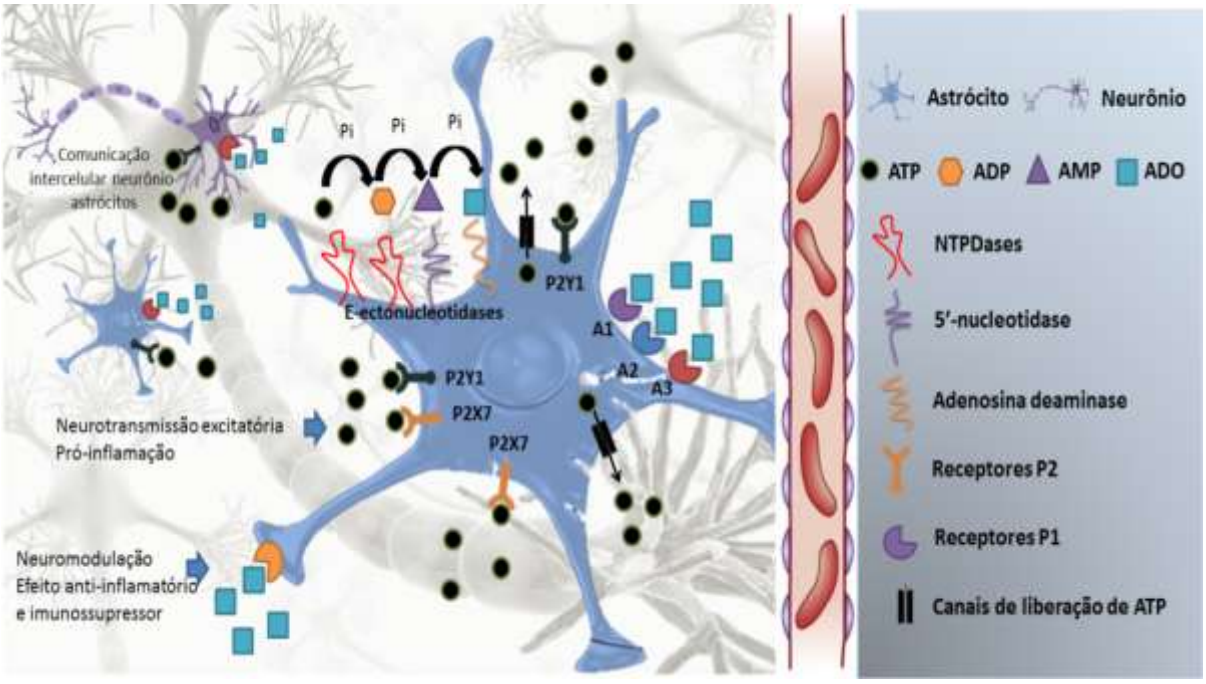

Figura 4: Componentes da sinalização purinérgica nos astrócitos. Os astrócitos possuem todos os componentes do sistema purinérgico incluindo receptores P2Y e P2X para trifosfato de adenosina (ATP) e adenosina difosfato (ADP) e receptores P1 para adenosina (ADO). Além disso, a membrana dessas células expressa uma cascata de ectoenzimas composta de trifosfato de nucleosideo fosfohidrolase (NTPDases) que convertem ATP e ADP em monofosfato de adenosina (AMP), que por sua vez é hidrolisado via 5′-nucleotidase em ADO e adenosina deaminase (ADA), que é responsável pela desaminação irreversível de ADO em inosina.

Cabe salientar que os astrócitos expressam em sua membrana toda a cascata de ectoenzimas que pertencem ao sistema purinérgico como, as NTPDases,

E-NPPs, fosfatases alcalinas, ecto-5'–nucleotidase e ADA (BURNSTOCK 2013, 2007) (Figura 4). Vuaden et al. (2014) demonstraram em um modelo experimental de hipermetioninemia, utilizando Zebrafish, que a Met é capaz de diminuir a atividade da NTPDase, e por outro lado, aumentar a expressão das NTPDase 1, 2 e 3 no cérebro desses animais. Além disso, os mesmos autores relataram um aumento na expressão dos receptores de ADO A1 e A2A (VUADEN et al., 2014). Com base na importância do sistema purinérgico no SNC, associado as evidências de que a Met altera alguns componentes do sistema purinérgico no cérebro, a investigação dos efeitos das Met e/ou MetO sobre a sinalização purinérgica nos astrócitos se torna extremamente relevante para o entendimento dos aspectos fisiopatolólogicos dessa desordem.

3.5 Sinalização Colinérgica

Um dos primeiros achados relatados em modelos experimentais de hipermetioninemia foram as alterações na atividade da enzima AChE em cérebro de ratos seguido de alterações cognitivas (STEFANELLO et al., 2007). Essa é uma enzima que pertence ao sistema colinérgico e é de extrema importância principalmente para o processo de aprendizado e memória. Entretanto, a AChE possui vários outros papeis no SNC, como ativação glial, diferenciação pós-sináptica, adesão celular e ativação de neurônios dopaminérgicos (SILMAN e SUSSMAN, 2005).

O sistema colinérgico inclui a sinalização mediada pela ACh e abrange todo o processo de síntese, armazenamento, transporte e degradação dessa molécula (Figura 5). A ACh além de ser um neurotransmissor clássico, é uma molécula que também pode ser liberada por células não neuronais como os linfócitos (PICCIOTTO et al., 2012; BECKMANN e LIPS, 2013). No SNC, a ACh age como neuromodulador envolvido em processos comportamentais, de cognição, aprendizado, memória e atenção. No sistema nervoso periférico desempenha um papel de neurotransmissor excitatório promovendo ativação dos músculos (PICCIOTTO et al., 2012). Em relação ao sistema colinérgico não neuronal, uma das principais funções é a modulação das respostas imunes e inflamatórias (BECKMANN e LIPS, 2013).

A síntese da ACh ocorre a partir de colina e acetilcoenzima A em uma reação catalisada pela enzima colina acetiltransferase. A ACh age através dos seus receptores muscarínicos e nicotínicos presentes na membrana das células (PICCIOTTO et al., 2012; BECKMANN e LIPS, 2013). Por fim, a ação da ACh é modulada pela ação das colinesterases: AChE e butirilcolinesterase (BuChE). Essas enzimas são constituintes ubíquos e diferenciam-se basicamente quanto à distribuição tecidual, propriedades cinéticas, especificidade por substratos e por inibidores seletivos (DAS, 2007).

A AChE é a principal enzima moduladora dos níveis de ACh no meio extracelular uma vez que é responsável pela hidrólise dessa molécula em acetato e colina. É uma glicoproteína classificada como homomérica com sítio ativo composto por uma tríade catalítica formada por resíduos de aminoácidos serina (Ser-200), histidina (His-440) e glutamato (Glu-327) (DAS, 2007; POHANKA, 2014). A AChE está presente majoritariamente no SNC, no entanto é encontrada em eritrócitos, linfócitos e plaquetas. Um aumento na atividade da AChE já foi observado em diversas patologias e são relacionadas com alterações em parâmetros inflamatórios (DAS, 2007; POHANKA, 2014). Além da função catalítica, a AChE possui diversas outras funções como a sua capacidade de neuritogênese, função sinaptogênica e de adesão celular, ativação de neurônios dopaminérgicos, além de possuir atividades hematopoiéticas e trombopoiéticas (SILMAN e SUSSUMAN, 2005).

A BuChE é uma colinesterase capaz de hidrolisar outros ésteres de colina além da ACh. É sintetizada pelo fígado e possui uma distribuição tecidual ampla podendo ser encontrada no soro, pâncreas, coração, células hematopoiéticas e SNC (DAS, 2007). Estruturalmente a BuChE é similar a AChE, tendo no seu sítio ativo uma tríade catalítica contendo os aminoácidos histidina, ácido glutâmico e serina o qual é essencial para a atividade catalítica (JOHNSON e MOORE, 2012). Também é encontrada em diferentes formas moleculares como monômeros (G1) e oligômeros (G2), as quais são simétricas, hidrofílicas e solúveis enquanto que a forma tetramérica (G4) pode ser assimétrica e ancorada à membrana (JOHNSON e MOORE, 2012). Quanto à função fisiológica da BuChE, ainda não foi completamente esclarecida entretanto nos últimos anos o seu papel vem sendo alvo de estudos em condições de inflamação sistêmica de baixo grau, além de desordens neuronais associadas a disfunções imune inflamatórias (DAS, 2007).

Estudos envolvendo modelos animais de hipermetioninemia demonstraram um estado pró-inflamatório, associados a mudanças no sistema colinérgico. Schulpis et al. (2006) demonstraram que Met, *in vitro*, é capaz de aumentar a atividade da AChE em hipocampo. Posteriormente, Stefanello et al. (2007) relataram que administração crônica de Met em ratos Wistar aumenta a atividade da AChE no córtex cerebral associada a uma disfunção cognitiva. Mais recentemente, estudos mostraram que a exposição a longo prazo ao Met causa um aumento importante na atividade da AChE no cérebro de Zebrafish associado a déficit de memória (VUADEN et al., 2012). Nos astrócitos, foi relatado que a injeção de AChE no cérebro induz hipertrofia dos astrócitos de hipocampo (CHACÓN et al., 2003).

Alguns pesquisadores correlacionaram o aumento da atividade da AChE com a neuroinflamação (SCHERER et al., 2014), o que também pode estar relacionado aos efeitos patogênicos encontrados na hipermetioninemia. Assim, considerando a intríseca relação do sistema colinérgico com o processo inflamatório e com a modulação de importantes funções no SNC como aprendizado, memória, controle no fluido cerebral e dos movimentos torna-se importante a investigação das enzimas envolvidas na degração da ACh frente a elevadas concentrações de Met e MetO.

3.6 Na^+,K^+-ATPase

A enzima Na^+,K^+-ATPase, também conhecida como bomba de sódio e potássio, é acoplada à membrana e a sua principal função é a manutenção do equilíbrio iônico regulando a entrada de potásio (K^+) com a saída de sódio (Na^+) das células (ARNAIZ e ORDIERES, 2014) (Figura 5). Um desbalanço na atividade dessa enzima pode causar uma despolarização neuronal com a entrada de cálcio (Ca^{2+}), promovendo liberação de neurotransmissores e desequilíbrio osmótico com consequente prejuízo funcional (ARNAIZ e ORDIERES, 2014; ZHANG et al., 2013). A modulação da atividade da Na^+,K^+-ATPase afeta diretamente a transmissão e plasticidade sináptica, e o processo de aprendizado e memória (MOSELEY et al., 2007; ZHANG et al., 2013; PETRUSHANKO et al., 2016).

A Na^+,K^+-ATPase possui três subunidades: α, β e γ, sendo a subunidade α a catalítica, e a responsável pela troca de íons Na^+ e K^+. Também é na subunidade α aonde se encontra o sítio de ligação para o ATP (ARNAIZ e ORDIERES, 2014). A subunidade β é a responsável por regular a atividade e a estabilidade

conformacional da subunidade α e a subunidade γ desempenha uma função reguladora de forma tecido-específica (ARNAIZ e ORDIERES, 2014). A subunidade α apresenta quatro diferentes isoformas: α1, α2, α3 e α4. Dessas, três isoformas são expressas no cérebro: a α1 é encontrada em vários tipos celulares do SNC; a α2 é predominantemente expressa em astrócitos; e a α3 é expressa apenas em neurônios (MOSELEY et al., 2007).

Stefanello et al. (2005) demonstraram que *in vitro* a Met é capaz de inibir a atividade da Na^+,K^+-ATPase em homogenatos de hipocampos (STEFANELLO et al., 2005). Mais tarde, em um modelo experimental *in vivo*, observou-se que a adminstração crônica de Met é capaz de inibir a atividade da Na^+,K^+-ATPase em córtex cerebral e hipocampo de ratos jovens (STEFANELLO et al., 2007a, 2007b). Recentemente, Schweinberger et al., (2016) demonstraram que altos níveis de Met inibiram a atividade da Na^+,K^+-ATPase mas não alteraram a expressão desta enzima em cérebro da prole de ratas submetidas ao modelo experimental de hipermetioninemia gestacional (SCHWEINBERGER et al., 2016). No entanto, os efeitos do metabólito MetO ainda não foram investigados. Além disso, esse é um importante alvo de estudo nessa patologia uma vez que um dos principais sintomas neurológicos relatados é o edema cerebral, o qual é bastante relacionado com disfunção na atividade da Na^+,K^+-ATPase (ARNAIZ e ORDIERES, 2014).

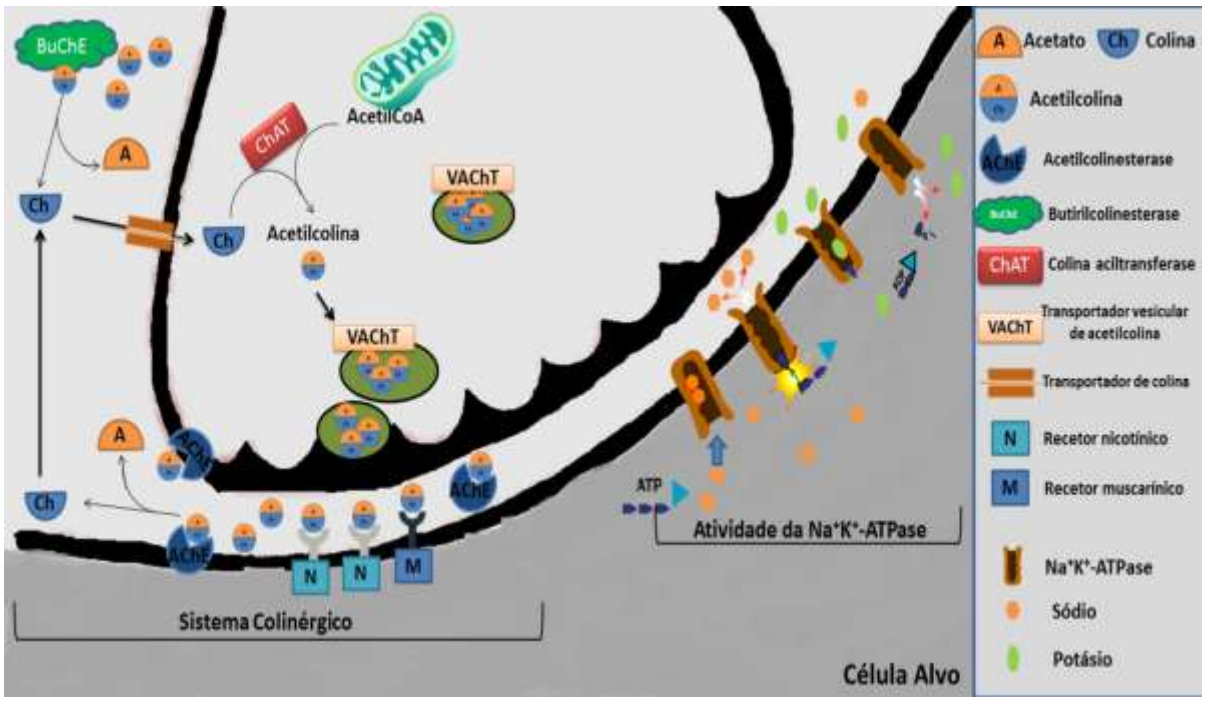

Figura 5: Componentes do sistema colinérgico e funcionamento da enzima Na^+,K^+-ATPase.

3.7 Fator neurotrófico derivado do cérebro (BDNF)

O fator neurotrófico derivado do cérebro (BDNF) é membro de uma família polipeptídica de fatores de crescimento, as neurotrofinas que desempenham um papel crucial nos processos de proliferação, diferenciação, sobrevivência e morte celular de neurônios e células não neuronais (SONG et al., 2017). Os papeis neurobiológicos do BDNF são essenciais desde o processo de desenvolvimento do SNC até a fase adulta, durante o qual desempenha um amplo espectro funcional como regulação de conexões sinápticas, estrutura sináptica, liberação de neurotransmissores e plasticidade sináptica (SONG et al., 2017).

O BDNF é secretado na sinapse, pelos neurônios pré-sinápticos, e posteriormente a sua liberação leva à ativação da cinase B do receptor de tropomiosina (TrkB) e consequentemente as suas cascatas de sinalização, as quais contribuem para eventos de transcrição gênica críticos para plasticidade sináptica e função cognitiva (SASI et al., 2017; SONG et al., 2017). Nesse sentido, o BDNF e sua cascata de sinalização são fatores reguladores nos processos de aprendizagem e memória, inclusive um grande número de desordens no neurodesenvolvimento, neurodegenerativa e neuropsiquiátrica são caracterizadas por anormalidade na plasticidade sináptica associada com déficits nos níveis e funções do BDNF (HEMPSTEAD, 2015; COHEN-CORY et al., 2010).

Portanto, muita atenção tem sido direcionada ao BDNF para entendimento de mecanismos neurofisiopatológicos de diversas doenças, uma vez que a sinalização específica induzida por essa proteína é considerada como uma estratégia interessante para estimular a plasticidade neuronal e sináptica para potenciais tratamentos protetores e funcionalmente restauradores para distúrbios neurológicos e psiquiátricos (SASI et al., 2017; SONG et al., 2017; HEMPSTEAD 2015). Sendo assim, esse é um relevante alvo de estudo na hipermetioninemia, uma vez que alterações cognitivas são relatadas associadas a falta de tratamento específico, principalmente por desconhecimento dos exatos mecanismos envolvidos patogênese dessa desordem.

4. RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de três artigos e dois manuscritos. As seções materiais e métodos, resultados, discussão e referências encontram-se nos próprios artigos e manuscritos e representam a íntegra deste estudo.

Os itens discussão e conclusões que se encontram no final desta tese apresentam interpretações e comentários gerais sobre os artigos contidos nesse trabalho.

As referências são referentes apenas às citações que aparecem nos itens introdução, revisão de literatura e discussão da tese.

Os artigos e os manuscritos estão estruturados de acordo com as revistas as quais foram publicados ou submetidos.

4.1 Artigo I

Chronic administration of methionine and/or methionine sulfoxide alters oxidative stress parameters and ALA-D activity in liver and kidney from young rats.

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ORIGINAL ARTICLE

Chronic administration of methionine and/or methionine sulfoxide alters oxidative stress parameters and ALA-D activity in liver and kidney of young rats

Mayara Sandrielly Pereira Soares[1] · Pathise Souto Oliveira[2] · Gabriela Nogueira Debom[1] · Bruna da Silveira Mattos[1] · Carla Roberta Polachini[3] · Jucimara Baldissarelli[3] · Vera Maria Morsch[3] · Maria Rosa Chitolina Schetinger[3] · Rejane Giacomelli Tavares[1] · Francieli Moro Stefanello[2] · Roselia Maria Spanevello[1]

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Abstract High levels of methionine (Met) and methionine sulfoxide (MetO) are found in several genetic abnormalities. Oxidative stress is involved in the pathophysiology of many inborn errors of metabolism. However, little is known about the role of oxidative damage in hepatic and renal changes in hypermethioninemia. We investigated the effect of chronic treatment with Met and/or MetO on oxidative stress parameters in liver and kidney, as lipid peroxidation (TBARS), total sulfhydryl content (SH), reactive oxygen species (ROS) and enzymes activities superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and delta aminolevulinic dehydratase (ALA-D). Serum biochemical parameters were evaluated. Wistar rats were treated daily with two subcutaneous injections of saline (control), Met (0.2–0.4 g/kg), MetO (0.05–0.1 g/kg) and the association between these (Met plus MetO) from the 6th to the 28th day of life. Our data demonstrated an increase of glucose and urea levels in all experimental groups. Cholesterol (MetO and Met plus MetO) were decreased and triglycerides (MetO) were increased. SOD (MetO and Met plus MetO) and CAT (Met, MetO and Met plus MetO) activities were decreased, while GPx was enhanced by MetO and Met plus MetO treatment in liver. In kidney, we observed a reduction of SH levels, SOD and CAT activities and an increase of TBARS levels in all experimental groups. ROS levels in kidney were increased in MetO and Met plus MetO groups. ALA-D activity was enhanced in liver (MetO and Met plus MetO) and kidney (Met plus MetO). These findings help to understand the pathophysiology of hepatic and renal alterations present in hypermethioninemia.

Keywords Methionine · Methionine sulfoxide · Oxidative stress · Delta aminolevulinic dehydratase

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✉ Francieli Moro Stefanello
fmstefanello@gmail.com

✉ Roselia Maria Spanevello
roseliabioquimica@gmail.com

[1] Programa de Pós-Graduação em Bioquímica e Bioprospecção, Laboratório de Neuroquímica, Inflamação e Câncer, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N, Capão do Leão, Pelotas, RS CEP 96010-900, Brazil

[2] Programa de Pós-Graduação em Bioquímica e Bioprospecção, Laboratório de Biomarcadores, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N, Pelotas, RS, Brazil

[3] Programa de Pós-Graduação em Bioquímica Toxicológica, Laboratório de Enzimologia Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário S/N, Santa Maria, RS, Brazil

Introduction

Methionine (Met) is a sulfur-containing essential amino acid which participates in the biosynthesis of important molecules such as *S*-adenosylmethionine (SAM) (Finkelstein 1990). Met is a direct target of reactive oxygen species (ROS) and is highly oxidation-susceptible resulting mainly in free and protein-bound methionine sulfoxide (MetO) (Moskovitz 2014). The biological effect of high levels of MetO has not been fully clarified, but its presence is associated with the loss of biological activity protein (Lee and Gladyshev 2011).

High plasma levels of Met and MetO are found in several genetic disorders mainly in methionine adenosyltransferase (MAT) I/III deficiency, which causes persistent hypermethioninemia (Hirabayashi et al. 2013; Mudd 2011). Hypermethioninemic patients have exhibited neurological alterations often associated with brain myelination abnormalities (Couce et al. 2013). Hepatic disorders, such as cirrhosis and steatosis, have also been found at high levels of Met and MetO (Avila et al. 2000; Mudd 2011).

It is well known that oxidative stress is an imbalance between pro-oxidants and antioxidant compounds which can cause irreversible damage to biomolecules and lead to cell dysfunction (Gonsette 2008; Ljubisavljevic 2016). The organism has the antioxidant machinery which is composed for non-enzymatic systems and endogenous enzymes such as superoxide dismutase (SOD) that catalyzes the dismutation of superoxide radicals to hydrogen peroxide, which can be degraded by catalase (CAT) and glutathione peroxidase (GPx) (Bó et al. 2015; Lowe 2014; Finkel and Holbrook 2000). In addition, delta aminolevulinic acid dehydratase (ALA-D) is a metalloenzyme which participates in heme biosynthesis and can be used as a marker sensitive to oxidative stress (Folmer et al. 2003). Therefore, studies have shown that this enzyme is a marker protein of oxidative stress because it is thiol-dependent and is highly sensitive to SH oxidation (Valentini et al. 2008).

Oxidative stress contributes to pathogenesis of several hepatic and renal diseases (Li et al. 2015; Ha et al. 2010; Nair et al. 2010). We demonstrated previously that chronic exposure to Met induces oxidative stress and promotes histological changes in the liver of young rats (Stefanello et al. 2009). In addition, Costa et al. (2013) showed that acute administration of Met and/or MetO alters oxidative stress parameters, such as thiobarbituric acid reactive substances (TBARS), total thiol content and enzymatic antioxidant defenses. However, the effect of chronic administration of MetO and the association of Met and MetO on redox status in the liver and especially kidney are still unknown.

Since there is evidence that high plasma levels of Met and MetO, as found in hypermethioninemic patients, can be toxic, the aim of this study was to investigate the effect of the chronic administration of Met and/or MetO on ALA-D activity, levels of TBARS, total sulfhydryl content (SH), ROS production and on the activity of antioxidant enzymes SOD, CAT and GPx in liver and kidney of young rats. Serum biochemical parameters were also evaluated.

Materials and methods

Chemicals

Methionine, methionine sulfoxide, epinephrine, thiobarbituric acid, 5.50-dithiobis (2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid and hydrogen peroxide were purchased from Synth® (Brazil). All other reagents used in the experiments were of analytical grade and the highest purity.

Animals

Wistar rats were obtained from the Central Animal House of the Federal University of Pelotas, Pelotas, RS, Brazil. The animals were maintained in a controlled temperature environment (22 ± 1 °C) on a 12/12 h light/dark cycle in colony room. Food (commercial chow) and water were provided ad libitum to the animals. All animal procedures were approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, Brazil under protocol number: CEEA 3527.

Treatment with methionine and/or methionine sulfoxide

Twenty-four rats, male and female, were used for chronic treatment. They were divided into four groups ($n = 6$ each): Group I (Control/saline), Group II (treated with Met), Group III (treated with MetO) and Group IV (treated with Met plus MetO). Met and MetO were dissolved in 0.9 % NaCl solution and were administered by subcutaneous injection twice a day at 8 h intervals between injections from the 6th to the 28th day of life as described by Stefanello et al. (2007a, b). During the first 8 days of treatment (6th to 14th day of life) the animals from group II received 0.2 g/kg body weight of Met and group III received 0.05 g/kg body weight of MetO. From the 15th to the 21st day group II received 0.3 g/kg body weight of Met and group III received 0.075 g/kg body weight of MetO and from the 22nd to the 28th day group II received 0.4 g/kg body weight of Met and group III received 0.1 g/kg body weight of MetO. Group IV received the combination of Met plus MetO at the same concentration according to week and control animals (group I) received saline solution in the same volume applied to the other groups (Fig. 1). The doses of Met and MetO administered were based on studies by Stefanello et al. (2007a, b) and Costa et al. (2013).

Serum preparation

Blood was collected without anticoagulant and immediately centrifuged at 2500*g* for 15 min at room temperature. The serum was stored at −80 °C for further biochemical analysis.

Tissue and homogenate preparation

All animals were submitted to euthanasia 21 days after treatment and liver and kidney were quickly dissected. For

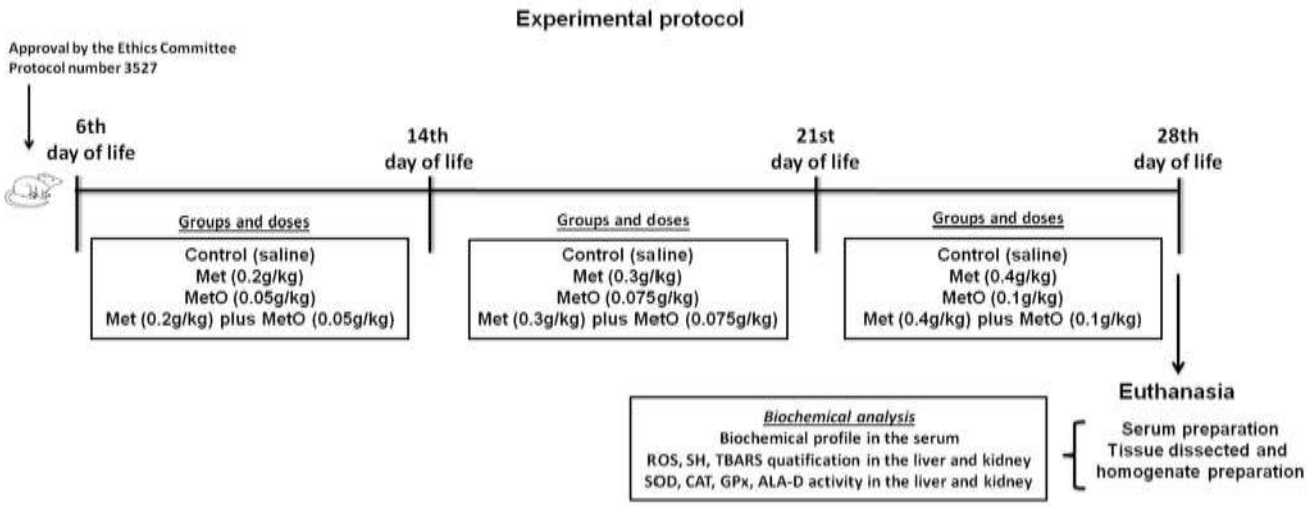

Fig. 1 Experimental protocol of Met and/or MetO administration

analysis of oxidative stress parameters, both tissues were homogenized in sodium phosphate buffer pH 7.4 containing KCl (1:10, w/v). The homogenates were centrifuged at 2500*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was separated and used for biochemical determination. For ALA-D assay, the samples were placed on ice and homogenized for 10 min in 50 mM Tris–HCl pH 7.4 (1/10, w/v). The homogenate was centrifuged at 2000*g*, at 4 °C for 10 min to yield the low speed supernatant that was used for ALA-D assay.

Biochemical analysis in serum

Glucose, total cholesterol, triglycerides (TAG) and urea were determined in serum using commercially available kits (Labtest® and Bioclin).

Oxidative stress parameters in liver and kidney

Thiobarbituric acid reactive substances (TBARS) quantification

TBARS levels were determined as described by Esterbauer and Cheeseman (1990). For the test, homogenates were mixed with trichloroacetic acid (10 %) and thiobarbituric acid (0.67 %) and were incubated in a dry block at 100 °C for 30 min. The result was a pink organic layer and TBARS levels were determined by the absorbance at 535 nm. Results were reported as nmol of TBARS per mg protein.

Total sulfhydryl content quantification

Total sulfhydryl content was measured according to Aksenov and Markesbery (2001) which is based on the reduction of DTNB by thiols, which in turn, becomes oxidized (disulfide) generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. Briefly, homogenates were added to PBS buffer pH 7.4 containing EDTA. The reaction was started by the addition of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Results were reported as nmol TNB per mg of protein.

Reactive oxygen species (ROS) quantification

The ROS formation was determined according to Ali et al. (1992), with some modifications. In this assay, the oxidation of dichloro-dihydro-fluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) was measured for the detection of intracellular RS. DCF fluorescence intensity emission was recorded at 525 and 488 nm excitation 60 min after the addition of DCFH-DA to the medium. ROS levels were expressed as µmol/g of tissue.

Superoxide dismutase (SOD) activity

Total SOD activity was measured by the method described by Misra and Fridovich (1972). This assay is based on the inhibition of superoxide dependent adrenaline auto-oxidation to adenochrome in a spectrophotometer adjusted at 480 nm. The intermediate in this reaction is superoxide, which is scavenged by SOD. One SOD unit was defined as the enzyme amount to cause 50 % inhibition of adrenaline autoxidation. The specific activity of SOD was reported as units per mg of protein (U/mg of protein).

Catalase (CAT) activity

CAT activity was assayed by the method of Aebi (1984). The decomposition of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) was continuously monitored

Table 1 Glucose, total cholesterol, triglycerides and urea levels in serum of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO)

Groups	Glucose (mg/dl)	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	Urea (mg/dl)
Control	96.3 ± 3.4	44.0 ± 2.4	26.2 ± 0.8	40.11 ± 4.4
Met	119.2 ± 4.9**	43.6 ± 1.1	32.2 ± 2.9	75.1 ± 5.6*
MetO	118.5 ± 1.6**	37.7 ± 1.1*	38.0 ± 1.8*	75.0 ± 7.4*
Met + MetO	128.6 ± 2.9***	39.44 ± 2.4*	33.2 ± 2.8	88.7 ± 10.3*

Values are expressed as mean ± SEM. All biochemical parameters were expressed as mg/dl

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ different from the control group ($n = 5$–6)

with a spectrophotometer at 240 nm for 180 s in a thermostat (37 °C). One unit of the enzyme is defined as 1 nmol of hydrogen peroxide consumed per minute and the specific activity was reported as units per mg protein.

Glutathione peroxidase (GPx) activity

GPx activity was measured using a commercial kit (RANSEL®; Randox Lab, Antrim, UK). GPx catalyzes glutathione (GSH) oxidation by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured. The specific activity of GPx was reported as units per mg of protein.

Delta Aminolevulinic acid dehydratase (ALA-D) activity

Hepatic and kidney ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of porphobilinogen (PBG) formation, except that in all enzyme assays the final concentration of ALA was 2.2 mM. An aliquot of 200 µl of liver sample was incubated for 30 min at 37 °C. The reaction was stopped by the addition of 250 µl of trichloroacetic acid (TCA). The reaction product was determined using modified Ehrlich's reagent at 555 nm. ALA-D activity was expressed as nmol porphobilinogen (PBG) mg/protein/h.

Protein determination

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

Statistical analysis

Statistical analysis was done using GraphPad Prism 5 software. Data were analyzed by analysis of variance (one-way ANOVA) followed by Tukey post hoc test for multiple comparison and $P < 0.05$ was considered to represent a significant difference in the analysis. All data were expressed as mean ± standard error (SEM).

Results

Serum biochemical parameters

First, we determined the effect of Met and/or MetO after chronic administration on serum biochemical parameters (Table 1). The glucose levels were significantly enhanced in the Met ($F_{(3-23)} = 10.98$, $P < 0.01$), MetO ($F_{(3-23)} = 10.98$, $P < 0.01$), and Met plus MetO($F_{(3-23)} = 10.98$, $P < 0.001$) groups when compared to the control group (Table 1). Additionally, it was observed that total cholesterol was decreased in the animals treated with MetO and Met plus MetO ($F_{(3-24)} = 6.5$, $P < 0.05$). Also, we observed an increase in the TAG levels caused by MetO administration ($F_{(3-24)} = 3.95$, $P < 0.05$). Urea levels were significantly increased in all experimental groups (Met, MetO and Met plus MetO) ($F_{(3-24)} = 4.27$, $P < 0.05$).

Oxidative stress parameters in liver

The levels of TBARS, total SH content and ROS in the liver of young rats 21 days after treatment with Met and/or MetO did not change (Fig. 2a, b). The activity of antioxidant enzymes was assayed in liver of rats subjected to chronic administration of Met and/or MetO. The SOD activity was significantly reduced in liver of young rats treated with MetO ($F_{(3-20)} = 14.87$, $P < 0.05$; Fig. 3a) and association of Met plus MetO ($F_{(3-20)} = 14.87$, $P < 0.001$; Fig. 3a). In addition, a decrease in CAT activity was observed in Met ($F_{(3-20)} = 13.45$, $P < 0.01$; Fig. 3b), MetO ($F_{(3-20)} = 13.45$, $P < 0.001$; Fig. 3b) and Met plus MetO ($F_{(3-20)} = 13.45$, $P < 0.001$; Fig. 3b) groups when compared to the control group. However, GPx activity was enhanced in liver of young rats 21 days after administration of MetO ($F_{(3-20)} = 18.99$, $P < 0.05$; Fig. 3c) and association of Met plus MetO ($F_{(3-20)} = 18.99$, $P < 0.001$; Fig. 3c).

Oxidative stress parameters in kidney

An increase in TBARS values was observed in all experimental groups, Met ($F_{(3-20)} = 4.90$, $P < 0.05$; Fig. 4a), MetO ($F_{(3-20)} = 4.90$, $P < 0.05$; Fig. 4a) and Met plus MetO ($F_{(3-20)} = 4.90$, $P < 0.01$; Fig. 4a) when compared

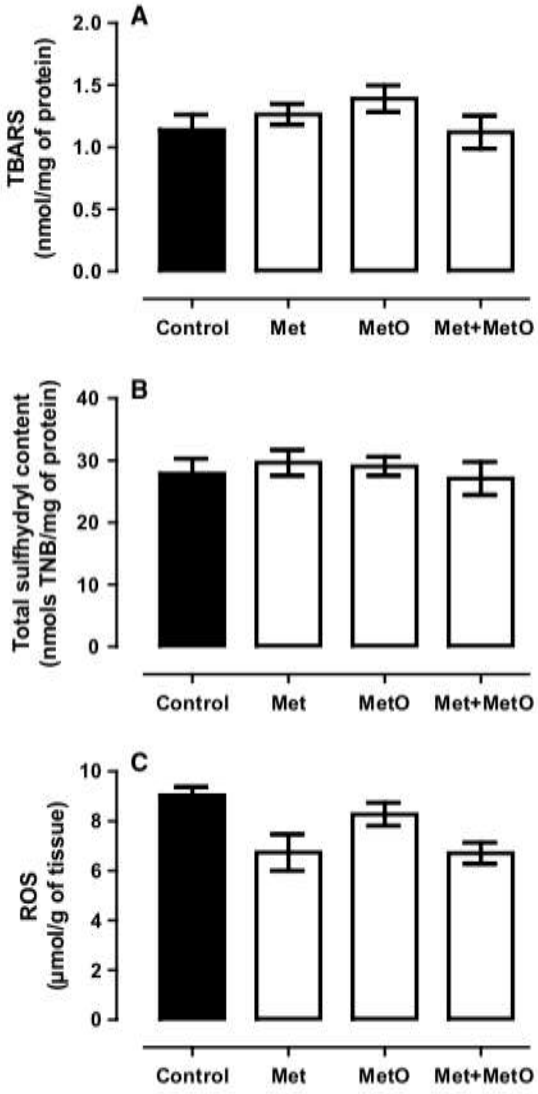

Fig. 2 TBARS (**a**), total sulfhydryl content (**b**) and ROS (**c**) levels in liver of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). Values are expressed as mean ± SEM ($n = 4$–6). TBARS levels were reported as nmol TBARS per mg protein, thiol content as nmol TNB per mg protein and ROS were expressed as µmol/g of tissue

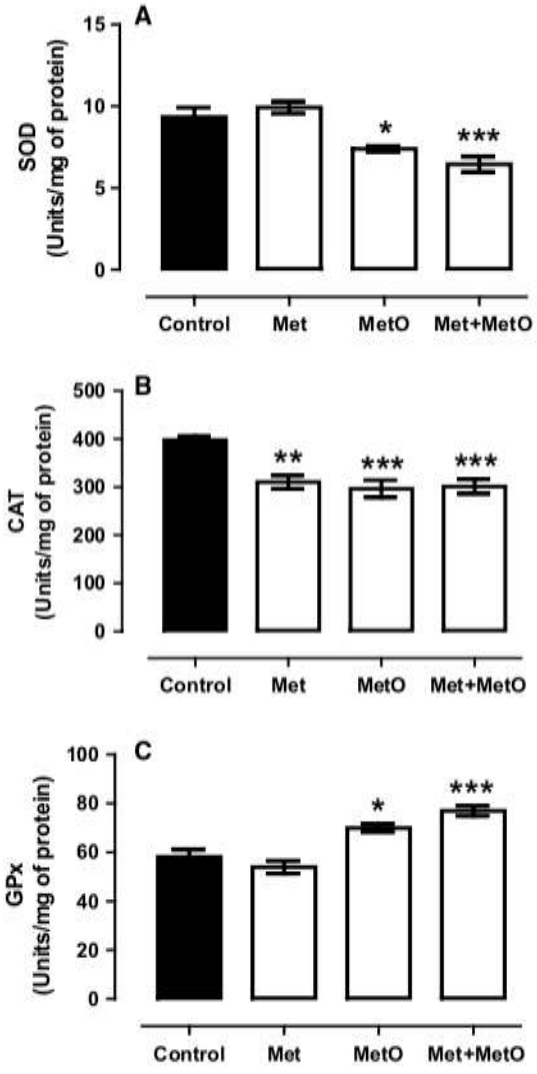

Fig. 3 Superoxide dismutase (**a**), catalase (**b**) and glutathione peroxidase (**c**) activities in liver of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). *Bars* represent mean ± SEM. CAT, SOD and GPx activities were reported as units per mg protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ different from the control group ($n = 4$–6)

to the control group. Besides, a decrease in total sulfhydryl content was observed in the kidney of young rats treated with Met ($F_{(3–20)} = 7.36$, $P < 0.05$; Fig. 4b), MetO ($F_{(3–20)} = 7.36$, $P < 0.05$; Fig. 4b) and association of Met plus MetO ($F_{(3–20)} = 7.36$, $P < 0.01$; Fig. 4b). ROS levels in kidney also were increased in MetO ($F_{(3–19)} = 10.63$, $P < 0.05$; Fig. 4c) and Met plus MetO ($F_{(3–19)} = 10.63$, $P < 0.01$; Fig. 4c) groups compared to the control group.

In the kidney, alterations were also observed in antioxidant enzyme activities. First, a reduction was observed in SOD activity in Met ($F_{(3–20)} = 5.53$, $P < 0.05$; Fig. 5a), MetO ($F_{(3–20)} = 5.53$, $P < 0.05$; Fig. 5a) and association of Met plus MetO ($F_{(3–20)} = 5.53$, $P < 0.05$; Fig. 5a) groups when compared to the control. Furthermore, there was also decreased CAT activity in kidney of rats treated with Met ($F_{(3–20)} = 9.16$, $P < 0.05$; Fig. 5b) MetO ($F_{(3–20)} = 9.16$, $P < 0.01$; Fig. 5b) and Met plus MetO ($F_{(3–20)} = 9.16$, $P < 0.001$; Fig. 5b). GPx activity in kidney was not affected by chronic administration with Met and/or MetO (Fig. 5c).

ALA-D activity in liver and kidney

Finally, liver ALA-D activity presented a significant increase in MetO ($F_{(3–24)} = 12.32$, $P < 0.05$; Fig. 6a) and association of Met plus MetO ($F_{(3–24)} = 12.32$, $P < 0.001$;

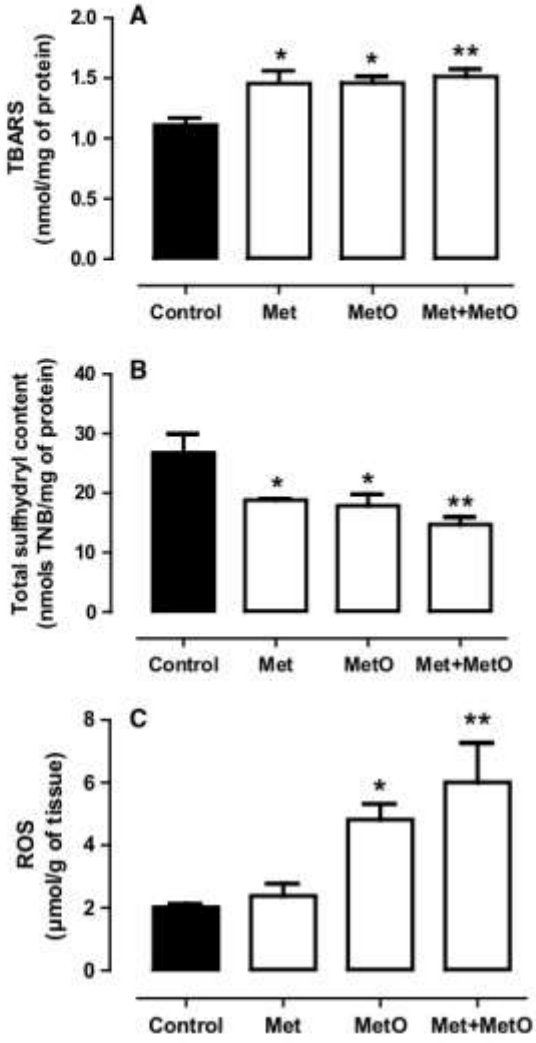

Fig. 4 TBARS (**a**), total sulfhydryl content (**b**) and ROS (**c**) levels in kidney of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). Values are expressed as mean ± SEM. TBARS levels were reported as nmol TBARS per mg protein, thiol content as nmol TNB per mg protein and ROS as µmol/g of tissue. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ different from the control group ($n = 4$–6)

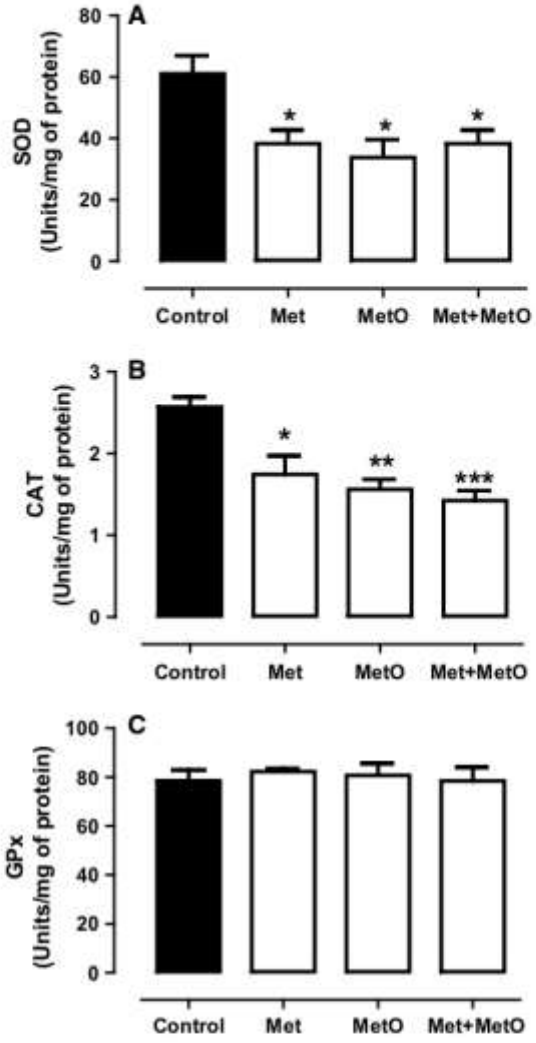

Fig. 5 Catalase (**a**), superoxide dismutase (**b**) and glutathione peroxidase (**c**) activities in kidney of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). *Bars* represent mean ± SEM. CAT, SOD and GPx activities were reported as units per mg protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ different from the control group ($n = 4$–6)

Fig. 6a) when compared to the control group. In kidney, ALA-D activity was enhanced only by Met plus MetO ($F_{(3\text{–}24)} = 5.79$, $P < 0.01$; Fig. 6b).

Discussion

Persistent hypermethioninemia is a metabolic disorder that can lead to serious brain and peripheral tissues injury which are still poorly understood (Mudd 2011). In vitro and in vivo (acute treatment) studies from our research group demonstrated that a high concentration of Met and/or MetO modifies liver homeostasis by altering the redox cellular state (Costa et al. 2013). In addition, it was demonstrated that chronic administration of Met promotes histological alterations and oxidative damage, and also alters serum biochemical parameters (Stefanello et al. 2009).

It is known that patients with liver cirrhosis have decreased SAM biosynthesis due to the expression of MAT1A and lower hepatic MAT activity (Mato and Lu 2007). Sanchez-Roman and Barja (2013) demonstrated that Met restriction increases longevity in rodents because decreased mitochondrial ROS production at complex I and restriction of all the dietary amino acids except Met do not

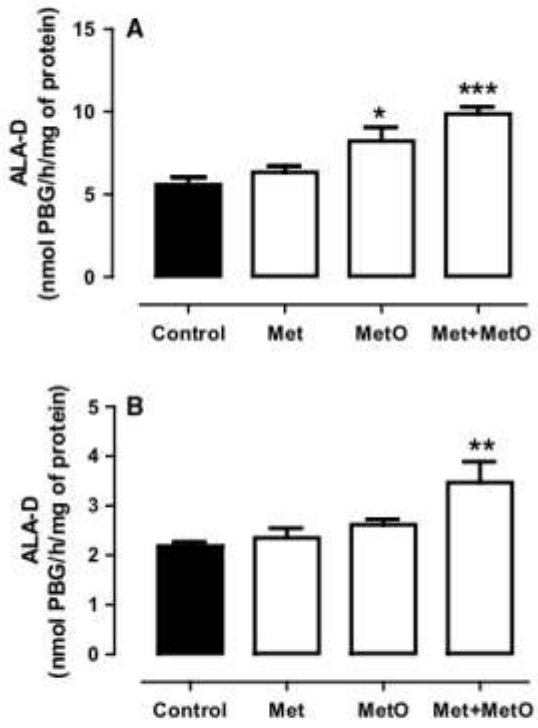

Fig. 6 ALA-D activity in liver (**a**) and kidney (**b**) of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). *Bars* represent mean ± SEM. ALA-D activity was expressed as nmol porphobilinogen (PBG)/h/ mg protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ different from the control group ($n = 6$)

modify mitochondrial ROS production, or oxidative damage to mtDNA (Sanchez-Roman and Barja 2013).

In the present study we evaluated the chronic administration of Met and/or MetO on liver and kidney oxidative damage parameters and metabolic markers in the serum of young rats. Our results showed that glucose levels were significantly higher in all animals treated with Met and/or MetO, which may be due to the increased production of glucose from amino acids in gluconeogenesis. Yet it is known that high levels of glucose may cause an increased production of ROS from mitochondria (Yu et al. 2011). The results showed decrease in total cholesterol in animals treated with MetO and Met plus MetO and an increase in the TAG levels caused by MetO administration. MetO can be reduced by reductases to Met and the increase of this amino acid in the bloodstream may lead to acetyl-CoA production and subsequent increase of TAG levels in the MetO group. As regard cholesterol levels, although we cannot establish the mechanism involved in this reduction, our findings are in agreement with previous data from our research group demonstrating a reduction in the concentration of cholesterol in rat brain (Stefanello et al. 2007a). On the other hand, Hidiroglou et al. (2004) showed that chronic and excessive Met supplementation increases LDL-cholesterol (Hidiroglou et al. 2004). After that, we observed an increase in blood urea levels in all groups, which was expected since urea is a substance produced in the liver as a result of the metabolism of amino acids such as Met and/or MetO. Furthermore, urea in the blood helps to assess renal function because it is a substance filtered by the kidneys (Enns 2008).

Results of the current study also showed that Met and/or MetO lead to renal and hepatic oxidative changes. We demonstrated that chronic treatment of these compounds significantly reduced SOD and CAT activities in the liver, which could result in an accumulation of superoxide and hydrogen peroxide radicals, since the antioxidant enzymes are involved in the detoxification of these species. The increase in GPx activity observed in the liver could be due to decreased CAT activity with consequent accumulation of hydrogen peroxide. This result might explain the normal ROS levels found in the liver and consequently homeostasis in TBARS and total SH content. In accordance with our results, Stefanello et al. (2009) demonstrated that chronic administration of Met decreased CAT activity and increased GPx activity, besides enhancing chemiluminescence levels in the hepatic tissue of rats (Stefanello et al. 2009). In this context, it should be emphasized that the Met metabolism occurs primarily in the liver, where the MAT enzyme catalyzes the degradation of Met to form SAM, thus high concentrations of this amino acid can be toxic and may lead to liver injury due to overload and the production of its potentially toxic metabolites such MetO (Martinov et al. 2010).

Regarding the kidney, TBARS levels was increased in the Met, MetO and Met plus MetO groups, indicating an increase in malondialdehyde concentrations, a product of lipid peroxidation. This lipid peroxidation may suggest an injury of the cell membrane initiated by ROS (Ayala et al. 2014). Moreover, it was possible to observe in Met, MetO and Met plus MetO groups a significant decrease of total sulfhydryl content showing that the protein-bound sulfhydryl status was impaired, probably by increased ROS (Davies 2016). The increase of TBARS levels as well as the reduction of SH content could be explained by high levels of ROS in MetO and Met plus MetO groups. ROS is involved in cell damage, necrosis and cell apoptosis due to the oxidation and nitration of cellular proteins, lipids and DNA, that can lead to loss of cell function (Bó et al. 2015).

In addition, SOD activity was reduced in renal tissue by Met and/or MetO, which have a deleterious effect on the cells mainly because of radical superoxide accumulation that can form the hydroxyl radical, which has a very short half-life making it difficult to detoxify. The hydroxyl radical often attacks biomolecules by hydrogen abstraction and addition to unsaturation (Lushchak 2015). With respect to CAT activity, the reduction observed in our findings could

be explained by reduction of SOD activity or because high levels of Met and/or MetO are impairing the enzymatic activity leading to accumulation of hydrogen peroxide. The compensatory mechanism observed in the liver against a possible accumulation of hydrogen peroxide was not observed in the kidney, since there was no significant difference in the GPx activity in any experimental group. Indeed, excessive Met supplementation increases plasma hydroperoxides (Hidiroglou et al. 2004). This H_2O_2 has a longer half-life, increased stability and the ability to diffuse freely through cell membranes. The decrease of antioxidant enzyme activity may contribute to the significant increase in ROS in the kidney of young rats treated with Met and/or MetO.

It is known that chronic Met supplementation induces vascular (Troen et al. 2003) and kidney damage with tubular hypertrophy (Kumagai et al. 2002). Although little is known about the effects of hypermethioninemia on the kidney, it is known that this tissue participates as a major regulator of plasma concentrations of amino acids and removing products such as urea and ammonia. Thus, high plasma concentrations of Met and MetO may increase the glomerular filtration rate progressively impairing renal function.

Finally, we evaluated the ALA-D activity in liver and kidney of young rats treated with Met and/or MetO. An increase was observed in the liver by MetO and Met plus MetO groups and in the kidney by Met plus MetO group. These results showed that the alterations in ALA-D activity are probably associated with MetO, a metabolite with potential toxicity. An enhancement of ALA-D activity has been demonstrated previously in other pathologies and was related to an increase in heme synthesis stimulated by the anemia or due to presence of chronic inflammatory processes, since the pro-inflammatory cytokine reduction of erythropoietin (Polachini et al. 2016; França et al. 2011; Zanini et al. 2014). Furthermore, Stefanello et al. (2009) demonstrated that chronic hypermethioninemia increases the number of inflammatory cells and alters the normal morphology of hepatic lobules (Stefanello et al. 2009).

Therefore, amino acids contribute significantly to the metabolic energy production and its metabolism is directly associated with liver and kidney tissues. Moreover, the high levels of Met, which are observed in hypermethioninemia, may overload these organs leading to tissue injury. An another important aspect to be discussed is that Met derivates such as homocysteine and MetO metabolites such as homocysteic acid and methionine sulfone may contribute to oxidative damage in liver and kidney observed in this study. So, there is strong evidence that Met and MetO, direct or indirectly, could lead to an imbalance in cellular redox homeostasis (Stefanello et al. 2011). In addition, studies have suggested that in vivo Met oxidation to MetO represent early posttranslational oxidative modification leading to the loss of protein function. However, elevated levels of MetO may serve as bio-markers for enhanced oxidative stress and are associated with the development of several diseases (Stadtman et al. 2003; Moskovitz 2014). Thus, studies aimed at verifying the changes present in hypermethioninemia are extremely relevant to help understand the pathophysiology of this disease and finally assist in an adequate therapeutic choice.

In conclusion, our data showed that chronic exposure to Met and/or MetO alters the cellular redox homeostasis in the liver and kidney of young rats (Fig. 7). The findings may contribute to a better understanding of the mechanisms

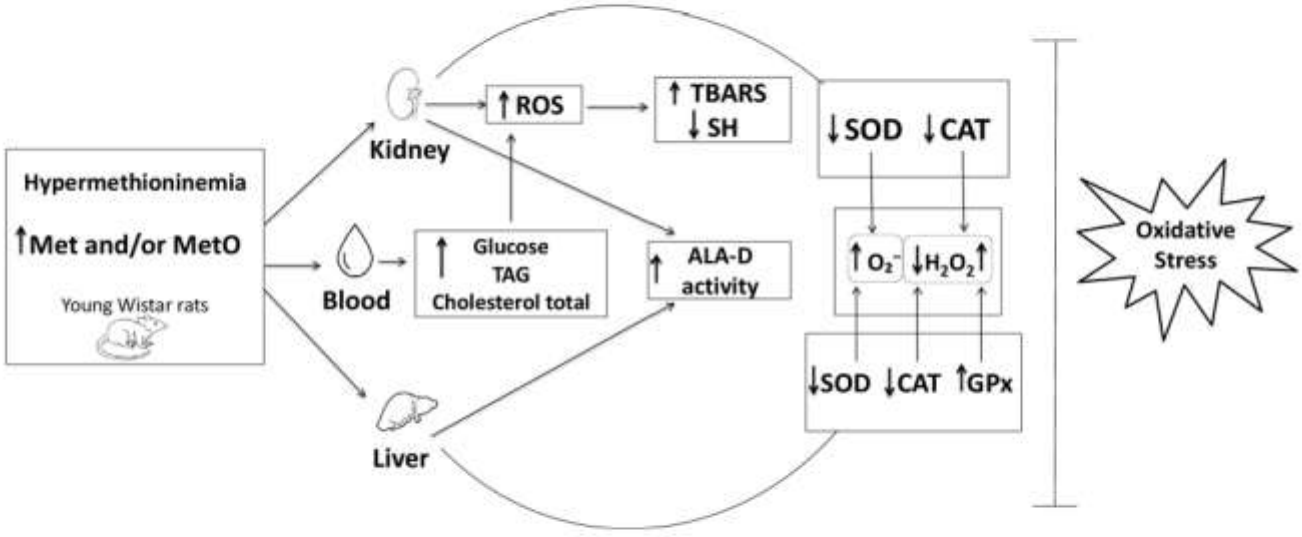

Fig. 7 Overview of the possible effects of Met and MetO administration on biochemical profile in serum and oxidative stress parameters in liver and kidney of young rats

involved in hepatic and renal complications associated with hypermethioninemia.

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Compliance with ethical standards

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

Ethical approval All animal procedures were approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, Brazil under protocol number: CEEA 3527.

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4.2 Artigo II

Acute administration of methionine and/or methionine sulfoxide impairs redox status and induces apoptosis in rat cerebral cortex

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ORIGINAL ARTICLE

Acute administration of methionine and/or methionine sulfoxide impairs redox status and induces apoptosis in rat cerebral cortex

Mayara Sandrielly Pereira Soares[1] · Cassiana Macagnan Viau[2] · Jenifer Saffi[2] · Marcelo Zanusso Costa[3] · Tatiane Morgana da Silva[3] · Pathise Souto Oliveira[3] · Juliana Hofstatter Azambuja[4] · Alethéa Gatto Barschak[4] · Elizandra Braganhol[4] · Angela T S Wyse[5] · Roselia Maria Spanevello[1] · Francieli Moro Stefanello[3]

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Abstract High plasma levels of methionine (Met) and its metabolites such as methionine sulfoxide (MetO) may occur in several genetic abnormalities. Patients with hypermethioninemia can present neurological dysfunction; however, the neurotoxicity mechanisms induced by these amino acids remain unknown. The aim of the present work was to study the effects of Met and/or MetO on oxidative stress, genotoxicity, cytotoxicity and to evaluate whether the cell death mechanism is mediated by apoptosis in the cerebral cortex of young rats. Forty-eight Wistar rats were divided into groups: saline, Met 0.4 g/Kg, MetO 0.1 g/Kg and Met 0.4 g/Kg + MetO 0.1 g/Kg, and were euthanized 1 and 3 h after subcutaneous injection. Results showed that TBARS levels were enhanced by MetO and Met+MetO 1 h and 3 h after treatment. ROS was increased at 3 h by Met, MetO and Met+MetO. SOD activity was increased in the Met group, while CAT was reduced in all experimental groups 1 h and 3 h after treatment. GPx activity was enhanced 1 h after treatment by Met, MetO and Met+MetO, however it was reduced in the same experimental groups 3 h after administration of amino acids. Caspase-3, caspase-9 and DNA damage was increased and cell viability was reduced by Met, MetO and Met+MetO at 3 h. Also, Met, MetO and Met+MetO, after 3 h, enhanced early and late apoptosis cells. Mitochondrial electrochemical potential was decreased by MetO and Met+MetO 1 h and 3 h after treatment. These findings help understand the mechanisms involved in neurotoxicity induced by hypermethioninemia.

Keywords methionine · methionine sulfoxide · oxidative stress · apoptosis · caspases · DNA damage

✉ Roselia Maria Spanevello
roselia.spanevello@ufpel.edu.br

✉ Francieli Moro Stefanello
francieli.stefanelo@ufpel.edu.br

[1] Programa de Pós-Graduação em Bioquímica e Bioprospecção - Laboratório de Neuroquímica, Inflamação e Câncer, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N CEP: 96010-900, Pelotas, RS, Brazil

[2] Laboratório de Genética Toxicológica, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil

[3] Programa de Pós-Graduação em Bioquímica e Bioprospecção - Laboratório de Biomarcadores, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N, Pelotas, RS, Brazil

[4] Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil

[5] Laboratório de Neuroproteção e Doença Metabólica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Introduction

High methionine (Met) levels have been encountered in various inherited disorders such as methionine adenosyltransferase (MAT) deficiency, in which metabolites such as methionine sulfoxide (MetO) and methanethiol can also be increased in the plasma and urine of affected patients (Gahl et al. 1988; Mudd 2011). Hypermethioninemic patients can present neurological symptoms, including mental retardation, cognitive deficit and cerebral edema; however, the precise mechanisms involved in these alterations remain poorly understood (Mudd et al. 2000, 2001).

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Despite many studies report the toxic effects of Met and its metabolites, the mechanisms behind these actions remain to be elucidated. However, studies have begun to identify some of the tissue-damage inducing actions of these compounds. In this context, we have previously demonstrated that Met induces oxidative stress, reduces brain energy metabolism and inhibits Na^+,K^+-ATPase activity in rat hippocampus *in vitro* (Stefanello et al. 2005; Streck et al. 2002, 2003). MetO is generated by Met oxidation by reactive oxygen species (ROS). This is a very important secondary pathway in hypermethioninemia (Dever and Elfarra 2008). The biological function of MetO is not clear, however it has been shown that this metabolite is associated with loss of protein function (Moskovitz 2014). In addition, studies showed that high MetO levels *in vitro* have a cytotoxic potential in mice hepatocytes (Dever and Elfarra 2008) and alter oxidative stress and inflammatory parameters in primary culture macrophages (Dos Santos et al. 2017). Costa et al. (2013) also demonstrated that acute treatment with Met and/or MetO alters lipid peroxidation, carbonyl content and ROS levels and also alters superoxide dismutase (SOD) and catalase (CAT) activities (Costa et al. 2013).

As demonstrated previously, oxidative stress, an imbalance between pro-oxidants and antioxidant, is found in *in vitro* and *in vivo* hypermethioninemia models (Stefanello et al. 2011, 2009, 2005; Costa et al. 2013; Dos Santos et al. 2017; Soares et al. 2017). An increase of ROS levels is a signal that can trigger apoptosis, a cell death process during which a cell undergoes self-destruction. This process is essential to organisms and their regulation is crucial for cell homeostasis. The accumulation of ROS can activate the intrinsic and extrinsic pathway of apoptosis, which involves mitochondrial, death receptor and endoplasmic reticulum pathways (Redza-Dutordoir and Averill-Bates 2016). These signaling pathways lead to the activation of cysteine-dependent aspartate specific proteases (caspases). Caspases have a central regulator of apoptosis, and consist of upstream initiators like caspase-9 and downstream effectors like caspase-3 (D'Amelio et al. 2010; Wurstle et al. 2012). Thus, it is important to highlight that oxidative stress and apoptosis deregulation have been associated with the pathophysiology of many pathologies such as chronic inflammation and neurodegenerative diseases.

Considering that pathophysiology of hypermethioninemia is as yet unknown and that high tissue levels of Met and MetO can be harmful, the aim of the present study was to investigate the *in vivo* effect of the acute administration of Met and MetO on some parameters of oxidative stress, DNA damage, cytotoxicity and apoptosis in cerebral cortex of young rats in hopes of clarifying the underlying mechanisms inducing neurotoxic effects of these compounds.

Materials and Methods

Drug and reagents

Methionine and methionine sulfoxide were purchased from Sigma (St. Louis, MO, USA). Primary antibodies anti-caspase 9, anti-caspase 3 and secondary antibodies anti-rabbit IgG (H+L) F(ab')2 fragment conjugated to Alexa Fluor® 488 were obtained from Cell Signaling Technology (USA) and Invitrogen (Grand Island, NY, USA), respectively. Annexin V-Phycoerythrin (PE) and 7-Amino-Actinomycin (7-AAD) were purchased from BD Bioscience (San Diego, CA). All other reagents used in the experiments were of analytical grade and the highest purity.

Animals

Wistar rats (29 days old), were obtained from the Central Animal House of the Federal University of Pelotas, Pelotas, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle at an air-conditioned constant temperature (22 ± 1°C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, Brazil under protocol number: CEEA 3527.

In vivo studies

Forty-eight Wistar rats were divided into four groups: Group I (control); Group II (treated with Met 0.4 g/kg of body weight); Group III (treated with MetO 0.1 g/kg of body weight) and Group IV (treated with Met 0.4 g/kg + MetO 0.1 g/kg of body weight). The rats received a single subcutaneous injection of Met and/ or MetO dissolved in saline. The animals in Group I received an equivalent volume of saline. The animals were euthanized 1 and 3 h after injection. The protocol and the doses of Met and MetO administered were based on previous studies performed by Stefanello et al. (2007a) and Costa et al. (2013) (Fig. 1).

Tissue and homogenate preparation

Animals were euthanized by decapitation. The cerebral cortex was dissected and homogenized in 10 volumes (1:10 w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 x g for 10 min at 4°C, the pellet was discarded and the supernatant was immediately separated and used for the measurements.

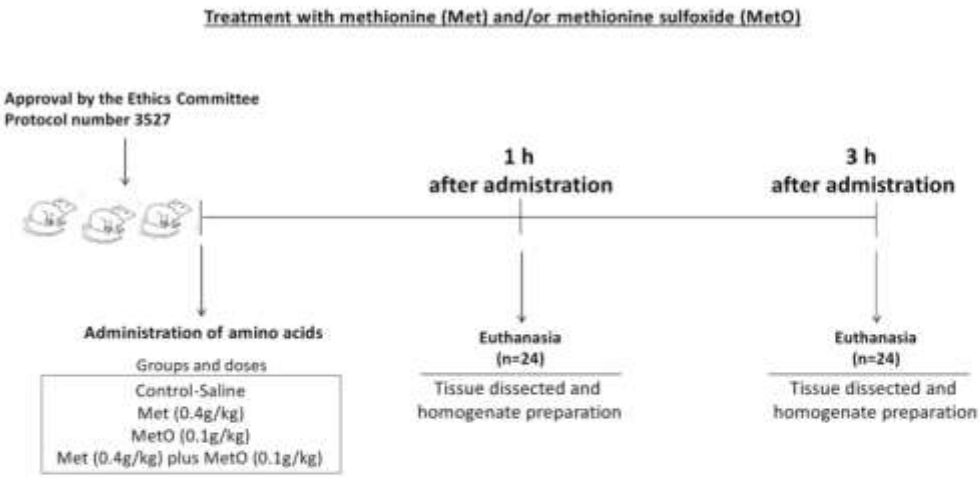

Fig. 1 Experimental acute protocol of Met and/or MetO administration

Tissue dissociation for obtaining ex vivo cell suspension

The cell suspension from cerebral cortex was incubated with Trypsin-EDTA solution 0.25% for 30 min at 37°C for tissue disintegration and isolation of the cells. After this, the tissue was allowed to settle for 5 min, and the top of suspended cells were transferred into a new tube.

Reactive oxygen species (ROS) detection by flow cytometric analysis

Levels of intracellular ROS were estimated following treatment using 2',7'-dichlorofluorescein diacetate (H_2DCFDA, Sigma) as a fluorescent probe. Detection of oxidative stress was done by incubating the cells with 20 μM of H_2DCFDA for 20 min at 37°C. Cells were then detached by trypsinization and washed twice with PBS. After filtration through a cell strainer cap, cells were analyzed using a FACS Calibur flow cytometer with CellQuest software in accordance with Bass et al. (1983). A total of 10,000 events were measured per sample. ROS production was expressed as percentage.

Thiobarbituric acid reactive substances (TBARS)

TBARS, a measure of lipid peroxidation, was determined according to Esterbauer and Cheeseman (1990). Homogenates were mixed with trichloroacetic acid 10 % and thiobarbituric acid 0.67 % and heated in a boiling water bath for 25 min. TBARS was determined by the absorbance at 535 nm. Results were reported as nmol of TBARS per mg protein.

Total sulfhydryl content

This assay was performed as described by Aksenov and Markesbery (2001), which is based on the reduction of DTNB by thiols and in turn becomes oxidized (disulfide) generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. Briefly, homogenates were added to PBS buffer pH 7.4 containing EDTA. The reaction was started by the addition of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Results were reported as nmol TNB/mg protein.

Carbonyl assay

Protein carbonyl was assayed by the method of Reznick and Packer (1994), which is based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm. Results were reported as nmol carbonyl/mg protein.

Superoxide dismutase (SOD) assay

SOD activity was measured by the method described by Misra and Fridovich (1972). This method is based on the inhibition of superoxide dependent adrenaline auto-oxidation in a spectrophotometer adjusted at 480 nm. The specific activity of SOD was reported as units per mg of protein.

Catalase (CAT) assay

CAT activity was assayed by the method of Aebi (1984). H_2O_2 disappearance was continuously monitored with a spectrophotometer as 240 nm for 90 s. One unit of the enzyme is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity was reported as units per mg protein.

Glutathione peroxidase (GPx) assay

GPx activity was measured using a commercial kit (RANSEL®; Randox Lab, Antrim, United Kingdom). GPx

catalyzes glutathione (GSH) oxidation by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340nm was measured. The specific activity of GPx was reported as units per mg of protein.

Alkaline Comet assay

The alkaline comet assay was performed as previously described (Singh et al. 1988). Briefly, 10 μL of cells were mixed with 90 μL of LMP agarose, spread on a normal agarose precoated microscope slide, and placed at 4°C for 5 min to allow for solidification. Cells were lysed in a high concentration of salt and detergent (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100 and 10% DMSO freshly added) for 2 h. Slides were removed from lysing solution and washed three times with PBS. Subsequently, cells were exposed to alkali conditions (300 mM NaOH/1 mM Na_2EDTA, pH >13, 30 min, 4°C) to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 25 min at 25 V and 300 mA (94 V/cm). After electrophoresis, the slides were neutralized and silver stained (Nadin et al., 2001). One hundred cells were scored visually according to the tail length and the amount of DNA present in the tail. Each comet was given an arbitrary value of 0–4 (0, undamaged; 4, maximally damaged), as described by Collins et al. (Collins et al. 1995). A damage score was thus assigned to each sample and can range from 0 (completely undamaged: 100 cells X 0) to 400 (with maximum damage: 100 cells X 4). International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it is highly correlated with computer-based image analysis (Burlinson et al. 2007; Collins et al. 1995; Nadin et al. 2001).

Cell viability assay

Relative cell viability determined by trypan blue dye-exclusion assay (TBDE) was employed as a cytotoxic measurement (Da Silveira et al. 2015). Trypan blue staining is a long-standing and widely used method to identify dead cells. Only cells with intact membranes can effectively exclude the dye, hence dead cells with compromised membranes become stained. For each group, homogenates were mixed with 0.4% trypan-blue solution which was then added. Cytotoxicity (the cellular growth inhibitory rate) was determined from the number of viable cells (no color) in treated samples as a percentage of the PBS control. We used the Countess® Automated Cell Counter (Invitrogen). The test was carried out according to the instructions of the manufacturer.

Assessment of apoptosis by flow cytometric analysis

Annexin V-PE was used in conjunction with a vital dye, 7-AAD, to distinguish apoptotic (Annexin V-PE positive, 7-AAD negative) from necrotic (Annexin V-PE positive, 7-AAD positive) cells. After treatment, cells were collected and resuspended in 40 μL of binding buffer with 2 μL Annexin V-PE. Cells were incubated for 15 min in the dark at room temperature. After incubation, 160 μL of binding buffer and 2 μL of 7-AAD were added. Cells were incubated for 5 min and an additional 200 μL of binding buffer were added. Before analysis, cells were filtered through a cell strainer cap fitted to a polystyrene round bottom flow cytometric tube. Data were collected and analyzed by a FACS Calibur flow cytometer with CellQuest software, in a total of 10,000 events per sample; fluorescence was measured and the percentage of viable, early apoptotic, late apoptotic and necrotic cells was determined (Viau et al. 2014)

Quantification of cleaved caspase 9 and cleaved caspase 3 by flow cytometric analysis

After treatment, cells were harvested and re-suspended in 25 μL PBS and fixed with 4% formaldehyde. After permeabilization and blocking (0.2% Triton X-100 in PBS and 1% BSA), cells were incubated with the primary antibodies diluted 1:1000 for 1 h at room temperature followed by incubation with anti-rabbit FITC secondary antibody at 1:1000 for 1 h at room temperature in the dark. Fluorescence intensity in arbitrary units was plotted in histograms, and the mean fluorescence intensity was calculated using CellQuest software.

Assay of the electrochemical potential

After treatment cells were incubated with 2 mM Rh123 (rhodamine 123) for 30 minutes at 37° C, washed, and then resuspended in 100 μL PBS. Mitochondrial electrochemical potential is correlated with the fluorescence intensity of Rho123 (with decreased fluorescence signifying loss of the mitochondrial electrochemical potential) (Emadi et al. 2010). Flow cytometry was performed using a FACS Calibur (Becton Dickinson, San Jose, California, United States) with excitation at 488 nm and emission read using a 525–550 nm filter (FL1).

Protein determination

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

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using the Tukey test, with $P \leq 0.05$ considered statistically significant using GraphPad Prism version 5.0 Program. All data were expressed as mean ± standard error (SEM).

Results

Initially, we investigated the effect of acute exposure of Met and/or MetO on some parameters of oxidative stress in young rat cerebral cortex. As shown in Table 1, Met, MetO and the association between these compounds significantly increased the ROS production at 3 h after administration [$F_{(3,16)} = 29.86$, $P<0.01$], but not at 1 h [$P>0.05$]. TBARS levels were enhanced by MetO and Met+MetO 1 h [$F_{(3,12)} = 6.97$, $P<0.05$] and 3 h [$F_{(3,13)} = 26.71$, $P<0.01$] after administration. No change was observed in sulfhydryl and protein carbonyl content in Met, MetO and Met+MetO groups compared to control group 1 and 3 h after treatment [$P>0.05$].

As regard antioxidant enzymes (Fig. 2), Met at 1 h increased SOD activity [$F_{(3,19)} = 15.73$, $P<0.001$], but not at 3 h [$P>0.05$]. MetO and Met+MetO also did not alter this enzyme activity [$P>0.05$]. CAT activity was significantly reduced by Met, MetO and Met plus MetO at 1 h [$F_{(3,17)} = 11.37$, $P<0.01$] and at 3 h [$F_{(3,18)} = 7.97$, $P<0.01$] after the injection of these amino acids (Fig. 2). In addition, GPx activity was increased by Met, MetO and Met+MetO 1 h after treatment [$F_{(3,14)} = 9.23$, $P<0.05$], but 3 h after administration with Met, MetO and Met+MetO the GPx activity was reduced compared with the control group [$F_{(3,12)} = 12.90$, $P<0.05$] (Fig. 2). In an attempt to assess the DNA damage, we performed an alkaline comet assay. Figure 3 shows that Met, MetO and Met+MetO significantly increased DNA damage at 3 h [$F_{(3,16)} = 12.69$, $P<0.05$], but not at 1 h [$P>0.05$] (Fig. 3).

Furthermore, cell viability was significantly reduced 3 h after by Met, MetO and Met+MetO [$F_{(3,16)} = 37.19$, $P<0.05$], but did not change at 1 h [$P>0.05$] (Fig. 4). The annexin V-PE/7-AAD combination allows quantifying cells in the process of apoptosis and necrosis in the same cell population through flow cytometry. Based on this, no changes were observed in Met, MetO and Met+MetO groups 1 h after administration when compared to the control group [$P>0.05$]. However, it was found that Met, MetO and Met + MetO 3 h after administration increased annexin V-PE and 7-AAD staining. We observed an increase in early apoptosis cells in Met, MetO and Met+MetO [$F_{(3,8)} = 5450$, $P<0.05$]. Also cells in late apoptosis were enhanced in Met, MetO and Met+MetO groups when compared with control group [$F_{(3,8)} = 156.6$, $P<0.05$]. No changes were observed in necrotic cell [$P>0.05$]. A remarkable decrease was found in the viable cells population in Met, MetO and Met+MetO when compared with control group [$F_{(3,8)} = 458.9$, $P<0.05$].

In order to delineate the cytotoxicity mechanism after acute treatment, we monitored the caspase-3 and caspase-9 activities (Fig. 5). There was no change in Caspase-3 at 1 h [$P>0.05$]. After 1 h of treatment only caspase-9 was increased by Met+MetO [$F_{(3,16)} = 6.65$, $P<0.01$] (Fig. 5). However, we observed a significant increase in caspase-3 [$F_{(3,15)} = 44.54$, $P<0.001$] and caspase-9 [$F_{(3,14)} = 48.91$, $P<0.01$] activities after 3 h of administration of Met, MetO and Met+MetO (Fig. 5). We also observed a decrease of mitochondrial electrochemical potential by MetO and Met+MetO 1 h [$F_{(3,16)} = 5.38$, $P<0.05$] and 3 h [$F_{(3,13)} = 23.34$, $P<0.05$] after treatment (Fig. 6). Met does not alter the mitochondrial electrochemical potential [$P>0.05$] (Fig. 6).

Table 1 Thiobarbituric acid reactive substances (TBARS), total thiol content, carbonyl content and reactive oxygen species (ROS) production in the cerebral cortex of young rats 1 h and 3 h after treatment with methionine (Met) and/or methionine sulfoxide (MetO)

Groups	TBARS	Total thiol content	Carbonyl content	ROS production
	1 h			
Control	1.45 ± 0.05	76.18 ± 2.10	3.00 ± 0.11	87.08 ± 4.47
Met	1.54 ± 0.08	84.60 ± 3.94	2.79 ± 0.33	95.64 ± 4.01
MetO	1.87 ± 0.03*	80.78 ± 3.28	2.48 ± 0.11	97.22 ± 3.09
Met+MetO	1.96 ± 0.15*	79.62 ± 3.46	2.47 ± 0.17	99.67 ± 2.74
	3 h			
Control	1.69 ± 0.06	76.18 ± 2.10	3.00 ± 0.11	77.51 ± 4.00
Met	1.59 ± 0.10	81.61 ± 4.78	2.68 ± 0.18	112.31 ± 4.24**
MetO	2.61 ± 0.10***	75.11 ± 3.30	2.40 ± 0.04	130.95 ± 6.69***
Met+MetO	2.22 ± 0.06**	85.47 ± 2.44	2.41 ± 0.12	151.65 ± 5.00***

Data are expressed as mean ± S.E.M. TBARS levels were reported as nmol TBARS per mg protein, thiol content as nmol TNB per mg protein, carbonyl content as nmol of carbonyl per mg protein and ROS production as percentage. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, compared to control (n=4-7)

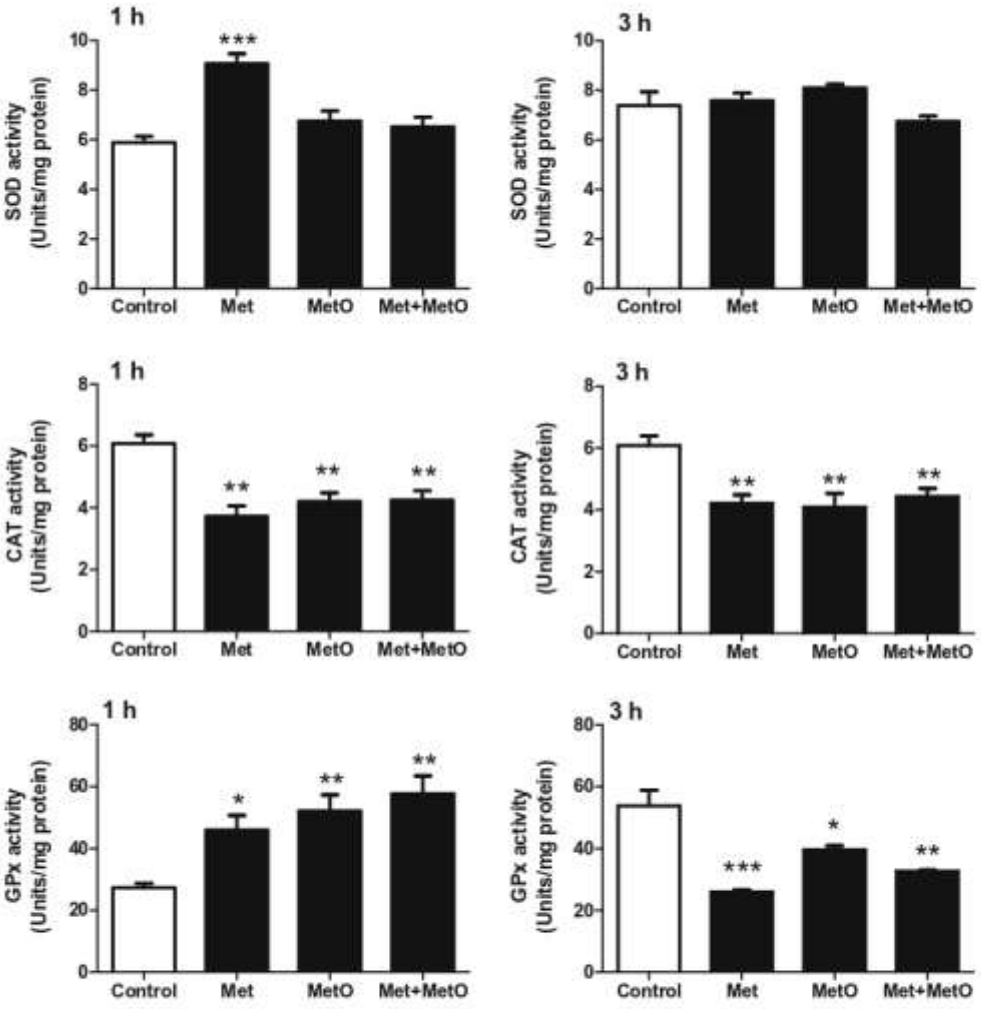

Fig. 2 *In vivo* acute effects of methionine (Met) and/or methionine sulfoxide (MetO) on catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in the cerebral cortex of young rats. CAT, SOD and GPx activities were reported as units per mg protein. Data are reported as mean ± S.E.M. **P*<0.05; ***P*<0.01; ****P*<0.001, compared to control group. (n=5-6)

Discussion

Hypermethioninemic patients exhibit various degrees of neurological symptoms, whose pathomechanisms are poorly understood. Therefore, this study aimed to evaluate the effect of acute administration of Met and its metabolite MetO, on some parameters of oxidative stress, DNA damage, cytotoxicity and apoptosis in the cerebral cortex of young rats. To our knowledge, this study is the first to investigate the contribution of MetO and the association between Met and MetO in brain alterations found in hypermethioninemia. The doses of Met and MetO administered were based on studies by Stefanello et al. (2007b), Costa et al. (2013) and Dos Santos et al. (2016). It is important to note that the plasma levels of Met and MetO observed in rats are similar to those found in hypermethioninemic patients (Stefanello et al. 2007a; Costa et al. 2013; Dos Santos et al. 2016).

Several studies by our research group have demonstrated that *in vitro* and *in vivo* hypermethioninemia can change oxidative stress parameters in the hippocampus (Stefanello et al. 2005), kidney (Soares et al. 2017), liver (Stefanello et al. 2011; Costa et al. 2013; Soares et al. 2017), macrophages (Dos Santos et al. 2017) and gastrocnemius skeletal muscle (Schweinberger et al. 2015). Moreover, Met restriction has shown positive effects in relation to the oxidative stress such as decreased oxidative damage and mitochondrial ROS production at complex I increasing the longevity of rodents (Sanchez-Roman and Barja 2013) and lifespan extension of fibroblasts followed by a significant decrease in the levels of subunits of mitochondrial complex IV and I (Ables and Hens 2016). The beneficial effects of Met restriction could be explained in part by its ability to reduce mitochondrial oxidative stress (Ables and Hens 2016).

Met participates in an endogenous system with local antioxidant capacity, and therefore an increase in MetO levels can lead to an imbalance in this oxidation / reduction cycle constituted by Met. In view of the intrinsic relationship between the Met metabolism and ROS production and consequent oxidative stress and the neurological effects caused by this imbalance in the redox status, it

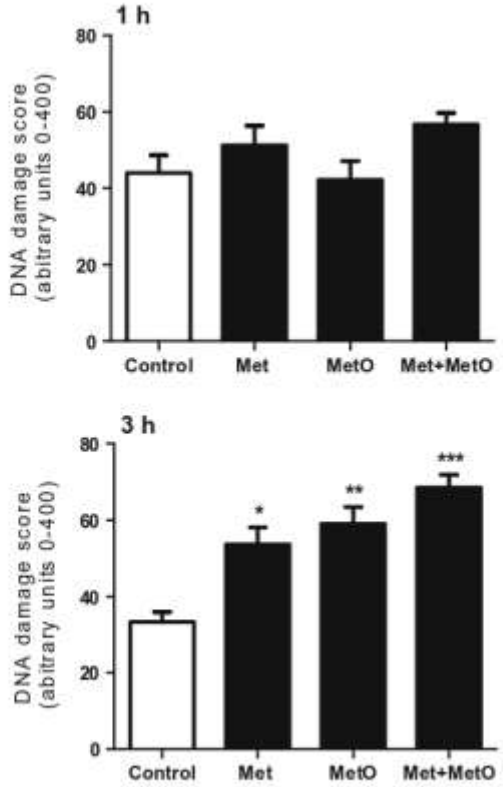

Fig. 3 *In vivo* acute effects of methionine (Met) and/or methionine sulfoxide (MetO) on DNA damage. DNA damage as arbitrary units 0-400. Data are reported as mean ± S.E.M. *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared to control group (n=4-6)

is relevant to investigate the pathological effects of hypermethioninemia on oxidative stress in cerebral structures. Initially, we assessed the state of oxidative damage markers as TBARS, total thiol and carbonyl content, as well as ROS in the cerebral cortex 1 h and 3 h after treatment with Met and/or MetO. TBARS levels are broadly used for malondialdehyde determination as index of lipid peroxidation (Esterbauer and Cheeseman 1990). We observed that MetO and Met+MetO lead to enhancement of TBARS levels at 1 h and 3 h. This result can be explained, at least in part, by an increase in ROS levels by Met and MetO at 3 h. Moreover, the increase in TBARS levels is indicative of damage to the lipids of plasmatic membrane in cerebral cortex, which could compromise the integrity, functionality and viability of cells. In fact, it was previously described that chronic administration of Met reduced gangliosides, phospholipids and cholesterol content in cerebral cortex (Stefanello et al. 2007b). No changes were observed in total thiol content and carbonyl content, which were major elements involved in causing protein damage (Reznick and Packer 1994; Aksenov and Markesbery 2001).

We then investigated the antioxidant enzyme activity (SOD, CAT and GPx) in cerebral cortex of young rats 1 h and 3 h after treatment with Met and/or MetO. The cascade made up by these enzymes is a major defense mechanism against the production and/or accumulation of ROS. The SOD enzyme removes superoxide radical (O_2•-) by accelerating its conversion to hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge 2007; Halliwell 2011). We observed that Met at 1 h increased SOD activity, although we have not found an increase in ROS levels 1 h after Met administration. Other studies showed similar results and suggested that this enhancement can be a metabolic adaptation to combat an increase of ROS (Viggiano et al. 2012). The H_2O_2 formed by SOD reaction, can be converted by CAT or GPx activity (Halliwell and Gutteridge 2007; Halliwell 2011). CAT activity was decreased by Met, MetO and Met+MetO 1 h and 3 h after treatment. In addition, GPx activity

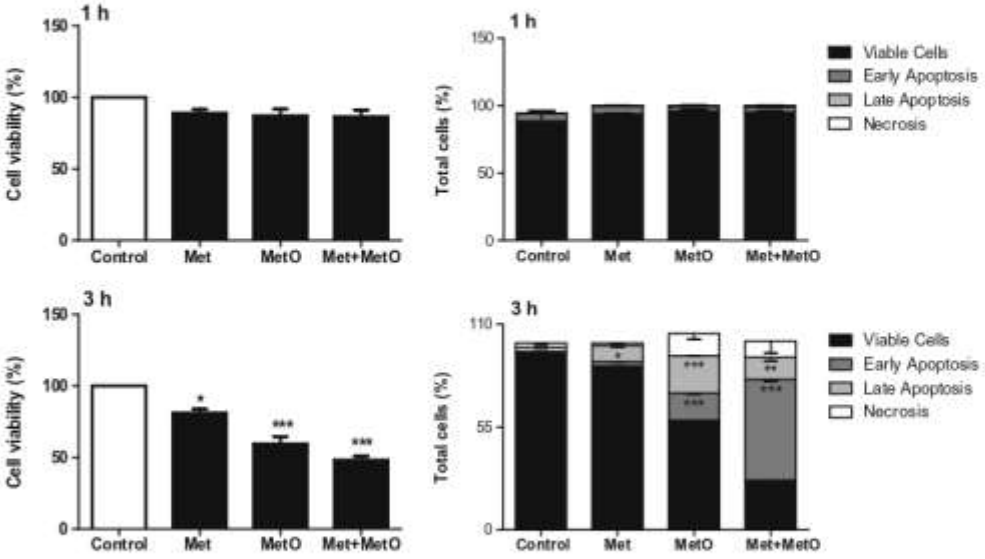

Fig. 4 *In vivo* acute effects of methionine (Met) and/or methionine sulfoxide (MetO) on cell viability and Annexin V assays in cerebral cortex of young rats .Cell viability is express as percentage of control and the sum of the percentages of Annexin V and 7-AAD-PE-positive cells was calculated. Data are reported as mean ± S.E.M (n=3-6). *$P<0.05$; **$P<0.01$, compared to control group

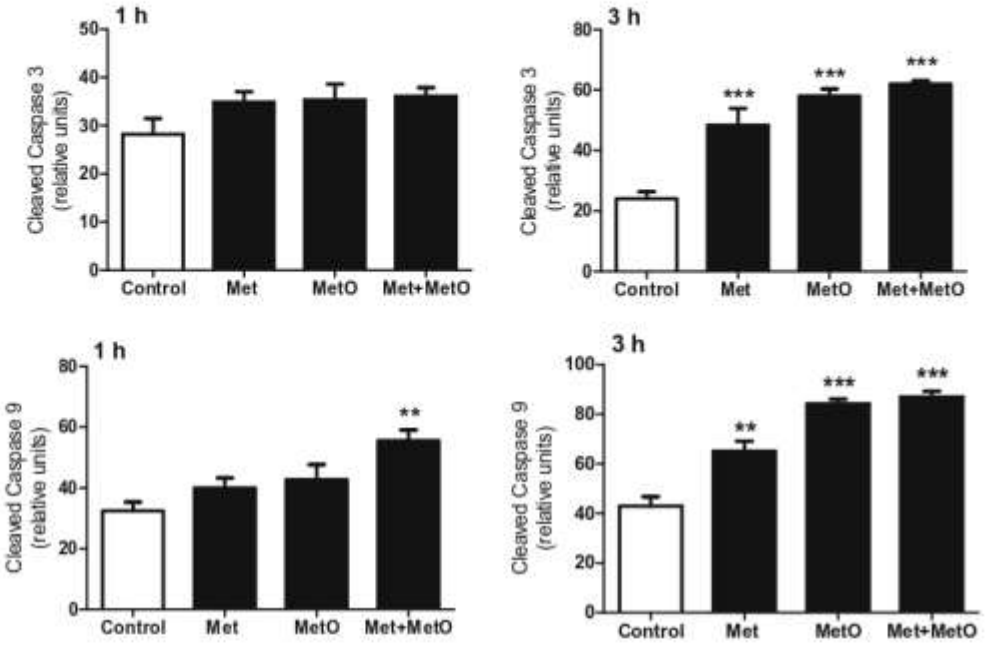

Fig. 5 *In vivo* acute effects of methionine (Met) and/or methionine sulfoxide (MetO) on cleaved caspase-3 and caspase-9 activities in the cerebral cortex of rats. Cleaved caspase-3 and caspase-9 activities were assessed by monitoring the change in fluorescence intensity. Data are mean ± S.E.M **P<0.01; ***P<0.001, compared to control group (n=4-6)

was increased at 1 h by Met, MetO and Met+MetO which may justify normal levels of ROS in those experimental groups. However, 3 h after administration of Met, MetO and Met+MetO there was an inhibition of GPx activity. The decrease of CAT and GPx activities at 3 h leads to accumulation of H_2O_2 which can be confirmed through the major ROS levels observed in the same experimental groups. Although we cannot explain precise mechanisms by which high doses of Met and/or MetO inhibit the CAT and GPx activities, it can be concluded that the reduction in the activity of these enzymes is strongly positively associated with the accumulation of ROS, especially H_2O_2, leading to lipid peroxidation observed by high levels of TBARS.

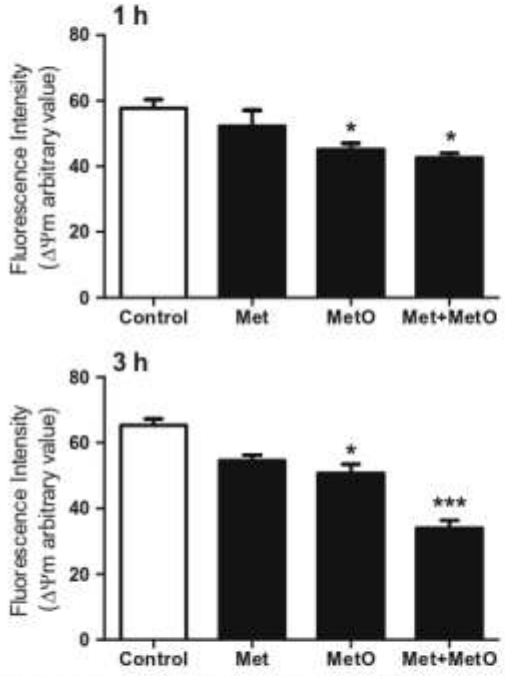

Fig. 6 *In vivo* acute effects of methionine (Met) and/or methionine sulfoxide (MetO) decrease of mitochondrial electrochemical potential, with decreased fluorescence signifying loss of the mitochondrial electrochemical potential. Mean data for Rho-123 fluorescence. Data are mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001, compared to control group (n=4-6)

Besides causing cellular injury, oxidative stress is a mechanism modulator of several crucial body processes, including metabolism, growth, differentiation and cell death, acting this way in all physiological or pathological procedural stages (Halliwell and Gutteridge 2007). Therefore, we investigated whether the imbalance between the levels of ROS and enzymatic antioxidant defenses would compromise cell viability, since it is already well reported in the literature that oxidative stress might lead to cell and tissue damage by lipid peroxidation, proteins and DNA damage. When we analyze cell viability, the results indicated a reduction in cell survivability by Met, MetO and Met+MetO at 3 h after treatment.

We also observed an increase in the cells of cerebral cortex in the apoptosis process along with an increase of caspase-9 and caspase-3 3 h after treatment with Met and/or MetO. The caspase-dependent cell death machinery is crucial to maintain cellular homeostasis and this system is mediated mainly by caspase activity (D'Amelio et al. 2010). Caspase-3 is activated by a death receptor or a mitochondria-mediated pathway (Mazarakis et al. 1997), while caspase-9 contains a caspase activation recruitment domain (CARD), which is essential for initiator caspase activation (Wurstle et al. 2012). Met and/or MetO at 3 h were capable of activating the caspase 3 and caspase-9. This result suggests a possible involvement of an

apoptotic mitochondrial pathway since activated caspase-9 is found after the release of proteins located in the mitochondrial intermembranous space. In the cytosol, caspase-9 is cleaved and participates in the apoptosome composition, which ultimately mediates the cleavage of caspase-3 responsible for DNA cleavage (Wurstle et al. 2012; Redza-Dutordoir and Averill-Bates 2016).

Baydas et al. (2005) also showed that a high-methionine (HM) diet activated caspase-3 and caspase-9 and this result is associated with the release of cytochrome c from mitochondria to the cytosol and fragmentation of DNA in neuronal cells of rats (Baydas et al. 2005). The mitochondrial outer membrane permeabilization occurs in the intrinsic pathway and is carried out by the Bcl-2 family. Bcl-2 is a protein which is divided into pro- (Bax, Bak) and antiapoptotic (Bcl-2, Bcl-XL) (Redza-Dutordoir and Averill-Bates 2016). Baydas et al. (2005) also reported that the HM diet increases proapoptotic Bax protein levels and decreases in antiapoptotic Bcl-2 levels in neuronal cells of rats (Baydas et al. 2005). Yet, Dever and Elfarra (2008) demonstrated that methionine-dl-sulfoxide decreases the cell viability of isolated mouse hepatocytes (Dever and Elfarra 2008). We also analyzed the DNA integrity and observed a significant increase in genotoxic effects 3 h after treatment. Met, MetO and Met+MetO groups present elevated DNA damage in cerebral cortex. These results support the hypothesis that increased ROS levels and decreased antioxidant defenses lead to damage in the DNA and a consequent apoptotic process.

To complement the ROS training, we evaluated mitochondrial membrane potential (MMP). Our data showed a reduction of MMP in MetO and Met+MetO 1 h and 3 h after treatment, which supports the idea that there is an increased intracellular production of ROS. The integrity of MMP is directly associated with energy production and is essential for the physiological function of the respiratory chain to generate ATP (Chen 1988). A dysfunction in MMP can lead to energy depletion with subsequent cell death. It is important to point out that the alterations observed in MMP seem to be related to the presence of the MetO metabolite.

It is important to note that the responses obtained in this work appear to be accentuated in the presence of the MetO metabolite. This suggests that the differences observed between the treated groups in some parameters are possibly associated with the metabolites formed from the oxidation of MetO, such as methionine sulfone and homocysteic acid. In addition, a point that should be considered is that during the Met metabolism, the first metabolite formed is S-adenosylmethionine (SAM), which is described to suppress oxidative stress in pathological conditions (Ghorbani et al. 2016; Stiuso et al. 2016; Yoon et al. 2016). Thus, we could speculate that the SAM formed attenuates the oxidative process in the Met group by decreasing the toxicity induced by this amino acid.

Some studies have already shown that oxidative stress may be associated with the long-term exposure of Met. However, few studies showed a possible relationship between oxidative stress with lower neuronal cell viability and the neurological dysfunction in the presence of high Met and MetO levels and the association between them as found in hypermethioninemia.

In conclusion, our results demonstrated that acute administration of Met and/or MetO induces oxidative damage, reduces cell viability and induces cellular death by apoptosis in the cerebral cortex of young rats (Fig. 7). These data help to understand the possible neurological pathophysiological changes present in hypermethioninemia, and may help perform the diagnosis and choosing the appropriate therapy.

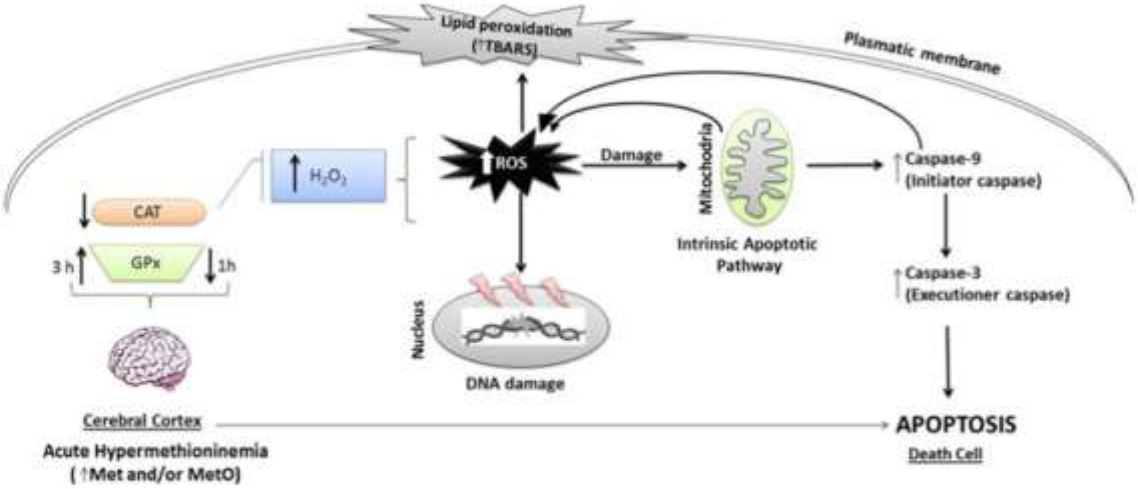

Fig. 7 Neurochemical changes found in young rats submitted to acute hypermethioninemia. Met - Methionine, MetO - Methionine sulfoxide, SOD - Superoxide dismutase, CAT - Catalase, GPx - Glutathione peroxidase, $O_2^{\bullet-}$ - Superoxide radical, H_2O_2 - Hydrogen peroxide, ROS - Reactive oxygen species, TBARS - Thiobarbituric acid reactive substances

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Compliance with ethical standards

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

Ethical approval All animal procedures were approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, Brazil under protocol number: CEEA 3527.

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4.3 Artigo III

High levels of methionine and methionine sulfoxide: impact on adenine nucleotide hydrolysis and redox status in platelets and serum of young rats

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RESEARCH ARTICLE

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High levels of methionine and methionine sulfoxide: Impact on adenine nucleotide hydrolysis and redox status in platelets and serum of young rats

Mayara Sandrielly Pereira Soares[1] | Bruna da Silveira de Mattos[1] | Anita Almeida Ávila[1] | Luiza Spohr[1] | Nathalia Stark Pedra[1] | Fernanda Cardoso Teixeira[1] | Natália Pontes Bona[2] | Pathise Souto Oliveira[2] | Francieli Moro Stefanello[2] | Roselia Maria Spanevello[1]

[1]Programa de Pós-Graduação em Bioquímica e Bioprospecção - Laboratório de Neuroquímica, Inflamação e Câncer, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, Brazil

[2]Programa de Pós-Graduação em Bioquímica e Bioprospecção - Laboratório de Biomarcadores, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, Brazil

Correspondence
Francieli Moro Stefanello and Roselia Maria Spanevello, Universidade Federal de Pelotas, Campus Universitário s/n, Capão do Leão, Pelotas, RS 96010-900, Brazil.
Email: rspanevello@gmail.com; francieli.stefanelo@ufpel.edu.br

Abstract

We investigated acute and chronic effects administration of methionine (Met) and/or methionine sulfoxide (MetO) on ectonucleotidases and oxidative stress in platelets and serum of young rats. Wistar rats were divided into four groups: control, Met, MetO, and Met + MetO. In acute treatment, the animals received a single subcutaneous injection of amino acid(s) and were euthanized after 1 and 3 hours. In chronic protocol, Met and/or MetO were administered twice a day with an 8-hour interval from the 6th to the 28th day of life. Nucleoside triphosphate phosphohydrolase and 5′-nucleotidase activities were reduced in platelets and serum by Met, MetO, and Met + MetO after 3 hours and 21 days. Adenosine deaminase activity reduced in platelets at 3 hours after MetO and Met + MetO administration and increased after 21 days in animals treated with Met + MetO. Superoxide dismutase and catalase activities decreased in platelets in MetO and Met + MetO groups after 3 hours, while reactive oxygen species (ROS) levels increased in same groups. Catalase activity in platelets decreased in all experimental groups after chronic treatment. Met, MetO, and Met + MetO administration increased plasmatic ROS levels in acute and chronic protocols; glutathione *S*-transferase activity increased by MetO and Met + MetO administration at 3 hours, and ascorbic acid decreased in all experimental groups in acute and chronic protocols. Thiobarbituric acid reactive substances increased, superoxide dismutase and catalase activities reduced in the Met and/or MetO groups at 3 hours and in chronic treatment. Our data demonstrated that Met and/or MetO induced changes in adenine nucleotide hydrolysis and redox status of platelets and serum, which can be associated with platelet dysfunction in hypermethioninemia.

KEYWORDS

ectonucleotidases, hypermethioninemia, methionine, methionine sulfoxide, oxidative stress, platelets

1 | INTRODUCTION

Methionine (Met) is an essential sulfur-containing amino acid that plays a key role in the maintenance of several functions in an organism. However, a condition of excessive Met, known as hypermethioninemia, can be toxic.[1] To date, six genetic alterations have been reported that may lead to hypermethioninemia, but methionine adenosyltranferase I/III (MATI/III) deficiency, which is due to mutations in the *MAT1A* gene, is the most frequent cause found in patients with isolated and persistent hypermethioninemia.[2,3] At least 37 different *MAT1A* mutations have been reported; however, the worldwide incidence rate of this disease has not yet been described. In the Iberian Peninsula, the incidence rate of MATI/III deficiency was 1 of 27 000 newborns.[4] Clinical manifestations of hypermethioninemia include neurological and liver dysfunctions.[5] However, the pathophysiological mechanisms involved in the origin and maintenance of these alterations are not elucidated in detail.

Previous studies have demonstrated that high levels of Met and/ or methionine sulfoxide (MetO) are associated with hepatic,[6,7] renal,[7] skeletal,[8] and neurological dysfunctions.[9] Oxidative stress,[6,7,9] inflammation,[10] brain enzymes changes,[11] decrease in neurons number,[11] and cell death by apoptosis[9] has been described as possible mechanisms involved in hypermethioninemia complications. However, little is known about the association of this pathological condition with platelet function.

Platelets are an important component for normal functioning of the vascular system because they participate in the maintenance of vascular integrity via adhesion, aggregation, and subsequent thrombus formation.[12] In addition, studies have also discussed the critical role of platelets in inflammatory and immune responses. Altered platelet function can lead to several pathological conditions[12]; thus, better understanding of the mechanisms underlying the role of platelets in thromboregulation and inflammation may result in new therapeutic strategies for many diseases, such as hypermethioninemia.

Platelets are stimulated by a variety of agonists, including adenosine diphosphate (ADP). Extracellular adenosine triphosphate (ATP), adenosine monophosphate (AMP), and adenosine also are able to modulate several effects on the vascular system by interacting with specific receptors in platelets.[13,14] Platelet membrane expresses a cascade of ectoenzymes composed of nucleoside triphosphate phosphohydrolase (NTPDases) that convert ATP and ADP to AMP via 5′-nucleotidase, which hydrolyzes AMP to adenosine (Ado) and adenosine deaminase (ADA), which is responsible for Ado irreversible deamination.[15] These enzymes play an important role in thromboregulation, and altered activities of these enzymes have been observed in various diseases.[14] We showed that the activities of these ectoenzymes are altered in the lymphocytes of rats treated with Met and MetO,[10] suggesting that purinergic signaling dysfunction may be involved with the pathogenesis of hypermethioninemia.

In addition, several studies have demonstrated that platelets can produce reactive oxygen species (ROS), which also affect the mechanism of platelet activation and aggregation,[12,14,16] An imbalance between ROS levels and antioxidant defense system, composed of enzymatic and nonenzymatic pathways, is known as oxidative stress.[12] This condition is observed in hepatic,[6,7] renal,[7] brain,[9] and skeletal muscle[8] dysfunctions in animal models of hypermethioninemia.

However, considering that the involvement of platelet alteration in the pathogenesis of hypermethioninemia is still poorly understood, the aim of the current study was to evaluate the acute and chronic effects of Met and/or MetO on purinergic enzymes and parameters of oxidative stress in platelets and serum of young Wistar rats.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Met, MetO, nucleotides, Trizma base, adenosine, dichlorodihydro-fluorescein diacetate (DCFH-DA), epinephrine, thiobarbituric acid (TBA), and 5.50-dithiobis (2-nitrobenzoic acid [DTNB]) were purchased from Sigma Chemical Co (St Louis, MO). Trichloroacetic acid (TCA) and hydrogen peroxide (H_2O_2) were purchased from Synth (São Paulo, Brazil). All other reagents used in the experiments were of analytical grade and highest purity.

2.2 | Animals and ethical aspects

Wistar rats used in this study were obtained from the Central Animal House of the Federal University of Pelotas, Pelotas, RS, Brazil. The animals were maintained at a constant temperature (22 ± 1°C) with a 12/12 hours light/dark cycle with ad libitum access to food and water. All animal procedures were approved by the Ethics Committee of Animal Experimentation from the Federal University of Pelotas, Brazil (protocol under number: CEEA 3527). The use of the animals was in accordance with the Brazilian Guidelines for the Care and Use of Animals in Scientific Research Activities and with the National Council of Control of Animal Experimentation.

2.3 | Acute protocol

Forty 29-day-old rats weighing 90 to 110 g were divided in four groups (n = 10): group I (control), group II (treated with Met 0.4 g/kg), group III (treated with MetO 0.1 g/kg), and

group IV (treated with Met 0.4 g/kg + MetO 0.1 g/kg). A single subcutaneous injection of Met and/or MetO was administered, and the control rats received an equivalent volume of saline. Five animals from each group were anesthetized with isoflurane and euthanized by cardiac puncture 1 hour after injection, and the remaining five animals were euthanized 3 hours after the amino acid treatment.[6,10,17] Blood was collected by cardiac puncture (Figure 1A).

2.4 | Chronic protocol

Forty 6-day-old rats weighing 10 to 15 g were divided into four groups (n = 10): group I (control), group II (treated with Met), group III (treated with MetO), and group IV (treated with Met + MetO). Met and MetO doses administered were chosen to induce high plasma levels similar to those found in patients affected by hypermethioninemia as described by previous studies.[6,10,17] Met and MetO were administered to rats subcutaneously twice a day with an interval of 8 hours between injections from day 6 to 28, as described previously by Stefanello et al.[17] Animals of groups II and III received 0.2 g/kg of Met or 0.05 g/kg of MetO during the first 8 days of treatment, 0.3 g/kg of Met or 0.075 g/kg of MetO from day 15 to 21, and 0.4 g/kg of Met or 0.1 g/kg of MetO from days 22 to 28. The animals of group IV received a combination of Met and MetO, whereas control rats (group I) received saline solution in the same volumes. After the treatment time, the animals were anesthetized with isoflurane, and blood was collected by cardiac puncture (Figure 1B).

2.5 | Platelet preparation

For platelet-rich plasma (PRP) preparation, total blood was collected with 0.120M sodium citrate as the anticoagulant as previously described by Lunkes et al.[18] The total blood was centrifuged at 160g for 10 minutes at room temperature. Next, PRP was centrifuged at 1400g for 30 minutes and washed twice with 3.5mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.0; PRP was then resuspended in 200 μL of HEPES buffer for further analysis.

2.6 | Serum preparation

Blood samples were collected in tubes without the anticoagulant and centrifuged at 2500g for 15 minutes at room temperature. The clot was removed, and the resulting serum was stored at −80°C and used for biochemical determinations.

2.7 | NTPDase, 5′-nucleotidase, and adenosine deaminase assays in platelets

NTPDase and 5′-nucleotidase enzymatic activities were performed as described by Pilla et al[19] and Lunkes et al.[18] For the NTPDase enzymatic assay, samples (20 μL) were preincubated for 10 minutes at 37°C in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 5 mM KCl, 6 mM glucose, and 50 mM Tris-HCl buffer, pH 7.4, at a final volume of 200 μL. For 5′-nucleotidase activity, 5 mM $CaCl_2$

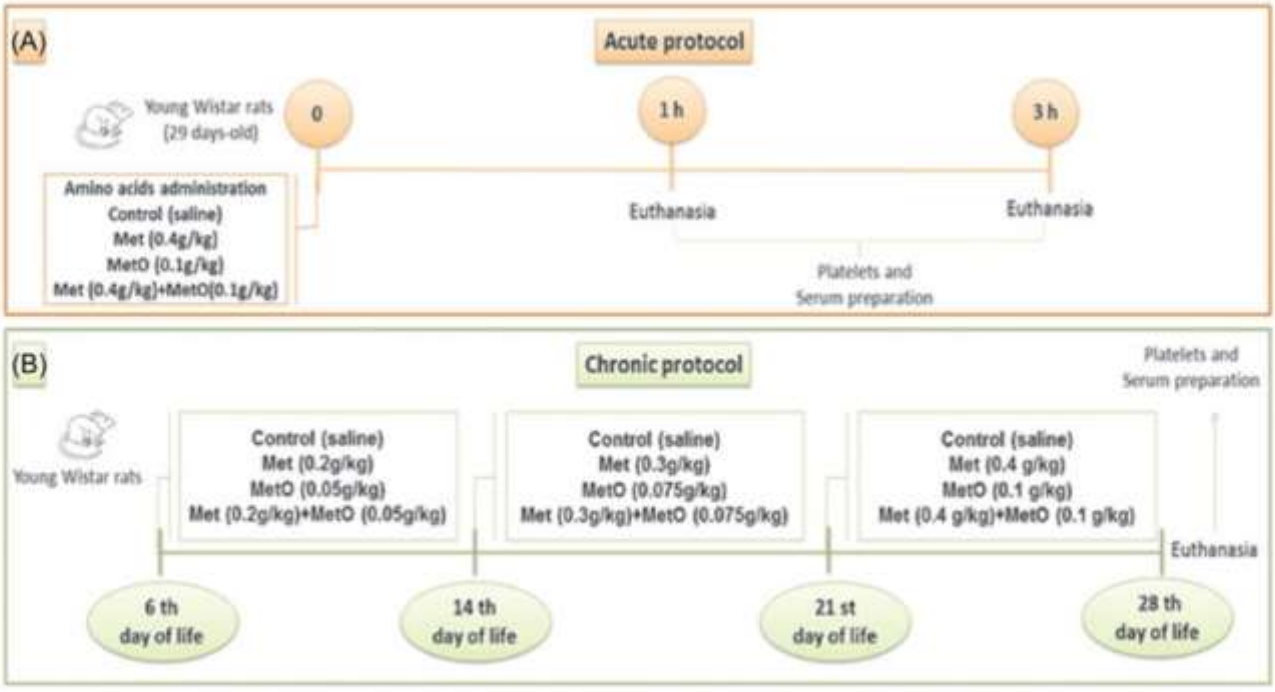

FIGURE 1 Experimental design for acute (A) and chronic (B) protocols for hypermethioninemia

was replaced with 10 mM $MgCl_2$ in the above-mentioned reaction medium. Reaction was initiated by addition of ATP or ADP at a final concentration of 1 mM and AMP at a final concentration of 2 mM. After 60 minutes of incubation, both enzyme assays were stopped by adding 200 μL of 10% TCA. The released inorganic phosphate (Pi) was assayed by the method of Chan et al[20] using malachite green as the colorimetric reagent and KH_2PO_4 as the standard. Controls were carried out to determine nonenzymatic Pi release. For this, the reaction medium (160 μL) was incubated, as previously described, only with substrate (20 μL). After 60 minutes of incubation, the reaction was stopped with 10% TCA and then, the platelet samples (20 μL) were added. To determine the specific enzymatic activity, the absorbance obtained in these control samples was discounted from the absorbance of tests (which the platelet samples were incubated for one hour with substrate). Enzymatic specific activities were reported as nmol Pi released/min/mg protein.

ADA activity was measured by Giusti and Galanti.[21] For this assay, 50 μL of platelets were subjected to reaction with 21 mmol/L of Ado, pH 6.5, and incubated at 37°C for 60 minutes. The ammonia formed, because of Ado hydrolysis by ADA activity, reacted with hypochlorite and phenol to form the intense blue indophenol. The specific ADA activity was expressed as U/mg protein. One unit (1 U) of ADA was defined as the amount of enzyme required to release 1 mmol ammonia/min from Ado at standard assay conditions.

2.8 | NTPDase and 5′-nucleotidase assays in serum

NTPDase and 5′-nucleotidase assays in serum were performed as described by Fürstenau et al.[22] Serum samples (20 μL) were preincubated for 10 minutes at 37°C in a reaction medium containing 112.5 mM Tris-HCl, pH 8.0. To start the reactions, substrates, ATP, ADP, and AMP were added to the medium at a final concentration of 3.0 mM. The incubation time was 40 minutes, and 10% TCA was used to stop the enzymatic reactions. The amount of Pi released was measured by the method of Chan et al.[20] Controls to correct for nonenzymatic substrate hydrolysis were performed by adding serum preparations after the reactions were stopped with TCA. Enzyme activities were expressed as nmol Pi released/min/mg protein.

2.9 | Oxidative stress parameters

2.9.1 | ROS detection

ROS formation was determined in platelets and serum according to the method of Ali et al[23] with minor modifications. Oxidation of DCFH-DA to fluorescent 2′,7′-dichlorofluorescein (DCF) was measured for detecting ROS production. DCF fluorescence intensity emission was recorded at excitation wavelengths of 525 and 488 nm 30 minutes after adding DCFH-DA to the medium containing 5 μL of the sample. ROS production was expressed as μmol DCF per mg of protein.

2.9.2 | Nitrite level quantification

Nitrite levels in serum were measured using the Griess reaction following the method of Stuehr and Nathan.[24] In brief, 50 μL of serum was incubated with 50 μL of 1% sulfanilamide and 50 μL of 0.3% *N*-1-naphthylethylenediamine dihydrochloride at room temperature for 10 minutes. Nitrite was quantified by spectrophotometry at 540 nm using sodium nitrite as the standard. Results were expressed as μM nitrite/mg protein.

2.9.3 | Ascorbic acid level quantification

Ascorbic acid concentration in serum was determined by the method described by Abdala et al[25] with minor modifications. One hundred microliter of serum was added in a solution containing 2,4-dinitrophenylhydrazine and TCA, and was incubated for 2 hours at 37°C. Next, 130 μL of 65% (v/v) H_2SO_4 was added to the medium, resulting in an orange-red compound that was measured at 520 nm and expressed as μmol/mg of protein.

2.9.4 | Total sulfhydryl content level quantification

Total sulfhydryl content was measured in platelets and serum. Samples (10 μL) were added to phosphate-buffered saline buffer (pH 7.4) containing EDTA. The reaction was started by adding DTNB, and incubating for 60 minutes in the dark. The reduction of DTNB by thiols due to oxidization (disulfide) generated an yellow derivative (2-nitro-5-thiobenzoic acid [TNB]) and was measured at 412 nm.[26] The results were reported as nmol TNB/mg protein.

2.9.5 | Thiobarbituric acid reactive substances level quantification

Firstly, serum samples were mixed with 10% TCA and centrifuged at 2500*g* for 5 minutes at room temperature. The resulting supernatant was mixed with TBA (0.67%) and incubated in a dry block at 100°C for 30 minutes. The resulting pink organic layer was measured at 535 nm. Thiobarbituric acid reactive substances (TBARS) quantification was performed as

described by Esterbauer and Cheeseman[27] and reported as nmol TBARS/mg protein.

2.9.6 | Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was evaluated in platelets and serum. This assay is based on the inhibition of superoxide-dependent adrenaline auto-oxidation to adenochrome. The intermediate in this reaction is superoxide, which is scavenged by SOD, and is measured using a spectrophotometer at 480 nm. SOD activity was measured according to the method of Misra and Fridovich[28] and was reported as U/mg protein.

2.9.7 | Catalase activity assay

Catalase (CAT) activity was evaluated in platelets and serum by decomposition of 30mM H_2O_2 in 50mM potassium phosphate buffer (pH 7.0), and was continuously monitored at 240 nm for 180 seconds at 37°C according to the method of Aebi.[29] CAT activity was reported as U/mg protein.

2.9.8 | Glutathione *S*-transferase activity assay

Glutathione *S*-transferase (GST) activity was measured in serum using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to the method of Habig et al.[30] The assay mixture contained 1 mM CDNB (in ethanol), 10 mM glutathione, 20 mM potassium phosphate buffer (pH 6.5), and 20 μL of serum. The activity was expressed as μmol GS-DNB min/mg protein.

2.10 | Protein determination

For oxidative stress parameters, protein level was measured by the method of Lowry et al[31] using bovine serum albumin as the standard. For NTPDase and 5′-nucleotidase activities, protein levels were measured by the Coomassie blue method according to Bradford.[32]

2.11 | Statistical analysis

Data were analyzed by one-way analysis of variance followed by Tukey's post hoc test for mean comparison

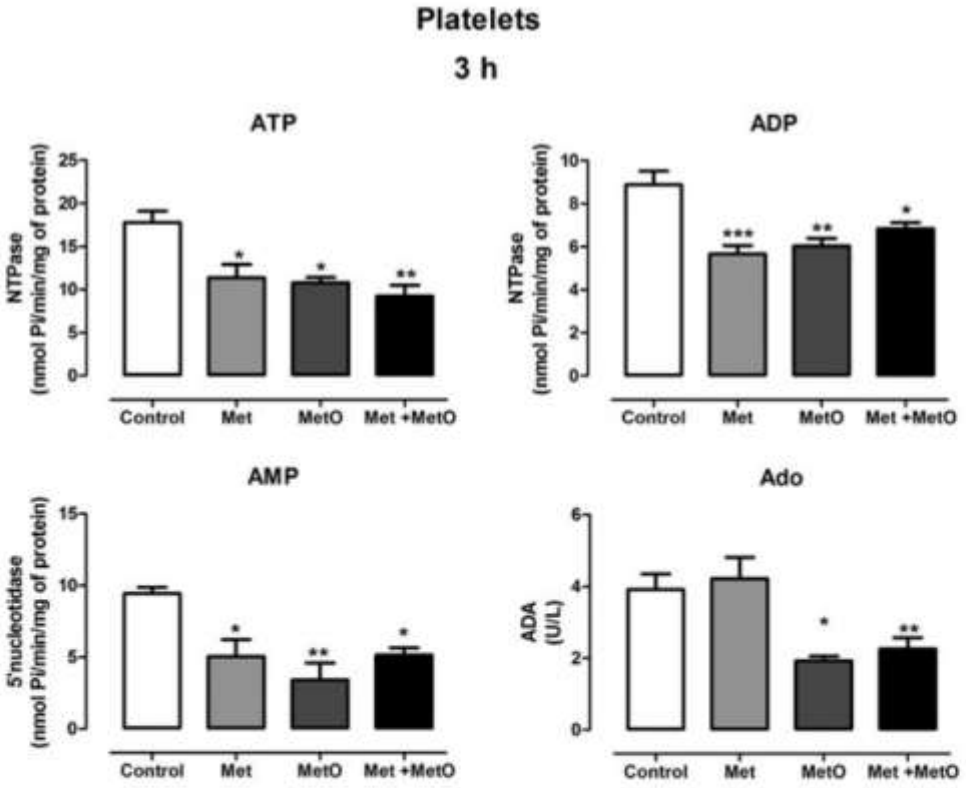

FIGURE 2 NTPDase activity using ATP and ADP as the substrates, 5′-nucleotidase activity using AMP as the substrate, and ADA activity using adenosine (Ado) as the substrate in platelets of young rats 3 hours after administration of Met and/or MetO. NTPDase and 5′-nucleotidase activities are expressed as nmol Pi/min/mg protein, and ADA activity is expressed as U/L. Bars represent mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ denote statistically significant difference from the control group ($n = 3$ to 5 each group). ADA, adenosine deaminase; Ado, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; NTPDase, nucleoside triphosphate phosphohydrolase; Met, methionine; MetO, methionine sulfoxide

using GraphPad Prism version 5.0 Program (Intuitive Software for Science, São Diego, CA). *A P* value less than or equal to 0.05 was considered as statistically significant difference. All data were expressed as mean ± standard error of mean (SEM).

3 | RESULTS

3.1 | Purinergic enzyme activities in platelets

The results of NTPDase, 5′-nucleotidase, and ADA activities in platelets after acute and chronic treatment with Met and/or MetO are demonstrated in Figures 2 and 3. Our findings showed that 3 hours after amino acid treatment, a significant reduction was observed in NTPDase (using ATP ($F_{3,12} = 9.22$; $P < 0.05$) and ADP ($F_{3,14} = 10.29$; $P < 0.05$) as substrate) and 5′-nucleotidase ($F_{3,12} = 8.23$; $P < 0.05$) activities in platelets of animals of the groups Met, MetO, and Met + MetO when compared with control group. In the animals that were treated with MetO and Met + MetO was observed a decrease in the ADA activity in platelets when compared with the control group ($F_{3,12} = 10.96$; $P < 0.05$) (Figure 2). In all groups evaluated in this study, no changes were observed in ATP, ADP, and AMP hydrolysis and ADA activity in platelets 1 hour after amino acid administration (data not shown).

After 21 days of the treatment, NTPDase activity, using ATP ($F_{3,20} = 5.34$; $P < 0.05$) and ADP ($F_{3,20} = 11.99$; $P < 0.01$) as the substrates was reduced in Met, MetO, and Met + MetO groups, whereas 5′-nucleotidase activity was decreased only in the Met + MetO group ($F_{3,20} = 8.92$; $P < 0.01$) in relation to control group. However, our results showed also an increase in the ADA activity in the Met + MetO group ($F_{3,20} = 3.96$; $P < 0.05$) when compared with control animals (Figure 3).

3.2 | Purinergic enzyme activities in serum

Figure 4 shows the results of NTPDase and 5′-nucleotidase activities in serum of rats after acute and chronic

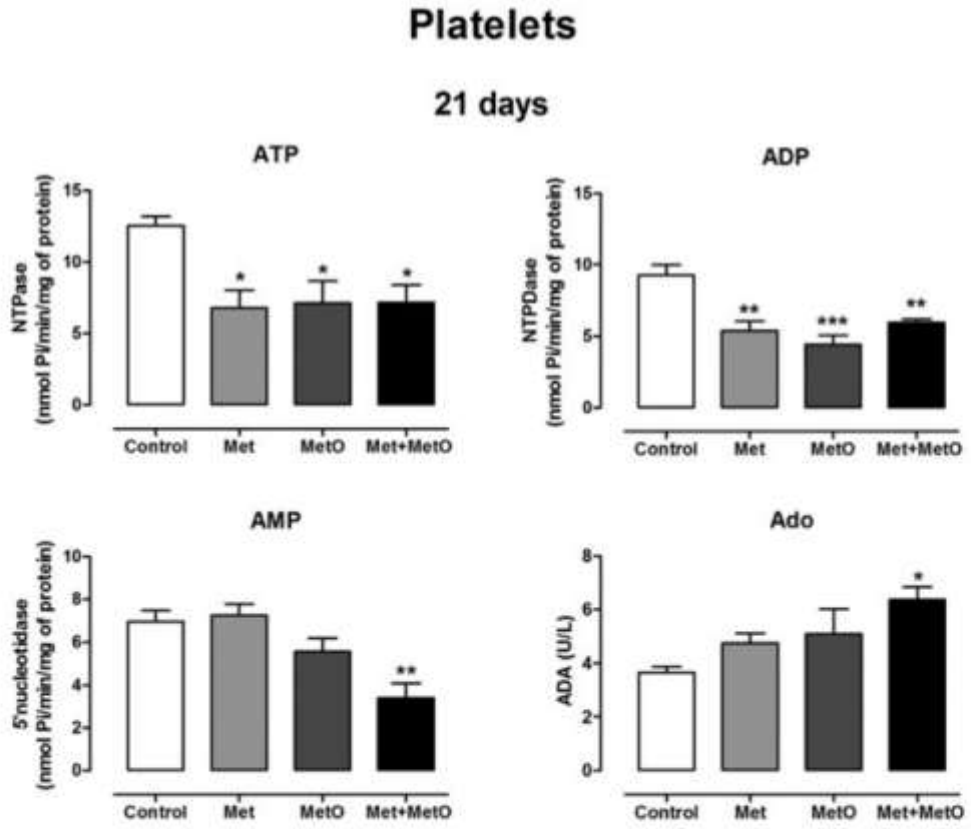

FIGURE 3 NTPDase activity using ATP and ADP as the substrates, 5′-nucleotidase activity using AMP as the substrate, and ADA activity using Ado as the substrate in platelets of young rats 21 days after administration of Met and/or MetO. NTPDase and 5′-nucleotidase activities are expressed as nmol Pi/min/mg of protein, and ADA activity is expressed as U/L. Bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ denote statistically significant difference from the control group ($n = 3$ to 5 each group). ADA, adenosine deaminase; Ado, adenosine; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Met, methionine; MetO, methionine sulfoxide; NTPDase, nucleoside triphosphate phosphohydrolase

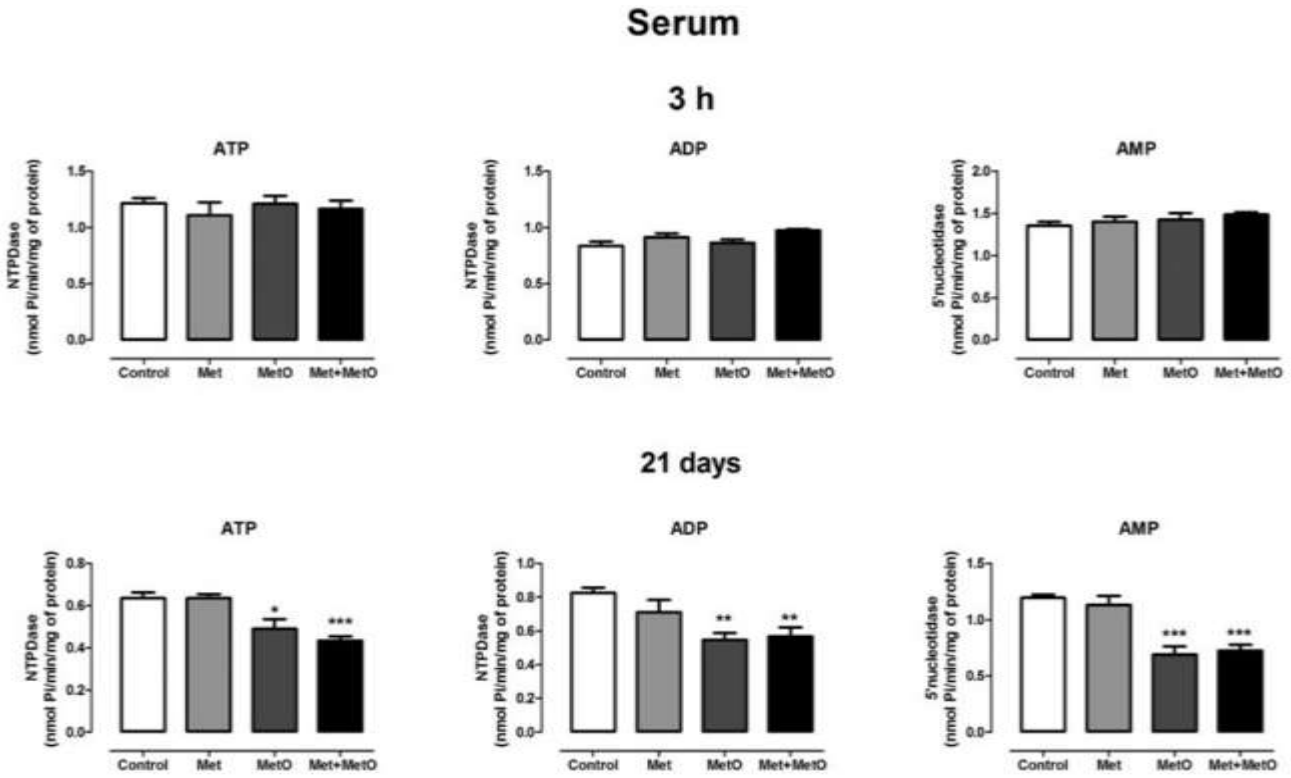

FIGURE 4 NTPDase activity using ATP and ADP as the substrates and 5′-nucleotidase activity using AMP as the substrate in serum of young rats 3 hours, and 21 days after treatment with Met and/or MetO. NTPDase and 5′-nucleotidase activities are expressed as nmol Pi/min/mg protein. Bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ denote statistically significant difference from the control group ($n = 7$ to 10 each group). ADP, adenosine diphosphate; ATP, adenosine triphosphate; NTPDase, nucleoside triphosphate phosphohydrolase; Met, methionine; MetO, methionine sulfoxide

treatment with Met and/or MetO. In the acute protocol, no changes were observed in NTPDase and 5′-nucleotidase activities in the serum the animals treated with Met, MetO, and Met + MetO after 1 hour (data not shown) and 3 hours [ATP ($F_{3,15} = 0.98$; $P > 0.05$); ADP ($F_{3,15} = 3.13$; $P > 0.05$); and AMP ($F_{3,15} = 0.38$; $P > 0.05$)] (Figure 4). After chronic treatment, NTPDase, determined using ATP ($F_{3,20} = 4.56$; $P < 0.05$) and ADP ($F_{3,20} = 7.19$; $P < 0.01$) as the substrates, and 5′-nucleotidase activities ($F_{3,20} = 18.02$; $P < 0.001$) were reduced significantly in the MetO and Met + MetO groups compared with those in the control group (Figure 4).

3.3 | Oxidative stress parameters in platelets

The results of oxidative stress in platelets after acute and chronic treatment are shown in Figures 5 and 6, respectively. ROS production ($F_{3,12} = 0.97$; $P > 0.05$), total sulfhydryl content ($F_{3,12} = 2.94$; $P > 0.05$), SOD ($F_{3,12} = 3.11$; $P > 0.05$), and CAT ($F_{3,12} = 1.57$; $P > 0.05$) activities remained unchanged 1 hour after amino acid treatment (Figure 5) in the animals treated with Met, MetO, and Met + MetO when compared with control group. However, 3 hours after amino acid administration, a decrease in SOD ($F_{3,12} = 8.88$; $P < 0.05$) and CAT ($F_{3,12} = 6.38$; $P < 0.05$) activities was observed in serum of animals treated with MetO and Met + MetO (Figure 5) when compared with control group. In all groups evaluated in this study, no changes were observed in ROS levels ($F_{3,12} = 0.34$; $P > 0.05$) and total sulfhydryl content ($F_{3,12} = 7.73$; $P > 0.05$) in platelets after 3 hours the amino acids treatment.

In the chronic treatment, we observed an increase in ROS levels in animals treated with MetO and Met + MetO compared with that in animals of the control group ($F_{3,16} = 7.34$; $P < 0.05$). In contrast, CAT activity decreased with Met, MetO, and Met + MetO administration ($F_{3,14} = 9.04$; $P < 0.05$; Figure 6) in relation of control group. Total sulfhydryl content ($F_{3,14} = 2.07$; $P > 0.05$) and SOD activity ($F_{3,14} = 0.27$; $P > 0.05$) remained unchanged in Met, MetO and Met + MetO groups in the chronic protocol (Figure 6) when compared with control group.

3.4 | Oxidative profile in serum

Table 1 presents the results of oxidative stress in serum of young rats exposed for 1 and 3 hours to Met and/or

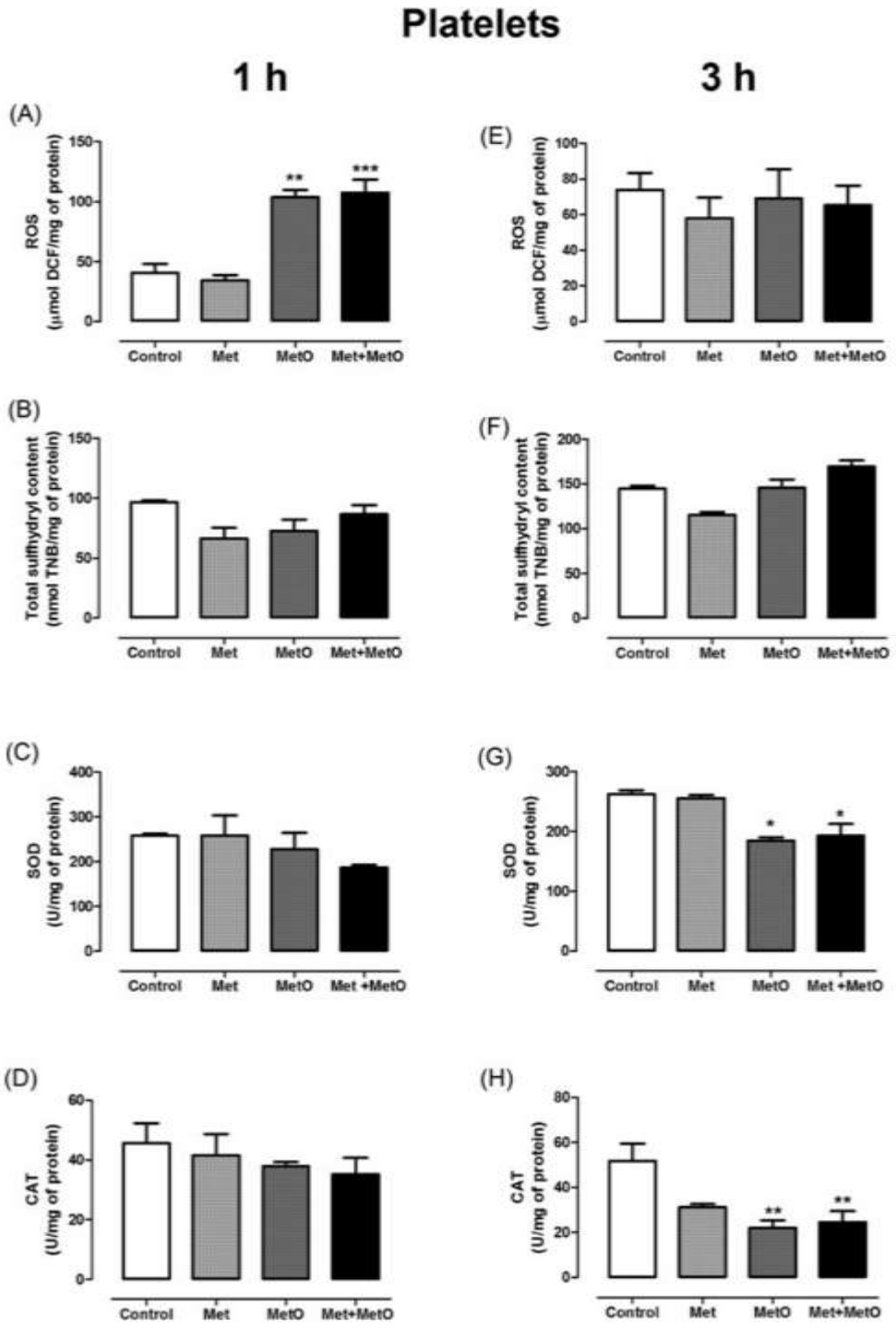

FIGURE 5 ROS (A) and total sulfhydryl content (B) levels and SOD (C) and CAT (D) activities in platelets of young rats 1 hour and 3 hours after treatment with Met and/or MetO. ROS levels are expressed as µmol DCF/mg protein, thiol content as nmol TNB/mg protein, and SOD and CAT activities as U/mg protein. Bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ denote statistically significant difference from the control group ($n = 4$ to 6 animals each group). CAT, catalase; Met, methionine; MetO, methionine sulfoxide; ROS, reactive oxygen species; SOD, superoxide dismutase

MetO. One hour after the treatment, only ROS production was elevated in the MetO and Met + MetO groups ($F_{3,12} = 6.94$; $P < 0.05$; Table 1) when compared with control group. Nitrite ($F_{3,12} = 1.02$; $P > 0.05$), total sulfhydryl content ($F_{3,12} = 0.90$; $P > 0.05$), ascorbic acid ($F_{3,9} = 3.92$; $P > 0.05$), and TBARS ($F_{3,9} = 4.45$; $P > 0.05$) levels as well as SOD ($F_{3,12} = 0.91$; $P > 0.05$), CAT ($F_{3,12} = 0.38$; $P > 0.05$), and GST ($F_{3,12} = 1.41$; $P > 0.05$) activities remained unchanged in Met, MetO and Met + MetO groups compared with those in control animals (Table 1). However, 3 hours after amino acid treatment, ROS production increased in the Met, MetO, and

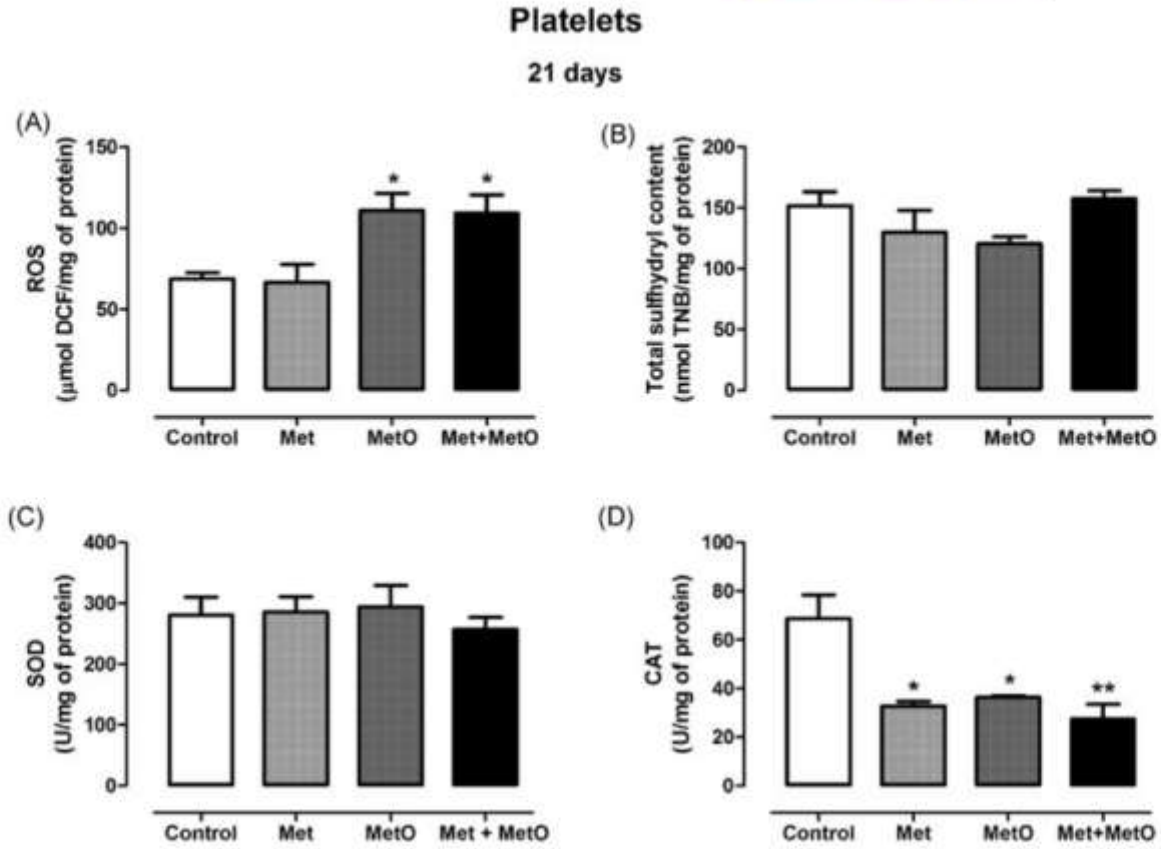

FIGURE 6 ROS (A) and total sulfhydryl content (B) levels and SOD (C) and CAT (D) activities in platelets of young rats 21 days after treatment with Met and/or MetO. ROS levels were expressed as µmol DCF/mg of protein, thiol content as nmol TNB/mg protein, and SOD and CAT activities as U/mg protein. Bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ denote statistically significant difference from the control group ($n = 4$ to 5 each group). CAT, catalase; DCF, 2′,7′-dichlorofluorescein; Met, methionine; MetO, methionine sulfoxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TNB, 2-nitro-5-thiobenzoic acid

Met + MetO groups ($F_{3,13} = 10.42$; $P < 0.05$), whereas in the same groups, ascorbic acid level reduced compared with that in the control group ($F_{3,11} = 5.06$; $P < 0.05$). TBARS levels also increased in animals treated only with MetO ($F_{3,12} = 4.11$; $P < 0.05$) in relation to animals of control group. SOD activity reduced in the MetO and Met + MetO groups ($F_{3,12} = 5.63$; $P < 0.05$), and CAT activity decreased in Met, MetO and Met + MEtO groups compared with that in the control group ($F_{3,12} = 8.41$; $P < 0.05$). Total sulfhydryl content ($F_{3,12} = 1.71$; $P > 0.05$), nitrite levels ($F_{3,12} = 0.24$; $P > 0.05$), and GST activity ($F_{3,15} = 0.55$; $P > 0.05$) remained unchanged in Met, MetO and Met + MetO groups in relation to control group.

In the chronic treatment, an increase in ROS production was observed in the Met, MetO, and Met + MetO groups ($F_{3,14} = 12.35$; $P < 0.05$; Figure 7). Moreover, TBARS level increased significantly in the MetO and Met + MetO groups ($F_{3,16} = 10.29$; $P < 0.05$; Figure 7), whereas ascorbic acid levels reduced ($F_{3,15} = 9.28$; $P < 0.01$) in Met, MetO and Met + MetO groups compared with those in control animals (Figure 7). Total sulfhydryl content ($F_{3,17} = 3.09$; $P > 0.05$) reduced in the Met + MetO group ($F_{3,17} = 3.09$; $P < 0.05$) compared with control (saline) group. Our results also showed a decrease in SOD activity in the Met, MetO, and Met + MetO groups ($F_{3,14} = 13.32$; $P < 0.01$), whereas CAT activity decreased only in the Met + MetO group ($F_{3,15} = 6.72$; $P < 0.01$; Figure 7) in relation to control (saline) group. In contrast, GST activity increased in the MetO and Met + MetO groups compared with that in the control (saline) group ($F_{3,16} = 4.93$; $P < 0.05$; Figure 7). Nitrite levels ($F_{3,15} = 1.10$; $P > 0.05$) remained unchanged in serum of animals subjected to chronic treatment with Met, MetO, and Met + MetO when compared with control animals (Figure 7).

4 | DISCUSSION

Clinical manifestations of persistent hypermethioninemia are variable, and there are many asymptomatic patients, making it difficult to achieve differential diagnosis and decide the most appropriate therapeutic

TABLE 1 ROS, nitrite, ascorbic acid, SH, and TBARS levels and SOD, CAT, and GST activities in serum of young rats 1 and 3 hours after treatment with Met and/or MetO

Groups	ROS	Nitrite	Ascorbic acid	SH	TBARS	SOD	CAT	GST
	1 h							
Control	2.98 ± 0.34	0.37 ± 0.05	4.87 ± 0.91	16.57 ± 3.95	42.28 ± 7.67	3.69 ± 0.23	0.55 ± 0.06	14.7 ± 1.55
Met	3.18 ± 0.28*	0.25 ± 0.04	3.63 ± 0.52	11.24 ± 2.40	35.73 ± 8.54	4.07 ± 013	0.4 ± 0.10	12.93 ± 4.87
MetO	5.76 ± 0.78*	0.37 ± 0.05	6.28 ± 0.28	11.67 ± 2.72	56.04 ± 3.22	3.81 ± 0.18	0.52 ± 0.14	18.68 ± 3.44
Met+MetO	5.55 ± 0.67*	0.33 ± 0.01	4.74 ± 0.27	14.85 ± 1.53	25.70 ± 2.51	3.60 ± 0.19	0.50 ± 0.09	22.33 ± 1.79
	3 h							
Control	4.67 ± 0.42	0.22 ± 0.03	13.44 ± 1.02	13.55 ± 3.60	36.59 ± 2.93	3.59 ± 0.17	0.61 ± 0.033	21.69 ± 1.60
Met	12.10 ± 1.65***	0.17 ± 0.04	7.42 ± 0.40*	9.24 ± 2.84	57.49 ± 7.79	3.37 ± 0.17	0.30 ± 0.04*	25.43 ± 2.25
MetO	8.74 ± 0.46*	0.22 ± 0.07	7.73 ± 1.043*	8.31 ± 0.59	72.75 ± 15*	2.53 ± 0.26*	0.30 ± 0.05*	24.82 ± 1.77
Met+MetO	8.56 ± 1.06*	0.24 ± 0.03	8.30 ± 1.30*	13.56 ± 2.19	45.62 ± 6.55	2.43 ± 0.34*	0.36 ± 0.06**	23.38 ± 2.98

Abbreviations: CAT, catalase; DCF, 2′,7′-dichlorofluorescein; GST, glutathione *S*-transferase; Met, methionine; MetO, methionine sulfoxide; ROS, reactive oxygen species; SH, total sulfhydryl content; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TNB, 2-nitro-5-thiobenzoic acid. ROS levels were expressed as μmol DCF/mg protein, nitrite levels as μM nitrite/mg protein, ascorbic acid as μmol/mg protein, TBARS levels as nmol TBA/mg protein, thiol content as nmol TNB/mg protein, SOD and CAT activities as U/mg protein, and GST as μmol GS-DNB min/mg protein. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate statistically significant difference from the control group (n = 4 to 5).

strategy.[2] Considering that the etiology of hypermethioninemia remains poorly understood, the use of animal models is important for understanding this condition. Therefore, we evaluated the effects of high Met and its metabolite, MetO levels on purinergic enzyme activities, and oxidative stress in platelets and serum of young rats.

Platelet dense granules contain molecules such as ADP and ATP. This secretory compartment plays an important role in primary hemostasis by acting as a feedback mechanism by stimulating the platelet receptors P2Y1 and P2Y12 via release of ADP.[13,33,34] However, Ado is a competitive inhibitor of platelet aggregation, induced by ADP by interacting with P1 receptor, which inhibits platelet function via activation of adenylate cyclase.[13,34] NTPDase, 5′-nucleotidase, and ADA are primarily responsible for controlling the extracellular concentration of adenine nucleotides and nucleosides; thus, these enzymes play crucial roles in the duration and magnitude of purinergic signaling for functions of platelets.[35,36] In fact, it has been demonstrated that inhibition of NTPDase activity contributes to the amplification of ADP-induced platelet aggregation.[37] Therefore, these enzymes have gained attention owing to their substantial contribution in inhibiting platelet activation and aggregation.

We showed for the first time that ATP, ADP, and AMP hydrolysis decreases in platelets after both acute (3 hours) and chronic treatment with Met and/or MetO. This inhibition in NTPDase and 5′-nucleotidase activities in platelets can be explained, at least in part, by Met metabolism. ATP transfers the adenosyl group to Met to form *S*-adenosylmethionine in a reaction catalyzed by MAT.[5] Thus, an increase in Met concentration demands higher ATP consumption for its metabolization, which can be associated with low levels of ATP in serum and reduced NTPDase activity in platelets. In addition, it is important to consider that elevated ROS production induced by Met and/or MetO treatment also can contribute toward the inactivation of ectoenzymes in the platelet membrane.[38]

The ADP released from platelets upon activation interacts with purinergic receptors (P2Y1, P2Y12, and P2X1) in the platelet membrane, leading to morphological change, granule content release, and platelet aggregation.[13,33,34] By hydrolyzing ADP to AMP, NTPDase plays a crucial role in modulating purinergic signaling for platelet control and, hence, in pathological thrombus formation and vascular occlusion.[15] Here, we demonstrated that Met and MetO treatment reduced NTPDase activity in platelets and serum, suggesting that these amino acids interfere with platelet activation and aggregation as ADP is accumulated in the extracellular milieu. Corroborating with these results, we recently demonstrated that ADP levels increase in the serum of young animals after 21 days of treatment with Met and/or MetO.[10]

In addition, the enzymes 5′-nucleotidase and ADA are critical for regulation of extracellular adenosine levels. Adenosine is produced from AMP by the action of 5′-nucleotidase and is converted via inosine by ADA.[15] Considering that extracellular Ado production occurs primarily through adenine nucleotide metabolism, the decrease in 5′-nucleotidase activity after acute treatment

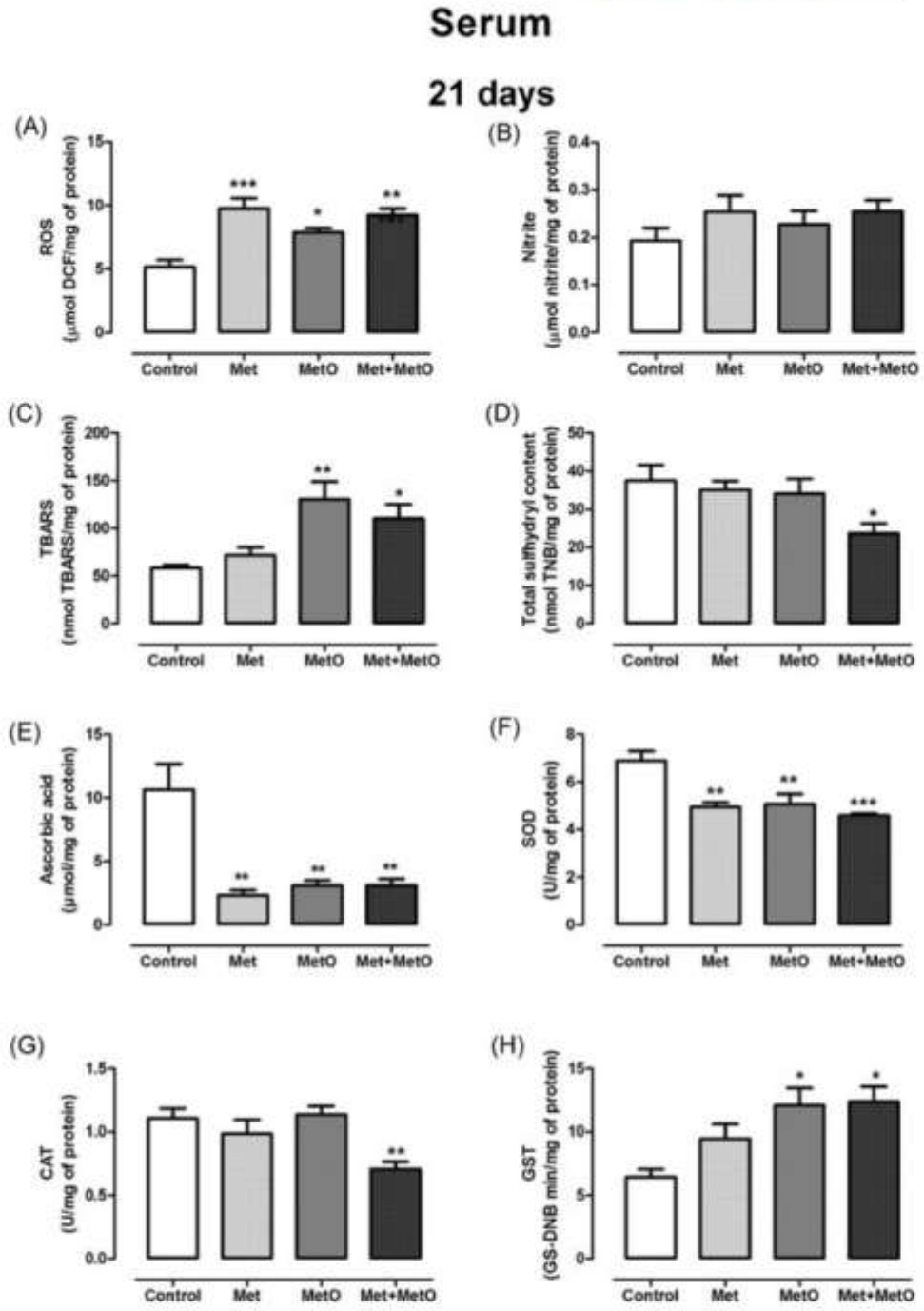

FIGURE 7 ROS (A), nitrite (B), TBARS (C), and total sulfhydryl content (D) and levels (E), SOD (F), CAT (G), and GST (H) activities in serum of young rats 21 days after treatment with Met and/or MetO. ROS levels were expressed as µmol DCF/mg of protein, nitrite levels as µM nitrite/mg of protein, TBARS levels as nmol TBA/mg of protein, thiol content as nmol TNB/mg protein, SOD and CAT activities as U/mg protein, and GST as µmol GS-DNB min/mg protein. Bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ denote statistically significant difference from the control group ($n = 4$ to 5 each group). CAT, catalase; DCF, 2′,7′-dichlorofluorescein; GST, glutathione *S*-transferase; Met, methionine; MetO, methionine sulfoxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TBA, thiobarbituric acid; TNB, 2-nitro-5-thiobenzoic acid

can be related to the decrease in AMP production by NTPDase. However, in chronic treatment, 5′-nucleotidase activity decreased in serum and platelets in the Met and Met + MetO groups, whereas ADA activity increased in platelets in the Met + MetO group. Here, an important point to be discussed is that during metabolization of Met, Ado molecules are formed.[5] Thus, the Ado production pathway can be associated with alterations in ADA activity 21 days after the treatment. However, our research group has shown that MetO administration decreases Ado levels in serum with

chronic treatment.[10] Taken together, these findings suggest that both acute and chronic treatment with Met and its metabolite MetO could alter platelet function by interfering with Ado levels.

Previous studies have demonstrated that Met and MetO increase the levels of proinflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor-α and alter cholinergic and purinergic enzyme activity in lymphocytes of young rats.[10] The platelet-lymphocyte axis may contribute to immune mechanisms through a reciprocal functional regulation between these cells.[39] Platelets are a source of chemokines, cytokines, and growth factors, which are important molecules for immune and inflammatory processes.[33] IL-6 is involved in the upregulation of tissue factors that initiate coagulation and exerts its effects on platelets by a membrane-bound β-receptor glycoprotein (gp130), which may be crucial in the development of inflammation within a damaged vessel and platelet thrombogenicity.[40] Thus, it is important to consider that an increase in IL-6 as well changes in ectonucleotidase activities in platelets could contribute to the dysfunction of pathways involved in inflammation and thromboregulation in hypermethioninemia.

Experimental findings have also demonstrated the intrinsic relation of high concentrations of Met and MetO and oxidative stress in several tissues[6,7,41] and cells.[42] MetO is formed from an oxidation reaction of Met.[43] Several proteins in blood contain oxidation-sensitive Met residues, including proteins involved in hemostasis and thrombosis.[43] The oxidation of Met residues to yield MetO can cause structural and functional changes with potential regulatory roles in the pathogenesis of vascular or thrombotic diseases.[43-45]

Our findings demonstrated an increase in ROS production in platelets and serum after both acute and chronic treatment with Met and/or MetO. The antioxidant cellular machinery is composed of nonenzymatic compounds, such as ascorbic acid, and enzymes, such as SOD, that protect the tissues from dismutation of superoxide radical (O_2^-) to H_2O_2, which is subsequently degraded by the enzymes CAT and GPx.[46-48] In platelets, it is important to observe that CAT activity decreased after 3 hours and 21 days of treatment with Met, MetO, and Met + MetO, which could have favored the accumulation of H_2O_2. Previous studies have demonstrated that H_2O_2 exerts multidirectional action on ADP-induced platelet aggregation.[49,50] Although high concentrations of this molecule potentially lead to platelet aggregation, low concentrations of H_2O_2 may inhibit platelet function.[49,50] Furthermore, high concentrations of H_2O_2 can also induce damages to platelet membrane.[50] When platelets exposed to

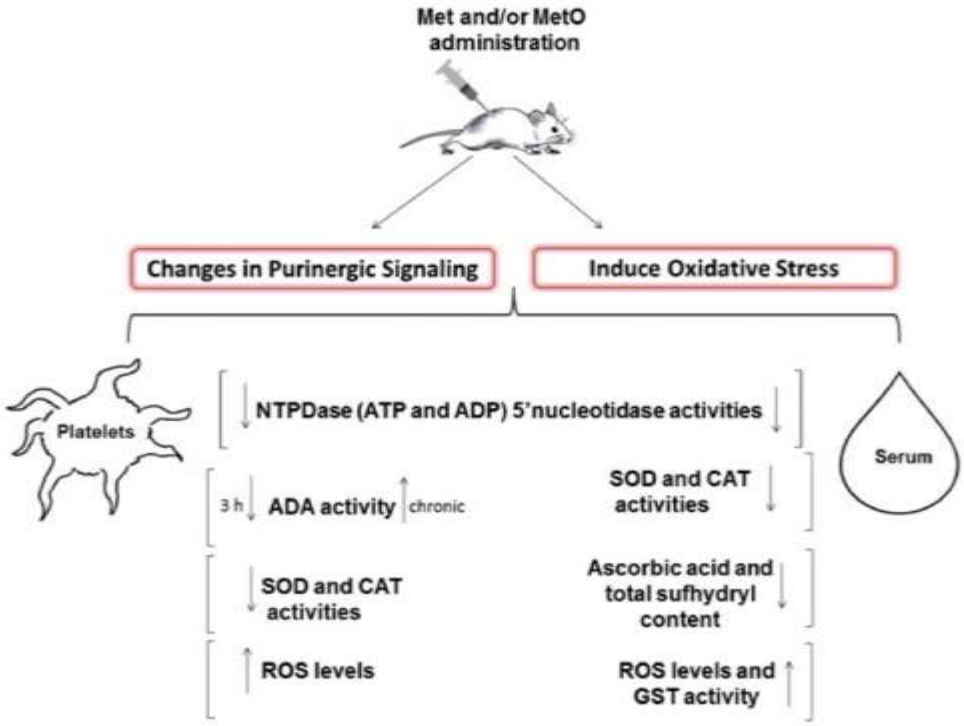

FIGURE 8 Overview of the possible effects of Met and MetO administration on purinergic enzyme activities and oxidative stress parameters in platelets and serum of young rats. ADP, adenosine diphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CAT, catalase; GST, glutathione *S*-transferase; Met, methionine; MetO, methionine sulfoxide; NTPDase, nucleoside triphosphate phosphohydrolase; ROS, reactive oxygen species; SOD, superoxide dismutase

ADP and H_2O_2 are treated with CAT, a decrease in platelet aggregation is observed.[50] Also was demonstrated that platelets treated with high concentrations of H_2O_2 present lipid and protein damage.[49] Thus, it is possible to infer that decrease in CAT activity by Met and MetO leads to the increase in ROS levels, such as H_2O_2, and this mechanism may contribute to ADP-induced platelet aggregation in hypermethioninemia.

A decrease in SOD and CAT enzyme activities was observed in serum after acute (3 hours) and chronic treatments. Moreover, a reduction in ascorbic acid levels and total sulfhydryl content, which are important nonenzymatic antioxidant defense compounds, can also contribute to the increase in ROS level and lipid peroxidation, subsequently leading to oxidative stress damage in experimental hypermethioninemia. With regard to the increase in GST activity in serum after chronic treatment, it is important to consider that Met is the essential component for glutathione synthesis and high concentration of this amino acid may stimulate the production of glutathione, and consequently high GST activity.[51]

5 | CONCLUSION

This study demonstrated that acute and chronic administration of Met and/or MetO alters the redox status and purinergic signaling in platelets and serum of young rats, demonstrating that these amino acids contribute to platelet disorders in hypermethioninemia (Figure 8). These findings help in understanding the pathogenesis of hypermethioninemia better, and may help to diagnose, select, and design appropriate therapy for improving early and long-term outcomes.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

Mayara Sandrielly Pereira Soares http://orcid.org/0000-0002-2181-3591

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4.4 Manuscrito I

O manuscrito será submetido à revista Amino Acids.

Methionine and/or methionine sulfoxide induce memory deficits and morphological changes in hippocampus of young rats: implications for the pathogenesis of hypermethioninemia

Mayara Sandrielly Pereira Soares[1*], Bruna da Silveira de Mattos[1], Anita Almeida Ávila[1], Luiza Spohr[1], Rejane Giacomelli Tavares[1], Cassiana Siebert[3], Daniella de Souza Moreira[3], Ângela Terezinha de Souza Wyse[3], Fabiano Barbosa Carvalho[4], Francine Rahmeier[4], Marilda da Cruz Fernandes[4], Francieli Moro Stefanello[2], Roselia Maria Spanevello[1]

[1]Programa de Pós-Graduação em Bioquímica e Bioprospecção - Laboratório de Neuroquímica, Inflamação e Câncer, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N, Pelotas, RS, Brazil.

[2]Programa de Pós-Graduação em Bioquímica e Bioprospecção - Laboratório de Biomarcadores, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N, Pelotas, RS, Brazil.

[3]Laboratório de Neuroproteção e Doença Metabólica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[4]Laboratório de Pesquisa em Patologia, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil.

*Address reprint requests to: Mayara Sandrielly Pereira Soares, Universidade Federal de Pelotas, Campus Universitário s/n, Capão do Leão, RS, Brazil, CEP: 96010-900, Phone: 55 53 32757355, Fax: 55 53 32757354. E-mail: mspereirasoares@gmail.com

Abstract

The aim of this study was to investigate the effect of the chronic administration of methionine (Met) and/or its metabolite, methionine sulfoxide (MetO), on the behavior and neurochemical parameters of young rats. Rats were treated with saline (control), Met (0.2–0.4 g/kg), MetO (0.05–0.1 g/kg), and or a combination of Met+MetO, subcutaneously twice a day from postnatal day 6 (P6) to P28. The results showed that Met, MetO, and Met+MetO impaired short-term and spatial memory ($P<0.05$), reduced rearing and grooming ($P<0.05$), but did not alter locomotor activity ($P>0.05$). Acetylcholinesterase activity increased in the cerebral cortex, hippocampus, and striatum following Met and/or MetO ($P<0.05$) treatment, while Na^+-K^+-ATPase activity reduced in the hippocampus ($P<0.05$). There was an increase in the level of thiobarbituric acid reactive substances (TBARS) in the cerebral cortex in Met-, MetO-, and Met+MetO-treated rats ($P<0.05$). Met and/or MetO treatment reduced superoxide dismutase, catalase, and glutathione peroxidase activity, total thiol content, and nitrite levels, and increased reactive oxygen species and TBARS levels in the hippocampus and striatum ($P<0.05$). Hippocampal brain derived neurotrophic factor was reduced by MetO and Met+MetO compared with the control group. The number of NeuN-positive cells decreased in the CA3 in Met+MetO group and in the dentate gyrus in the Met, MetO, and Met+MetO groups compared to control group ($P<0.05$). Taken together, these findings further increase our understanding of changes in the brain in hypermethioninemia by elucidating behavioral alterations, biological mechanisms, and the vulnerability of brain function to high concentrations of Met and MetO.

Key words: Methionine plus methionine sulfoxide, oxidative status, neurons number, CA3, dentate gyrus, behavior

1. Introduction

Hypermethioninemia is a metabolic disorder characterized by the accumulation of the essential amino acid, methionine (Met), in tissues (Mudd et al. 2001). Six genetic conditions can lead to elevated Met levels; however, methionine adenosyltransferase I/III (MAT I/III) deficiency is the most common cause of isolated and persistent elevated Met levels (Couce et al. 2008, Mudd 2011). Hypermethioninemics patients can present hepatic and neurological manifestations, such as cognitive deficits, demyelination, memory impairments, and cerebral edema (Chamberlin et al. 1996, Mudd et al. 2000, Mudd et al. 2001, Couce et al. 2008, Mudd 2011). Recently, it was demonstrated that the secondary metabolite, methionine sulfoxide (MetO), is closely associated with alterations in hypermethioninemia (Costa el al. 2013, Dos Santos et al. 2016, Soares et al. 2017a). Studies have shown that the formation of MetO via reactive oxygen species (ROS)-induced oxidation of Met modifies its physicochemical properties, alters its function, and leads to a loss of protein biological activity (Tarrago et al. 2015, Suzuki et al. 2016).

There is an intrinsic relationship between Met, MetO, and oxidative stress because they are all sulfur amino acids (Tarrago et al. 2015, Suzuki et al. 2016). Oxidative stress is a component of the pathophysiology of several human diseases and contributes to the development and/or progression of these pathologies (Patel 2016, Suzuki et al. 2016). High levels of Met and/or MetO alter the oxidative status in the liver (Stefanello et al. 2009, Costa et al. 2013, Soares et al. 2017a), kidney (Soares et al. 2017a), macrophages (Dos Santos et al. 2016), skeletal muscle (Schweinberger et al. 2015), and brain (Stefanello et al. 2005, 2007, Soares et al. 2017b) of rats.

Cholinergic signaling is mediated by the neurotransmitter, acetylcholine (ACh), which is essential for cognitive performance, such as learning, behavior, and memory (Ferreira-Vieira et al. 2016, Roy et al. 2016). Inactivation of this signaling is promoted by acetylcholinesterase (AChE), which cleaves ACh into choline and acetate (Ferreira-Vieira et al. 2016). Stefanello et al. (2007) have demonstrated that acute and chronic treatment with Met increases AChE activity in the cerebral cortex (Stefanello et al. 2007). However, little is known about the effects of MetO on AChE activity.

Sodium–potassium adenosine triphosphatase (Na^+,K^+-ATPase), an essential transmembrane enzyme responsible for the electrochemical gradient across the cell membranes (Moseley et al.2007), is also important for learning and memory-related processes. High levels of Met inhibit the Na^+,K^+-ATPase activity but increase the expression and immunocontent of Na^+,K^+-ATPase α1, α2, and α3 subunits in the synaptic plasma membrane and brain of rat offspring (Stefanello et al. 2011, Schweinberger et al. 2018).

We have previously demonstrated that acute administration of Met and/or MetO cause oxidative stress, reduce cell viability, induce DNA damage, and cause cell death by apoptosis in the cerebral cortex of young rats (Soares et al. 2017b); however, the precise mechanisms involved in the pathophysiology of chronic hypermethioninemia remain unclear. Based on these studies, we aimed to investigate the effect of chronic administration of Met and/or MetO on memory, AChE, Na^+,K^+-ATPase activity, and redox status, brain-derived neurotrophic factor (BDNF) and neuronal cell number in the cerebral cortex, hippocampus, and striatum in young rats.

2. Materials and Methods

2.1 Chemicals

Met and MetO were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and the highest purity.

2.2 Animals and ethical approval

All animal experimental protocols in this work were approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, RS, Brazil (protocol number: CEEA 3527). Wistar rats, male and female (6 days old) weighing 5-10 g were obtained from the Central Animal House of the Federal University of Pelotas. The litters (6-10 animals) and the progenitor were kept in a density of four rats per cage (acrylic boxes measuring 40cmx34cmx17cm) in the same room under a constant temperature (23 ± 2°C), humidity (50% - 65%) and illuminated from 7:00 a.m. to 7:00 p.m., with food and water available ad libitum. All animals were arbitrarily assigned before starting treatment without specific tools to achieve randomization in the animal facility room. The use of the animals was in

accordance with the Brazilian Guidelines for the Care and Use of Animals in Scientific Research Activities (DBCA), National Council of Control of Animal Experimentation (CONCEA) and with the NIH Guide for Care and Use of Laboratory Animals.

2.3 Hypermethioninemia protocol

Forty male and female Wistar rats (6 days old) were divided into four groups: Group I (Control/saline), Group II (Met), Group III (MetO), and Group IV (Met+MetO). Met and MetO were dissolved in 0.9% NaCl and were administered by subcutaneous injection twice a day at 8 h intervals between postnatal day 6 (P6) and P28 (Stefanello et al. 2007, Costa et al. 2013, Soares et al. 2017). From P6–P14, group II and III received 0.2 g/kg Met and 0.05 g/kg MetO, respectively. From P15–P21, group II and III received 0.3 g/kg Met and 0.075 g/kg MetO, respectively. From P22–P28, group II and III received 0.4 g/kg Met and 0.1 g/kg MetO, respectively. Group IV received a combination of the concentrations of Met+MetO in the same concentrations. Group I was administered an equivalent volume of saline from P6–P28 (Figure 1). The doses of Met and MetO were based on those reported in previous studies (Stefanello et al. 2007, Costa et al. 2013, Soares et al. 2017). Animals were directly euthanized when they met the exclusion criteria: weight loss in combination or not with behavioral impairment indicating pain and/or stress and suffering. No animals had to be excluded before the end of the study due to complications.

2.4 Behavioral procedure

2.4.1 Open-field test

All behavioral tests were performed by a blinded experimenter. Behavioral tests were performed between P23 and P25. Locomotor behavior was evaluated using open-field apparatus, which consisted of a wooden box. The floor of the arena was divided into 16 equal squares (18 × 18 cm) and placed in a sound free room. Rats were placed in the rear left square and allowed to explore freely for 5 min. The total number of squares crossed with all paws (crossing) and fecal droppings were manually counted; the degree of grooming and rearing was also evaluated. The apparatus was cleaned and dried with a 40% alcohol solution after testing with each

rat. This test was carried out to identify motor disabilities, which might influence the other behavioral tests performed.

2.4.2 Object recognition test

Rats were habituated to the experimental arena 24 h before the test. Objects, made of waterproof plastic, were placed on the sand floor. This test was performed in two stages. During training, rats were placed in the arena with two identical objects (A1 and A2) and allowed to explore for 5 min. The session was valid if rats explored each object for at least 30 s. Following this, rats were tested 120 min after training to evaluate their short-term memory. For the testing, one of the objects was changed to a different, novel object (B). The rats were introduced into the arena for 5 min and allowed to explore freely. The positions of the objects (familiar or novel) were randomly exchanged. Exploration was defined as smelling or touching the object with the nose and/or forelegs. Sitting on or around the object was not considered exploratory behavior. The apparatus and the objects were cleaned and dried with a 40% alcohol solution after each rat. In this task was evaluated the total exploration time (s) in the training and test; Time spent on each object – Training (s) (A1, A2); Time spent on each object – Test (s) (A1 and B); Exploratory preference time for the novel object expressed as a percentage evaluated in the object recognition test (Rossato et al. 2007).

2.4.3 Y-maze test

This test was measures spatial recognition memory. The apparatus has three arms: start arm, in which rats were placed to start to explore (always open); novel arm, which was blocked during the first trial, but open during the second trial; and other arm (always open).

Briefly, the task was performed as follows: first, rats underwent training: they were placed in the start arm and could explore the start and other arm. The third arm (novel arm) remained closed during training. After 2 h, testing was performed as follows: rats were placed in the start arm with free access to all three arms for 5 min. The number of entries in each arm and time spent exploring each arm (expressed as a percentage of the time spent in each arm) was recorded (Dellu et al. 1997). The apparatus was cleaned with 40% alcohol solution and dried after each session with each animal.

Rats were euthanized by cardiac puncture 12 h after the final amino acid or saline injection. The brain was removed and quickly dissected to obtain the cerebral cortex, hippocampus, and striatum samples. Biochemical analyses were performed by a blinded experimenter and all samples were run in duplicate.

2.5 Acetylcholinesterase activity

Brain samples were placed in a solution of Tris–HCl 10 mM, pH 7.4, homogenized and centrifuged at 1300 *g* for 10 min at 4 °C. The supernatant was used for the AChE assay, as previously described by Ellman et al. (1961). The reaction mixture comprised 10 mM 5,5-dithiobis(2-nitrobenzoic acid)**,** 100 mM phosphate buffer (pH 7.5), 15 µl supernatant, and 0,8 mM acetylthiocholine. The absorbance at 412 nm was read on a spectrophotometer at 30-s intervals for 2 min at 27 °C. AChE activity was expressed as µmol AcSCh/h/mg protein.

2.6 Na^+, K^+-ATPase activity

Brain sections were homogenized (1/10 w/v) in 0.32 mM sucrose containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenates were centrifuged at 1000 *g* for 10 min, and the supernatants were removed for further analysis. The reaction mixture for this assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, and 40 mM Tris-HCl (pH 7.4). The reaction was initiated by the addition of ATP to a final concentration of 3 mM. The control samples were generated under the same conditions with the addition of 1 mM ouabain. Na^+,K^+-ATPase activity was calculated by measuring the difference in absorbance between the study samples and the controls, as described by Wyse et al. (2007). The level of released inorganic phosphate (Pi) was measured, as described by Chan et al. (1986). Specific enzyme activity was expressed as nmol Pi released/min/mg protein.

2.7 Oxidative stress parameters in the brain structures

Brain regions were homogenized (1/10 w/v) using 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The homogenates were centrifuged at 2500 *g* for 10 min at 4 °C. The supernatants were collected and used in further analyses.

2.7.1 Reactive oxygen species (ROS) assay

The oxidation of DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF) was measured. Briefly, DCF fluorescence intensity emission was recorded at excitation wavelengths of 525 and 488 nm 30 min after the addition of DCFH-DA to the medium. ROS formation was expressed as µmol DCF/mg protein (Ali et al. 1992).

2.7.2 Thiobarbituric acid reactive substances (TBARS) assay

For this assay, the supernatants were mixed with 10% TCA and centrifuged. The supernatant was collected and mixed with TBA (0.67%) and incubated in a dry block at 100 °C for 30 min. TBARS levels were determined by absorbance at 535 nm and reported as nmol of TBARS/mg protein (Esterbauer et al. 1990).

2.7.3 Total sulfhydryl content assay

Supernatants were added to PBS buffer (pH 7.4) containing EDTA. The reaction was started by the addition of DTNB. One hour after incubation in the dark, DTNB reduced by thiol groups was oxidized (disulfide) and a yellow derivative (TNB) was generated, whose absorbance at 412 nm was measured. The results were reported as nmol TNB/mg protein (Aksenov et al. 2001).

2,7.4 Nitrite quantification

Nitrite was measured by Griess reaction (Stuehr et al. 1989). In brief, 50 µl homogenate was incubated with 50 µl 1% sulfanilamide and 50 µl 0.3% N-1-naphthylethylenediamine dihydrochloride at room temperature (RT) for 10 min. Nitrite was measured at 540 nm using sodium nitrite as standard. Results were expressed as µM nitrite/mg protein.

2.7.5 Superoxide dismutase (SOD) activity

This assay is based on the inhibition of superoxide dependent adrenaline auto-oxidation to adenochrome. The intermediate in this reaction is superoxide, which is scavenged by SOD and the absorbance is measured at 480 nm on a spectrophotometer. SOD activity was measured, as previously described by Misra and Fridovich (1972), and reported as units/mg protein.

2.7.6 Catalase (CAT) activity

The decomposition of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) was continuously monitored at 240 nm for 180 s at 37 °C, as previously reported by Aebi (1984). CAT activity was reported as units/mg protein.

2.7.7 Glutathione peroxidase (GPx) activity

GPx activity was determined according to manufacturer instructions (RANSEL®; Randox Lab, Antrim, United Kingdom). GPx activity was reported as units/mg protein.

2.8 Protein determination

Protein concentration was measured as previously described by Lowry et al. (1951), except for AChE activity, which was used the method previously described by Bradford (1976).

2.9 Brain derived neurotrophic factor (BDNF) assay

Hippocampus BDNF concentration was measured using the ChemiKine BDNF Sandwich ELISA Kit, CYT306 (Chemicon / Millipore, Billerica, MA, USA), according to the manufacturer's instructions. The results were expressed as pg BDNF/mg protein.

2.10 Histological procedures and immunohistochemistry techniques

Four rats from each group were euthanized by transcardial perfusion. Briefly, rats were anesthetized with isoflurane and perfused with 0.9% saline for 10 min, followed by 4% paraformaldehyde diluted in phosphate buffered saline (PBS; 0.1 M, pH 7.2–7.4) for 30 min. The brain of the animals was removed and stored for 24 h in 4% buffered paraformaldehyde followed by 70% ethanol for at least 24 h. Following this, the brain samples were embedded in paraffin. Coronal sections were cut using a rotary microtome (4-µm thickness) in the hippocampus. Six sections were used for the analysis (12 hippocampi), at a distance of 50 µm between each cut.

After 30 min at 80 °C, sections prepared for NeuN staining. Briefly, they were cleared using xylene (10 min, 5 min, and 5 min), followed by dehydration with ethanol 100% (5 min), 95% (5 min), 70% (5 min), 50% (5 min), and distilled water. Following this, the sections were rehydrated in PBS (pH 7.4) with 0.5% Triton X-100 (PBS-TX)

for 15 min, and then incubated in citrate buffer (pH 6.00) for 20 min at 98 °C. Endogenous peroxidase was blocked using 5% hydrogen peroxide (30V) in methanol for 10 min (3 times). Nonspecific proteins were blocked with 1% BSA; Sigma®) in PBS-tx for at least 1 h at RT. Then, the sections were incubated with primary monoclonal antibody (NeuN: Dako® 1:1000) 1 h RT and overnight at 4 °C. After 3 × PBS for 15 min, sections were incubated with conjugated secondary antibody for 40 min. Finally, the immunohistochemical reaction was revealed with 0.06% 3.3'-diaminobenzidine (DAB; Dako®) in PBS-tx for 5 min and mounted on slides using Entellan® (Merck®). NeuN-positive cells were visualized in the CA1, CA3, and dentate gyrus (DG) (Figure 5A). The number of positive cells within a 10.083 × 150 μm^2 square (Figure 5B) was counted using Image Pro Plus® 6.3 (Media Cybernetics®) software.

2.11 Statistical analysis

Data were analyzed by one or two-way analysis of variance (ANOVA) followed by Tukey or Bonferroni post-hoc tests using GraphPad Prism 5.0 (Intuitive Software for Science, São Diego, CA, USA). $P < 0.05$ was considered statistically significant. All data were expressed as mean ± standard error of the mean (SEM).

3. Results

3.1 Methionine and/or methionine sulfoxide does not alter locomotion, but reduces grooming and rearing

The parameters evaluated in the open field test are listed in Table 1. Locomotion was not significantly different in any experimental group compared with that in the control group ($F_{3,36} = 0.52$, $P > 0.05$). However, MetO and Met+MetO reduced the amount of grooming ($F_{3,36}= 3.78$, $P < 0.05$), while Met, MetO, and Met+MetO decreased the amount of rearing when compared with the control group ($F_{3,36}= 13.50$, $P < 0.05$). There was no difference in the number of fecal droppings among the groups ($F_{3,36}= 2.66$, $P > 0.05$; Table 1)**.**

3.2 Methionine and/or methionine sulfoxide impairs short-term and spatial memory

Figure 2A shows that all rats showed increased total exploration time in the testing *vs.* training phase of the object recognition test. Interestingly, MetO and Met+MetO-treated rats had longer total exploration times when compared with the control group. During the training phase, there was no difference in the exploration times between objects in all groups (Figure 2B). During testing, Met-, MetO-, and Met+MetO-treated rats explored the familiar object (A1) more than the rats in the control group. Furthermore, the MetO group showed reduced exploration of the novel object (B) compared with the control group (Figure 2C). We found that the control group explored the novel object more than the familiar object (Figure 2D). In contrast, the MetO group explored the novel object significantly less compared with the familiar object (Figure 2D). Taken together, these findings indicated that Met, MetO, and Met+MetO treatment impaired short-term object recognition memory ($F_{3,36}$= 5.12, $P < 0.05$; Figure 2E).

Similar results were observed in the Y-maze test, indicating an impairment in spatial memory; Met, MetO, and Met+MetO treatment decreased exploratory time ($F_{3,36}$= 6.72, $P < 0.05$) and number of entries ($F_{3,36}$= 3.30, $P < 0.05$) into the novel arm compared with those in the control group (Figure 2F). There were no differences in the exploratory time in the start ($F_{3,36}$= 0.51, $P > 0.05$) or other ($F_{3,36}$ = 0.70, $P > 0.05$; Figure 2F) arms or in the number of entries in the start ($F_{3,36}$ = 2.00, $P > 0.05$) and other ($F_{3,36}$= 0.90, $P > 0.05$; Figure 2G) arms.

3.3 Methionine and/or methionine sulfoxide alter acetylcholinesterase and Na^+,K^+ -ATPase activity

AChE activity was increased in the cerebral cortex ($F_{3,18}$= 10.77, $P < 0.05$) and hippocampus ($F_{3,13}$= 6.39, $P < 0.05$) following Met, MetO, and Met+MetO treatment when compared with control rats (Figure 3). Furthermore, there was an increase in AChE activity in the MetO and Met+MetO groups in the striatum compared with the control group ($F_{3,15}$= 4.79, $P < 0.05$, Figure 3).

As showed in Figure 4, Na^+,K^+-ATPase activity was significantly decreased in the MetO and Met+MetO groups in the hippocampus ($F_{3,17}$= 8.91, $P < 0.05$, Figure 3). However, no change was observed in Na^+,K^+-ATPase activity in the cerebral cortex ($F_{3,17}$= 1.88, $P > 0.05$) and striatum ($F_{3,17}$= 0.17, $P > 0.05$) following Met and/or MetO treatment (Figure 3).

3.4 Methionine and/or methionine sulfoxide induces oxidative stress in the cerebral cortex, hippocampus, and striatum

Table 2 shows the results of the oxidative stress analyses. Met, MetO, and Met+MetO treatment increased the level of TBARS in the cerebral cortex ($F_{3,22}$ = 5.23, $P < 0.05$); however, there was no change in ROS ($F_{3,15}$ = 0.42, $P > 0.05$), total thiol ($F_{3,17}$ = 3.13, $P > 0.05$), or nitrite ($F_{3,18}$ = 0.15, $P > 0.05$) levels. Furthermore, there was no difference in antioxidant enzyme activity, such as SOD ($F_{3,20}$ = 0.95, $P > 0.05$, Figure 4A), CAT ($F_{3,21}$ = 1.51, $P > 0.05$, Figure 4B), and GPx ($F_{3,23}$ = 0.87, $P > 0.05$, Figure 4C), compared with the control group.

In the hippocampus Met, MetO, and Met+MetO treatment increased ROS ($F_{3,14}$ = 8.42, $P < 0.05$), and TBARS ($F_{3,19}$ = 8.29, $P < 0.05$) levels, while MetO and Met+MetO treatment reduced the total thiol ($F_{3,19}$ = 6.05, $P < 0.05$) and nitrite ($F_{3,17}$= 7.40, $P < 0.05$; Table 2) content. In addition, administration of Met/MetO combined or alone reduced SOD ($F_{3,19}$ = 6.66, $P < 0.05$, Figure 4D), and GPx ($F_{3,16}$ = 12.30, $P < 0.05$, Figure 4F) activity in the hippocampus. CAT activity was reduced in the rats that received MetO or Met+MetO ($F_{3,15}$ = 8.69, $P < 0.05$, Figure 4E) when compared with the control group.

Met, MetO, and Met+MetO enhanced ROS levels ($F_{3,15}$ = 34.5, $P < 0.05$) in the striatum (Table 2). MetO and Met+MetO treatment increased TBARS levels ($F_{3,16}$ = 8.86, $P < 0.05$) and reduced total thiol content ($F_{3,19}$ = 7.93, $P < 0.05$) when compared with the control group (Table 2). Furthermore, reduced nitrite was observed in the striatum in all treatment groups ($F_{3,19}$ = 5.19, $P < 0.05$, Table 2). Figure 4 shows that MetO and Met+MetO reduced SOD ($F_{3,17}$ = 3.92, $P < 0.05$, Figure 4G) and CAT ($F_{3,16}$ = 4.24, $P < 0.05$, Figure 4H) activity in the striatum; however, no changes were observed in GPx activity ($F_{3,20}$ = 2.16, $P > 0.05$, Figure 4I).

3.5 Effect of methionine and/or methionine sulfoxide on BDNF in the hippocampus of young rats

Figure 5 showed that the concentration of BDNF was reduced in the hippocampus in MetO and Met+MetO groups when compared with the control group ($F_{3,17}$ = 6.84, P < 0.05, Figure 5).

3.6 Methionine and/or methionine sulfoxide reduces the number of NeuN-positive cells in the CA3 and DG

Figure 6A shows the CA1, CA3, and DG (area within the red square, objective lenses 5× scale bar 500 μm): CA1 (objective lenses 20×, scale bar 100 μm), CA3 (objective lenses 20×, scale bar 100 μm) and DG (objective lenses 10×, scale bar 250 μm). Areas were delimited in the hippocampus and the number of NeuN positive cells were counted (objective lenses 40×, scale bar 50 μm, Orange square [area 10,083 × 150 μm^2], figure 5B). Quantitative analysis of NeuN+ cells in the CA1 (Figure 5C), CA3 (Figure 6D), and DG (Figure 6E). We found a significant reduction in NeuN+ cells in the CA3 following Met+MetO treatment when compared with controls ($F_{3,12}$ = 4.26, P < 0.05, Figure 6D). In addition, there was a reduction in NeuN+ cells in the DG in Met, MetO, and Met+MetO groups when compared with the control group ($F_{3,12}$ = 7.52, P < 0.05, Figure 6E). No changes was founded in NeuN+ cells in the CA1 ($F_{3,12}$ = 0.10, P > 0.05, Figure 6C)

4. Discussion

Hypermethioninemia is characterized by high levels of Met and its metabolite, MetO. In patients with MAT I/III deficiency, Met plasma levels increase to up to 30-fold higher, and MetO levels may increase to 460 μM in the plasma (Gahl et al.1988). Although global epidemiological data have not been obtained to date, previous studies have shown that the incidence of MAT I/III deficiency is 1 in 27,000 newborns in the Iberian Peninsula (Marcão et al. 2015). We have previously assessed the effects of acute and chronic exposure of high levels of Met and MetO in experimental models. Based in these studies, we have shown the toxic potential of these amino acids in *in vitro* and *in vivo* (Costa et al. 2013, Dos Santos et al. 2016, Soares et al. 2017a 2017b, Stefanello et al. 2005, 2007a, 2007b, 2009, 2011, Schweinberger et al. 2015, 2018). In this study, we have demonstrated that Met and/or MetO induced memory deficits and biochemical alterations in important brain structures.

First, we assessed whether treatment affected behavior using an open-field test for motor abnormalities evaluation (Belzung et al. 2001). We found that Met and/or MetO did not alter the spontaneous locomotor activity of the animals. However, Met, MetO, and Met+MetO showed reduced rearing, indicating a lower

exploratory activity. Rearing behavior is associated with improvements in spatial cognitive map formation and space defense (Borta and Griebel 2001), which can facilitate learning and spatial memory. Therefore, it is plausible that a decrease in rearing may explain the findings of the Y-maze and object recognition tests (Pawlak and Schwarting 2002). The decrease in rearing and grooming may be associated with Met- and MetO-induced anxiolytic behavior. In line with this, Hrncic et al. (2016) demonstrated that methionine-enriched diet reduces rearing and promotes anxiety-like behavior.

We have shown that administration of Met and MetO alone, or in combination, impaired short-term object recognition memory. In addition, impairment in the Y-maze test was observed. Our results are in agreement with other studies that demonstrate memory impairment with long-term Met exposure in rats (Stefanello et al. 2007) and zebrafish (Vuaden et al. 2012). The brain is organized into multiple memory systems that are associated with different brain structures. The cerebral cortex, and more specifically, the perirhinal cortex and hippocampus contribute to recognition memory (Vann and Albasser 2011, Antunes and Biala 2012). In addition, the hippocampus plays a crucial role in spatial memory (Baste et al. 2009, Vann and Albasser 2011). Corroborating this evidence, Bast et al. (2009) demonstrated that animals with hippocampal damage were not able to learn new spatial locations within a familiar environment, similar to the results in the present study (Bast et al. 2009). Furthermore, the interaction between the hippocampus and striatum significantly contributes to the initial learning and sequential motor behavior (Ghiglieri et al. 2011, Albouy et al. 2013). Thus, damage to the cerebral cortex, hippocampus, and striatum can directly affect neural memory systems.

Our results showed that Met, MetO, and Met+MetO induced oxidative damage in the brain. The treatments increased ROS and TBARS levels and decreased the SH content and SOD, CAT, and GPx activities in the hippocampus and striatum. The enzyme and non-enzyme antioxidant defenses play an important role as ROS and reactive nitrogen species (RNS) scavengers. ROS and RNS may contribute to brain injury, activating several intracellular signaling cascades, such as mitochondrial and proteasomal dysfunction, and inflammation (Patel 2016, Suzuki et al. 2016). In this context, it is plausible that a Met and/or MetO-induced decrease antioxidant enzymatic activity could lead to an excess of reactive species, such as anion

superoxide and hydrogen peroxide, resulting in lipid peroxidation and a disruption of cellular homeostasis in brain structures.

Furthermore, nitrite levels were reduced in the hippocampus and striatum of young rats by Met and/or MetO treatment. Nitrite is the product of oxidation of nitric oxide (NO), which is an important intra and extracellular signaling molecule involved in learning and memory-related processes, and mediation of excitatory responses (Paul and Ekambaram 2011, Gasparovic et al. 2016). Decreased nitrite levels, as found in this study, may be an indirect measure of reduced levels of NO. This may be associated with the increase in ROS levels, leading to a reduction in the bioavailability of NO. This is associated with neurodegeneration, cognitive failure, and memory loss, similar to the behavior found in this present investigation (Toda and Okamura 2016).

We have evaluated the effect of chronic treatment of Met and/or MetO on AChE activity. AChE is a crucial enzyme in synaptic transmission as it rapidly hydrolyzes acetylcholine at cholinergic synapses. In addition, AChE has several other important roles in the central nervous system, such as glial activation, postsynaptic differentiation, cell adhesion, and the activation of dopaminergic neurons (Silman and Sussman 2005). Here, we showed that Met and MetO when administered alone or in combination altered the AChE activity in all brain structures evaluated. Similar results were founded by Stefanello et al. (2007), which showed an increase in AChE activity in the cerebral cortex of rats treated with Met.

The increase in the AChE activity in the cerebral cortex and hippocampus may reduce acetylcholine levels, leading to the memory deficits we observed in this study. In addition, data have demonstrated that dysfunctions on cholinergic signaling in the striatum are associated with the pathophysiology of Parkinson's disease and dystonia (Bohnen and Albin 2010). Considering the key physiological role of AChE, alterations in this enzyme may represent an important mechanism associated with the symptoms, such as cognitive deficits, delayed psychomotor development, and dystonia, noted in patients with hypermethioninemia (Mudd 2011, Schweinberger and Wyse 2016, Nashabat et al. 2018)

The rats treated with MetO and Met+MetO showed a decrease in Na^+K^+-ATPase activity in the hippocampus. This alteration may be associated with high ROS levels or reduced the neuron number observed in the same groups. High ROS production can lead to a conformational change in Na^+K^+-ATPase and induce the

endocytosis, thereby reducing plasma membrane density, and Na^+K^+-ATPase activity at the cell surface (Zhang et al. 2008). Schweinberger et al., (2016) showed that although high Met levels inhibited Na^+K^+-ATPase activity, the expression and content of this enzyme is increased in the brain of offspring (Schweinberger 2016). This result indicates that high ROS can directly influence Na^+K^+-ATPase activity.

The Na^+K^+-ATPase α3 isoform is highly expressed in neuronal projections, which suggest that it may be the main isoform affected by MetO and Met+MetO, because there is a reduction in the number of neurons (Clausen et al. 2017). Also, it was demonstrated that the Na^+K^+-ATPase α3 isoform has functional significance in the control of spatial learning and memory (Holm et al. 2016).

In addition to the decrease in Na^+K^+-ATPase activity in the hippocampus, we have demonstrated that MetO and Met+MetO treatment reduced the levels of BDNF. BDNF is essential for hippocampal long-term potentiation (LTP) (Leal et al. 2015, 2016). This is the main form of synaptic plasticity and is indicative of the efficacy of the synaptic information storage, which is the main cellular mechanism correlated learning and memory (Leal et al. 2015, 2016). In addition, BDNF can control differentiation and neuronal survival. In this sense, a decreased hippocampal BDNF concentration corroborates the reduction in NeuN, which also have an important cognitive function (Leal et al. 2015, 2016). Schweinberger el al., (2018) also demonstrated that Met administration in pregnant rats alters the ultrastructure of neurons in the brain of offspring, corroborating the data found in this study (Schweinberger et al. 2018).

The hippocampal formation is sensitive to pathophysiological changes, which can affect hippocampal-dependent functions and reduce the production of new neurons and structural changes (Huang et al. 2015). The mechanism that leads to cognitive dysfunction is associated with the hippocampus is complex; however, persistent oxidative stress plays an important role in this function (Huang et al. 2015). Another point to be considered is that DG is a region with the presence of precursor cells of neurons which seems to be the most affected, demonstrating reduction of neurons and increase of apoptosis under toxicological conditions (Rahmeier et al. 2016, Huf et al. 2018). Thus, alterations in this region, as observed in this work, could interfere in the other regions of the hippocampus.

The neurons generated postnatally in the subgranular region of the hippocampus are involved in spatial learning and memory. The generation of new neurons in dentate gyrus is important for hippocampal functioning, and has been implicated in spatial memory. Furthermore, it has been demonstrated that MetO and Met+MetO reduce the number of neurons in the DG, suggesting that these amino acids may impact neurogenesis of the hippocampus (Erasso et al. 2013).

In conclusion, the results obtained in the present study showed that chronic exposure to Met and/or MetO induced memory deficits in young rats *via* increased oxidative stress and AChE activity in the cerebral cortex, hippocampus, and striatum. MetO alone or in combination with Met also decreased Na^+,K^+-ATPase and BDNF levels, and the number of neurons in the hippocampus (Figure 7). These data assist with understanding the neurological changes found patients with hypermethioninemia. In addition, these results provide new perspectives for future studies that seek therapeutic targets for this pathology.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure and tables

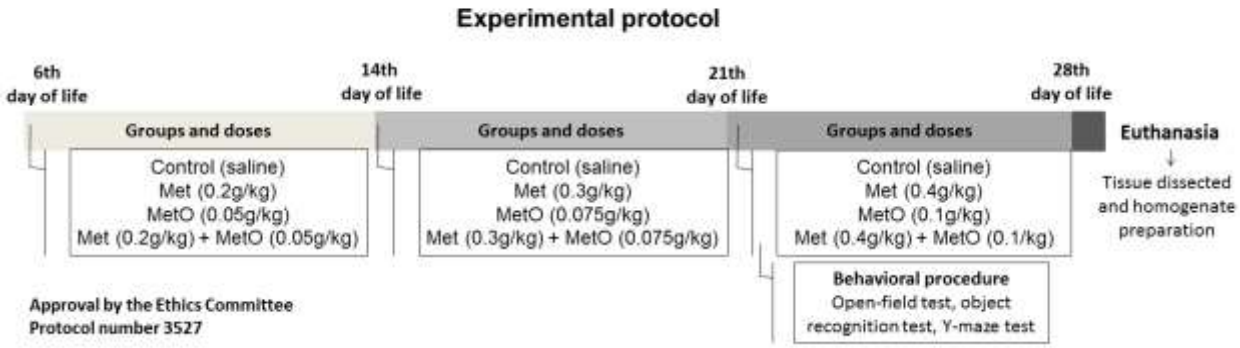

Figure 1 - Schematic of the experimental protocol

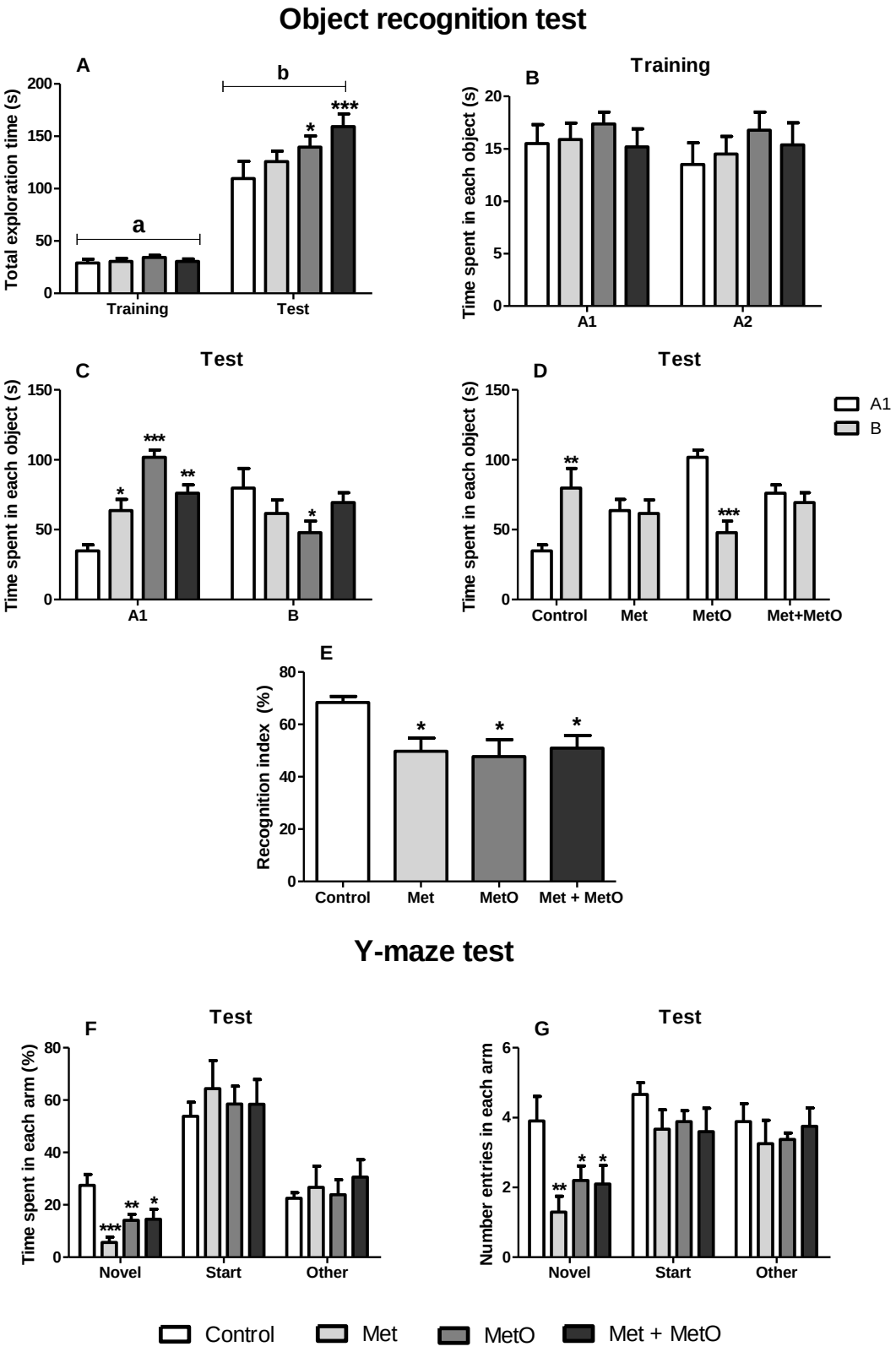

Figure 2 - Methionine (Met) and/or methionine sulfoxide (MetO) impairs both short-term and spatial memory in young rats 21 days after treatment. (**A**) Total exploration time (s); (**B**) Time spent on each object – Training (s); (**C**) Time spent on each object – Test (s); (**D**) Total time

spent during the test session on each object; **(E)** Exploratory preference time for the novel object expressed as a percentage evaluated in the object recognition test. (**F**) Exploratory preference time in the novel, start, and other arms, expressed as a percentage of the total exploration time in the Y-maze test and (**G**) Number of entries in the novel, start, and other arms evaluated in the Y-maze test. Two-way ANOVA and post-hoc Bonferroni multiple comparisons test were used for A–D. One-way ANOVA, and post-hoc Tukey's multiple comparisons tests were performed for E-G. A1 and A2 report the familiar object and B refers to new objet. Bars represent mean and ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, compared with control group (n = 9–10). In the figure 2A, **a** and **b** denote a significant difference between training and test *P < 0.05.

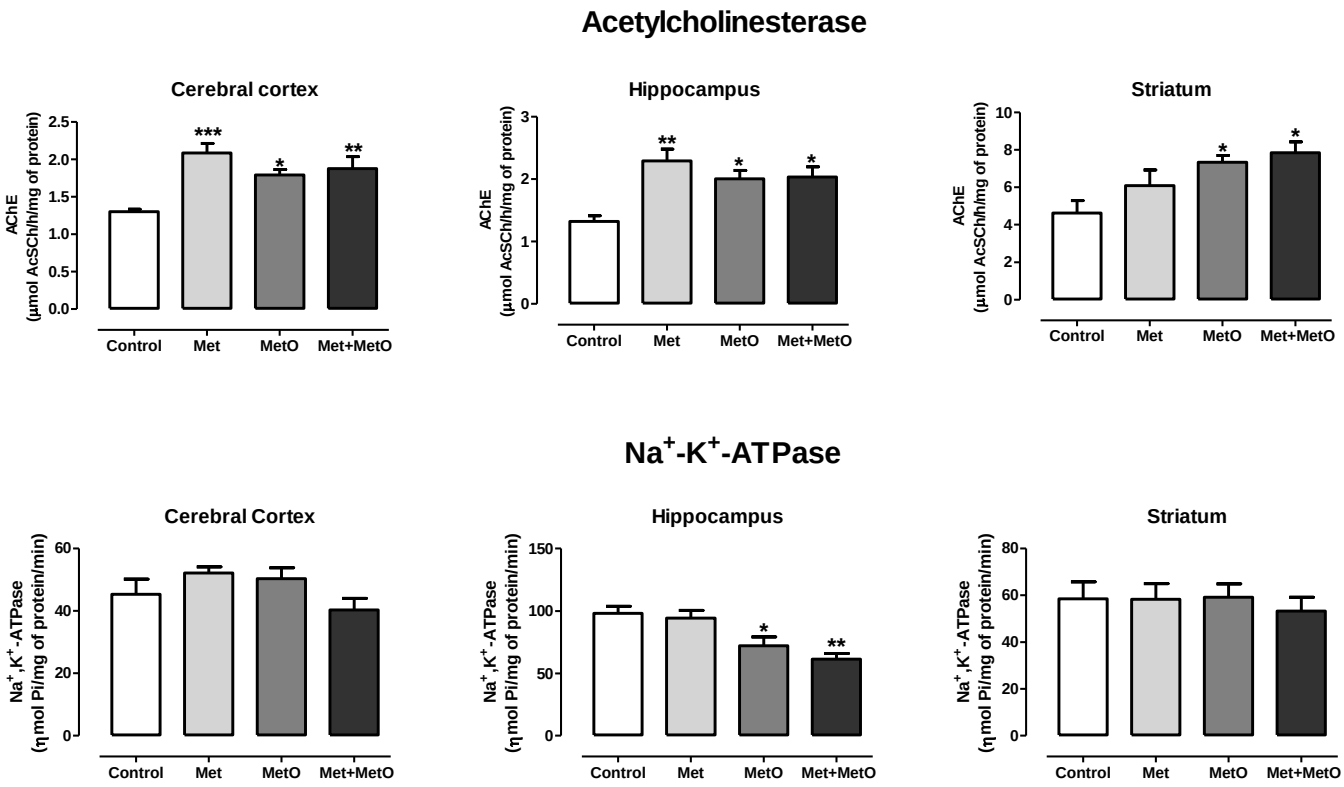

Figure 3 - Acetylcholinesterase and Na^+, K^+-ATPase activity in the cerebral cortex, hippocampus, and striatum of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). AChE activity is expressed in µmol AcSCh/h/mg protein and Na^+, K^+-ATPase activity as nmol/Pi released/min/mg protein. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Bars represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with the control group (n = 4–7).

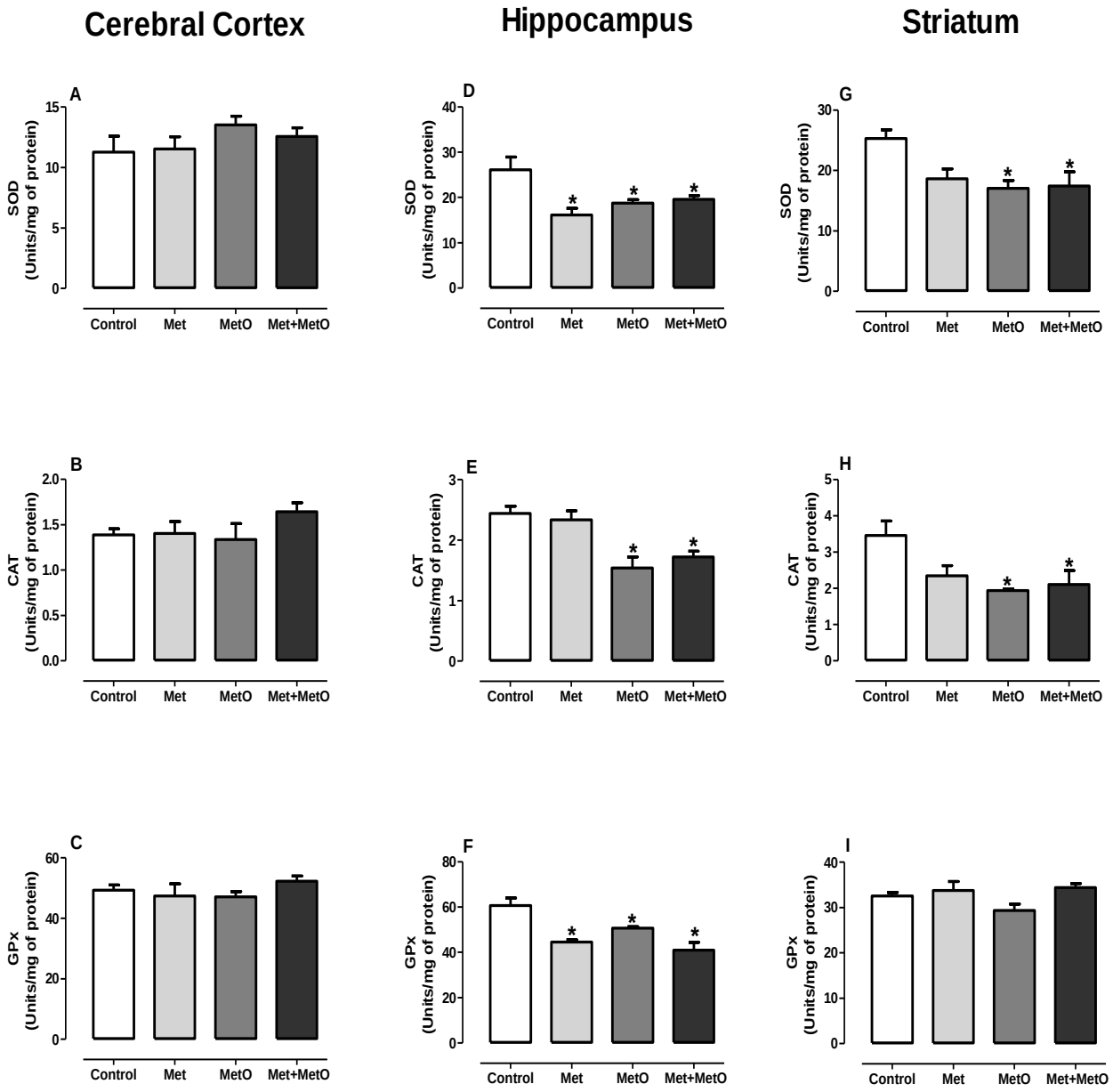

Figure 4 - Superoxide dismutase (SOD) catalase (CAT) and glutathione peroxidase (GPx) activity in the cerebral cortex (A–C), hippocampus (D–F), and striatum (G–I) of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). CAT, SOD, and GPx activity are reported as units/mg protein. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Bars represent mean ± SEM. *P < 0.05, compared with the control group (n= 4–7).

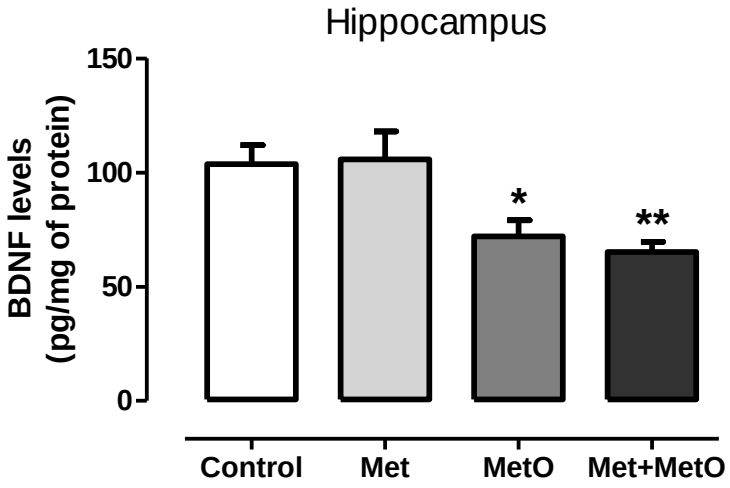

Figure 5 - Concentration of brain-derived neurotrophic factor (BDNF) in the hippocampus of young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). BDNF is reported as pg/mg protein. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Bars represent mean ± SEM (n = 4–7). *P < 0.05, **P < 0.01 compared with the control group (n = 5–6).

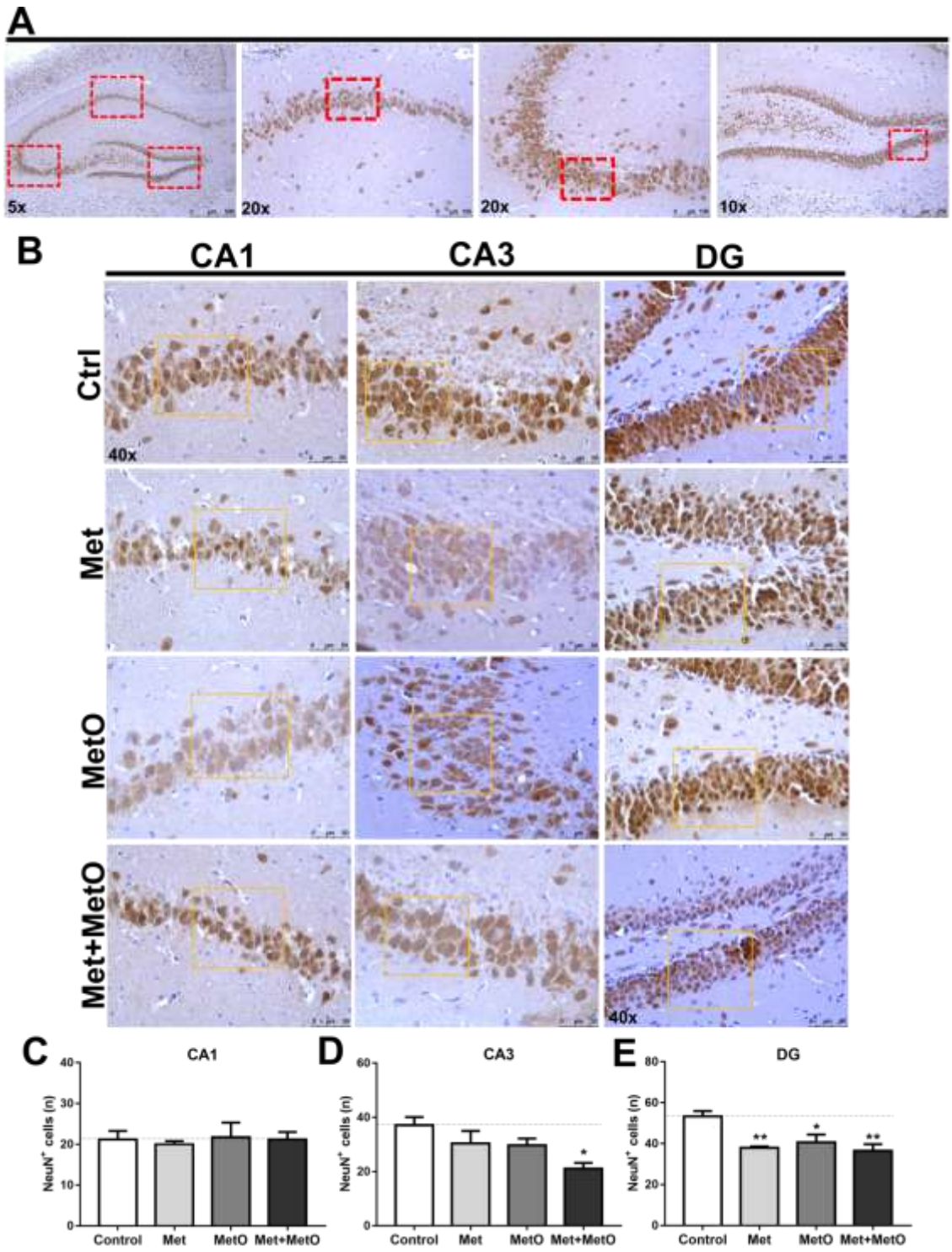

Figure 6 - Number of NeuN-positive cells in the CA1, CA3, and DG from young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO). Data are reported as mean ± SEM. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. **P* < 0.05 and ***P* < 0.01, compared with the control group (n = 4).

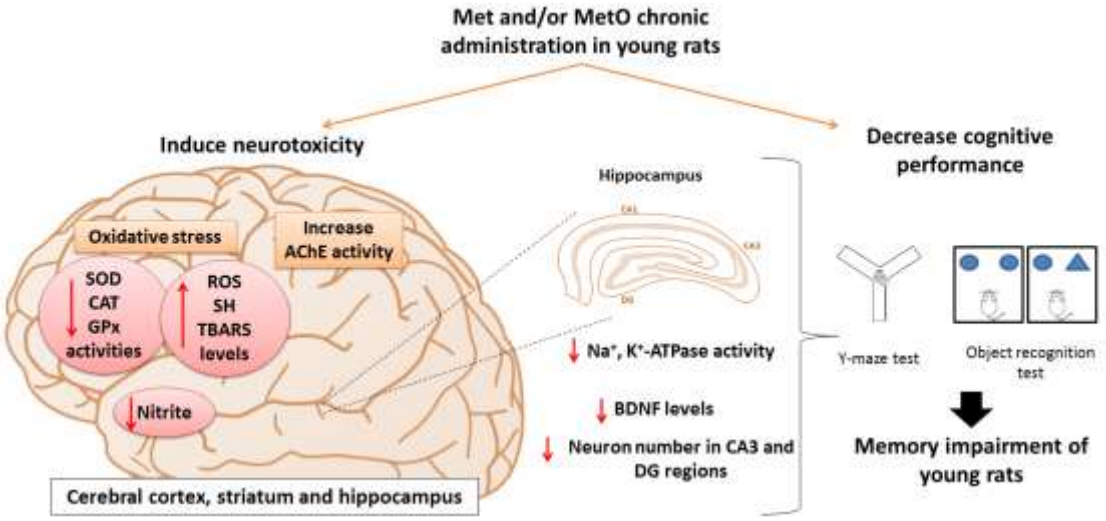

Figure 7- Effect of chronic administration of methionine (Met) and methionine sulfoxide (MetO) on the behavioral and neurochemical parameters in young rats. SOD, Superoxide dismutase; CAT, Catalase; GPx, Glutathione peroxidase; ROS, reactive oxygen species; SH-total, thiol content; TBARS, thiobarbituric acid reactive species; AChE, acetylcholinesterase; BDNF, Brain-derived neurotrophic factor.

Table 1 - Effect of methionine (Met) and/or methionine sulfoxide (MetO) on crossing, rearing, grooming, and number of fecal droppings in the open field-test

	Groups			
	Control	**Met**	**MetO**	**Met+MetO**
Total crossing	42.32 ± 8.01	40.03 ± 2.05	42.40 ± 5.44	46.00 ± 9.96
Rearing	30.10 ± 4.52	8.0 ± 1.30***	12.13 ± 1.98**	9.50 ± 2.14***
Grooming	3.77 ± 0.27	3.18 ± 0.29	2.50 ± 0.26*	2.50 ± 0.37*
Feces	2.34 ± 0.36	1.09 ± 0.39	1.20 ± 0.35	1.62 ± 0.32

One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Data were reported as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, compared with the control group (n = 9–10).

Table 2 - ROS, TBARS, SH, and nitrite levels in the cerebral cortex, hippocampus and striatum from young rats 21 days after treatment with methionine (Met) and/or methionine sulfoxide (MetO).

	Groups			
	Control	**Met**	**MetO**	**Met+MetO**
Cerebral Cortex				
ROS	102.0±17.5	120.6±14.33	107.7±10.47	99.74±5.0
TBARS	0.66±0.03	0.80±0.03*	0.82±0.02*	0.81±0.08*
SH	27.85±1.07	25.20±1.82	27.23±0.70	30.48±1.09
Nitrite	15.50±0.70	14.52±1.77	15.60±1.54	15.6±0.90
Hippocampus				
ROS	24.05±2.1	64.68±10.07**	69.23±5.1**	63.36±9.3*
TBARS	0.48±0.06	0.66±0.01*	0.65±0.04*	0.73±0.01*
SH	29.10± 1.34	26.24±2.4	20.39±0.73*	19.60±1.63*
Nitrite	15.50± 1.29	13.72±1.54	8.42±0.78**	10.50±0.70*
Striatum				
ROS	29.10±5.74	149.3±14.0***	64.62±2.74*	67.26±9.3*
TBARS	0.57±0.01	0.59±0.03	0.87±0.07*	0.77±0.04*
SH	29.08±2.63	29.46±2.43	18.57±0.71*	19.51±1.36*
Nitrite	26.86± 2.44	18.47±2.69*	17.63±0.93*	14.78±1.55**

ROS levels are expressed as µmol/DCF protein, TBARS levels are reported as nmol TBARS/mg protein, thiol content are expressed as nmol/TNB/mg protein, and nitrite levels are expressed as µM nitrite/mg of protein. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Values are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, compared with the control group. (n = 4–7).

4.5 Manuscrito II

O manuscrito será submetido à revista Cellular and Molecular Neurobiology.

Hypermethioninemia induces neurochemical and morphological dysfunction in cultured astrocytes: involvement of ion pump activity, oxidative status, and cholinergic and purinergic signaling

Mayara Sandrielly Pereira Soares[1*], Nathalia Stark Pedra[1], Natália Pontes Bona[2], Anita Almeida Ávila[1], Juliana Hofstatter Azambuja[3], Elizandra Braganhol[3], Francieli Moro Stefanello[2], Roselia Maria Spanevello[1]

[1] Programa de Pós-Graduação em Bioquímica e Bioprospecção - Laboratório de Neuroquímica, Inflamação e Câncer, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N, Pelotas, RS, Brazil.
[2] Programa de Pós-Graduação em Bioquímica e Bioprospecção - Laboratório de Biomarcadores, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N, Pelotas, RS, Brazil.
[3] Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

*Address reprint requests to: Mayara Sandrielly Pereira Saores, Universidade Federal de Pelotas, Campus Universitário s/n, Capão do Leão, RS, Brazil, CEP: 96010-900, Phone: 55 53 32757355, Fax: 55 53 32757354. E-mail: mspereirasoares@gmail.com

Abstract

The aim of this work was to investigate the *in vitro* effects of methionine (Met) or methionine sulfoxide (Met-SO) on viability, proliferation, necrotic death, morphology, and neurochemical parameters in primary culture of cortical astrocytes, after treatment with 1 or 2 mM Met or 0.5 mM Met-SO, for 24, 48, and 72 h. Although Met or Met-SO did not affect cell viability and proliferation, they induced astrocyte hypertrophy. Acetylcholinesterase activity increased, while Na^+, K^+-ATPase activity decreased after 24, 48, and 72 h incubation with 2 mM Met, Met-SO, or Met (1 and 2 mM) + Met-SO ($P < 0.05$). ATP and AMP hydrolysis decreased by Met (1 and 2 mM), Met-SO and Met (1 and 2 mM) + Met-SO treatment, while ADP hydrolysis enhanced by Met-SO and Met (1 and 2 mM) + Met-SO ($P < 0.05$) treatment. Superoxide dismutase activity increased by Met-SO and Met (1 and 2 mM) + Met-SO after 24 h of exposure ($P < 0.05$). Catalase and glutathione S-transferase activities reduced by Met (1 and 2 mM) or Met-SO treatment for 48 and 72 h ($P < 0.05$). Reactive oxygen species and total thiol content reduced by Met (1 and 2 mM) or Met-SO treatment for 24, 48, and 72 h of exposure, while nitrite and thiobarbituric acid reactive substance levels increased under the same experimental conditions ($P < 0.05$). These findings elucidate hypermethioninemia induced changes in brain astrocyte function, in the presence of high concentrations of Met and Met-SO.

Key Words: Astrocytes, purinergic signaling, oxidative status, acetylcholinesterase, Na^+, K^+-ATPase, hypermethioninemia

1. Introduction

Hypermethioninemia is characterized by high plasma concentrations of methionine (Met) and its metabolites such as methionine sulfoxide (Met-SO) (Mudd 2011, Mudd et al. 2001), and is caused by genetic and non-genetic conditions (Mudd 2011; Schweinberger and Wyse 2016). Patients with this pathology may present severe neurological symptoms such as cognitive deficits, demyelination, memory impairments, and cerebral edema (Couce et al. 2008; Mudd 2011; Schweinberger and Wyse 2016; Nashabat et al. 2018). However, little is known about the cellular and molecular changes underlying these symptoms.

Studies using experimental models have shown toxicity due to high concentrations of Met or Met-SO, and involvement of oxidative stress and inflammation in neuronal and non-neuronal changes in both *in vitro* and *in vivo* experiments (Costa et al. 2013; Dos Santos et al. 2017; Soares et al. 2018). In the brain, high Met concentration alters the antioxidant enzyme activity, acetylcholinesterase (AChE), and Na^+, K^+-ATPase activities, induces lipid peroxidation and reduces membrane lipid content (Stefanello et al. 2007b; Stefanello et al. 2007c). Hitherto, our research group showed that acute administration of Met or Met-SO causes oxidative stress, reduces cell viability, induces DNA damage, and causes apoptotic cell death in the cerebral cortex of young rats (Soares et al. 2017).

Astrocytes are specialized glial cells that participate in brain circuitry and processing, in addition to many essential functions in the brain such as the formation, maturation, and elimination of synapses, ionic homeostasis, clearance of neurotransmitters, regulation of extracellular space volume, and modulation of synaptic activity and plasticity. However, changes in astrocyte functions have been described in many neuropathologies (Sofroniew and Vinters, 2010; Sica et al. 2016; Vasile et al. 2017). Nevertheless, the effect of high concentrations of Met and Met-SO on astrocyte cultures have not yet been reported in the literature.

Astrocytes have many proteins, including essential enzymes for proper functionality, and maintenance of homeostasis in the central nervous system (CNS) (Sofroniew and Vinters, 2010; Sica et al. 2016; Vasile et al. 2017). For example, astrocytes express α2 isoform of sodium-potassium adenosine triphosphatase (Na^+, K^+-ATPase), which is essential for modulation of the electrochemical gradient in CNS cells (Moseley et al. 2007). In addition, AChE, which cleaves acetylcholine (ACh) into

choline and acetate, also plays a critical role in the modulation of astrocytic activation against neuronal insults (Chacón et al. 2003).

In addition, astrocytes are the first line of defense against oxidative insults in the CNS, as evidenced in several neuropathology conditions (Bhatia et al. 2019). This condition is characterized by high concentrations of reactive species, and markers of protein and lipid damage associated with the decrease in the enzymatic and non-enzymatic antioxidant defenses (Bhatia et al. 2019). Considering several studies showed that oxidative stress plays a role in hypermethioninemia, investigation of these markers in the astrocytes becomes interesting.

Both acute and chronic CNS damage releases large amounts of ATP, which in excess binds to the P2X and P2Y receptors located in the astrocytes, and generates purinergic signaling related to the onset of astrogliosis (Franke and Illes 2014; Cisneros-Mejorado 2015). The released ATP acts as an excitotoxic and neuroinflammatory molecule. In contrast, adenosine, produced by the ecto-enzymatic cleavage of ATP, is released by astrocytes directly into the extracellular environment (Burnstock 2007, 2013; Frankis and Illes, 2014; Illes and Verkhratsky 2016). Adenosine exerts an anti-ATP effect, acting as an anti-inflammatory and immunosuppressant, through its interaction with P1 receptors to play an essential role in presynaptic neuromodulation. Purinergic signaling is emerging as a major means of integrating functional activity between neurons and glial cells in the CNS (Burnstock 2007, 2013; Franke and Illes 2014; Cisneros-Mejorado 2015).

In the context of hypermethioninemia, investigation of enzyme activities that modulate the extracellular levels of nucleotides and nucleosides of adenine in the astrocytes is vital for understanding the neuropathological mechanisms of this disorder. Astrocytes express the whole cascade of ecto-enzymes belonging to the purinergic system, such as ecto-nucleoside triphosphate diphosphohydrolase (NTPDase), ecto-5'-nucleotidase, and adenosine deaminase (Burnstock 2007, 2013). Thus, this work aimed to investigate the *in vitro* effects of Met or Met-SO on cell viability, proliferation, and morphology, parameters of oxidative stress, AChE, Na^+, K^+- ATPase, and the ATP, ADP, and AMP hydrolysis in primary culture of cortical astrocytes derived from brains of *Wistar* rats.

2. Materials and Methods

2.1 Animals and ethical procedures

Wistar rats (1–2 days old) from Central Animal House of the Federal University of Pelotas, Pelotas, RS, Brazil, were kept in a room under a standard 12/12 h light/dark cycle and controlled temperature (22 ± 2 ºC). All animal procedures were approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, Brazil, under the protocol number CEEA 6210-2017. The use of the animals is by following the Brazilian Guidelines for the Care and Use of Animals in Scientific Research Activities (DBCA - 2013) and the National Council of Control of Animal Experimentation (CONCEA).

2.2 Astrocytic Cultures

Primary astrocytic cultures were prepared as described by Gottfried et al. 1999. Firstly multi-well plates used for growing cultures were pre-treated with poly-l-lysine. The newborn (1–2 days old) *Wistar* rats were euthanized by decapitation, and the cerebral cortex was removed and mechanically dissociated with calcium and magnesium-free balanced salt buffer (pH 7.4). After that, the meninges were removed, and the cerebral cortex was mechanically dissociated by sequential passage through a pipette. The cell suspension was settled through centrifugation at 1000 rpm for 10 min, and the pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), pH 7.6. Subsequently, the cells were seeded in plates (6, 48, and 96-well plates) and 4 h after seeding, the plates were washed, and fresh medium was added. Cultures were maintained at 5% CO_2, 37 ºC, and humidified atmosphere for 20 days until confluence, and the medium was replaced every four days (Figure 1).

2.3 Culture treatment with methionine and methionine sulfoxide

Met and Met-So were dissolved in water and subsequently mixed with DMEM with 10% FBS. Astrocytes were treated with concentrations of 0.5 mM Met-SO and 1

or 2 mM Met in combination or alone. For 24, 48 and 72 h. The control cells were maintained only in DMEM with 10% FBS (Figure 1).

2.4 Cell viability assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were washed with PBS, and 0.5 mg/mL MTT was added and incubated for 90 min at 37 °C and 5% CO_2. The MTT solution was removed and the precipitate dissolved with dimethyl sulfoxide (DMSO). The absorbance determined at 492 nm was proportional to the number of cells with active mitochondria.

2.5 Cell proliferation assay

Cell density was determined by the sulforhodamine B (SRB) assay by measurement of cell protein. Cultures were washed and fixed in trichloroacetic acid (50%) for 45 min at 4 °C. After washing five times with distilled water, 0.4% SRB in acetic acid was added and incubated for 30 min. The plates were washed five times with 1% acetic acid for the complete removal of unbound dye. Finally, the dye was eluted with 10 mM Tris solution and the absorbance measured at 530 nm.

2.6 Assessment of cell death pathway by propidium iodide (PI)

Flow cytometry with the use of propidium iodide (PI), allows verification of cell death by necrosis. Cell death was followed by fluorescence image analysis after incorporation of PI. After treatment with Met or Met-SO, the cells were incubated with PI (7.5 µM) for 1-h. The fluorescence excitation was at 515-560 nm using an inverted microscope (Olympus IX71, Tokyo, Japan) equipped with a standard rhodamine filter. The images were captured using a digital camera connected to the microscope.

2.7 Acetylcholinesterase (AChE) activity

AChE activity was determined as previously described (Ellman et al. 1961). First, the reaction system composed of 10 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), 100 mM phosphate buffer (pH 7.5), and 15 µL of cell lysate, was incubated for 2 min at 27 °C. Subsequently, 8 mM acetylthiocholine was added and the absorbance determined at 412 nm for 2 min at 30-sec intervals at 27 °C. AChE activity was expressed as the percentage of control.

2.8 Na^+, K^+-ATPase activity

Buffer containing 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, was used for Na+, K+-ATPase activity assay along with 50 µL of cell lysate. The reaction was initiated by adding ATP for a final concentration of 3.0 mM. Control assays were carried out under the same conditions with the addition of 1.0 mM ouabain. Na+, K+-ATPase activity was calculated from the difference between the two assays, according to the method of Wyse et al. 2007 and the released inorganic phosphate (Pi) was measured by the method of Chan et al. 1986. The specific activity of the enzyme was expressed as a percentage of control.

2.9 Ecto-nucleotidase assay

The NTPDase activity using ATP and ADP as substrates, and 5'-nucleotidase activity using AMP as substrate, were measured in 48-well plates containing astrocytes that were washed three times with phosphate-free incubation medium in the absence of nucleotides. The enzymatic reaction was started by the addition of 200 µL of incubation medium containing 2 mM $CaCl_2$ (2 mM $MgCl_2$ for AMPase assay), 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES (pH 7.4), and 2 mM ATP, ADP, or AMP as substrates. Following 10 min incubation at 37 °C, the reaction was stopped by transferring an aliquot of the incubation medium to a pre-chilled tube containing TCA (final concentration 5 % w/v). The release of Pi was measured by the malachite green method (Chan et al., 1986), using KH_2PO_4 as a Pi standard. Controls to determine the non-enzymatic Pi release was performed by

incubating the cells in the absence of the substrate, or the substrate in the absence of the cells. All assays were in triplicate.

2.10 Oxidative stress parameters

2.10.1 Reactive Oxygen Species (ROS)

ROS generation was measured by oxidation of 2′-7′-dichlorodihydrofluorescein diacetate (DCFHDA) to dichlorofluorescein (DCFH) catalyzed by the esterase in the sample. DCFHDA reacts with ROS emitting fluorescence. In brief, after treatment with Met and Met-SO, cultures were incubated with 1 mM DCFHDA for 30 min, and fluorescence measured at 488/525 nm in a microplate reader (SpectraMax M3). ROS production was reported as a percentage of control (Dos Santos et al. 2016).

2.10.2 Nitrite levels

Nitrite levels were measured after 24, 48, and 72 h treatment with Met or Met-SO by a colorimetric reaction with Griess reagent (Stuehr and Nathan 1989), and 100 µL of sulfanilamide in 5% phosphoric acid was added to 100 µL cell-culture supernatants and maintained at room temperature for 10 min, followed by addition of 100 µL of Griess reagent (0.1% N-[1 naphthyl]ethylenediamine dihydrochloride). After incubation for 10 min in the dark, the absorbance was measured at 540 nm. Nitrite levels reported as a percentage of control.

2.10.3 Total sulfhydryl (SH) Content

The total SH content in cell lysates was determined using the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent as described by Aksenov and Markesbery (2001). The reaction was based on the reduction of DTNB by thiols resulting in a yellow derivative (TNB) whose absorption is read at 412 nm. SH levels were reported as a percentage of control.

2.10.4 Thiobarbituric acid reactive substances (TBARS)

Determination of TBARS was according to Esterbauer and Cheeseman (1990) with minor modifications. Lysates were mixed with trichloroacetic acid and thiobarbituric acid, and heated in a boiling water bath for 30 min. Determination of TBARS was by measuring the absorbance at 535 nm. Results reported as a percentage of control.

2.10.5 Superoxide Dismutase (SOD)

The SOD activity was measured in the lysates, according to Misra and Fridovich (1972). This method is based on inhibition of superoxide-dependent adrenaline auto-oxidation measured using a spectrophotometer adjusted to 480 nm. Results reported as a percentage of control.

2.10.6 Catalase (CAT)

CAT activity is based on the decomposition of H_2O_2 monitored at 240 nm at ambient temperature and was performed according to Aebi (1984). The CAT activity reported as a percentage of control.

2.10.7 Glutathione S-transferase (GST)

GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the method of Habig et al. (1974). The assay medium consisted, 1 mM CDNB (in ethanol), 10 mM glutathione (GSH), 20 mM potassium phosphate buffer (pH 6.5), and 20 µL cell lysate. The activity expressed as a percentage of control.

2.11 Protein determination

AChE, Na+, K+-ATPase, and ecto-nucleotidase proteins in the assays were determined using the Bradford (1976) method, while the protein concentration for

oxidative stress parameters was measured by Lowry et al. (1951). In both methods, the bovine serum albumin was used as the standard.

2.12 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 and one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Furthermore, all data were expressed as mean ± standard error, and the differences between mean values were considered significant at $P < 0.05$.

3 Results

3.1 Cell viability, proliferation, and morphological analysis

As shown in Figure 2, no significant change in cell viability (Figure 2A) or proliferation (Figure 2 B) was observed in astrocytes exposed to Met and / or Met-SO after 24, 48 and 72 h of treatments ($P > 0.05$). Similarly, no significant change was seen in PI incorporation into astrocytes exposed for 72 h to Met and/or Met-SO, indicating no necrotic cell death (Figure 2C). However, interestingly, exposure of astrocytes to Met and/or Met-SO under all experimental conditions and times (24, 48 and 72 h) showed significant morphological alterations as seen by a significantly larger cell size when compared to control cells (Figure 3).

3.2 Acetylcholinesterase (AChE) and Na^+, K^+- ATPase activities

Figure 4 shows, enhancement in AChE activity in astrocytes after 24 and 48 h exposure to 0.5 mM Met-SO ($P < 0.01$ and $P < 0.05$ respectively), 1 mM Met + 0.5 mM Met-SO ($P < 0.05$ and $P < 0.01$ respectively), and 2 mM Met + 0.5 mM Met-SO ($P < 0.05$ and $P < 0.01$ respectively) when compared to control cells (Figure 4). After 72 h exposure to 0.5 mM Met-SO ($P < 0.01$), AChE activity remained elevated when compared to the control cells (Figure 4). The Na^+, K^+-ATPase showed no changes in activity during 24 h ($P > 0.05$) (Figure 4). However, after 48 h exposure to 2 mM Met

($P < 0.05$), 0.5 mM Met-SO ($P < 0.01$), 1 mM Met + 0.5 mM Met-SO ($P. < 0.01$), and 2 mM Met + 0.5 mM Met-SO ($P < 0.001$) showed a significant reduction of Na^+, K^+-ATPase activity when compared to control cells (Figure 4). Similar results were seen under all the experimental conditions ($P < 0.05$) when the astrocytes were exposed for 72 h, with a significant reduction in the Na^+, K^+-ATPase activity when compared to the control cells (Figure 4).

3.3 ATP, ADP, and AMP hydrolysis

ATP hydrolysis was reduced in the three experimental times evaluated (Figure 5). After 24 h, there was a reduction in the ATP hydrolysis when the astrocytes were exposed to 2 mM Met ($P < 0.01$), 0.5 mM Met-SO ($P < 0.01$), 1 mM Met + 0.5 mM Met-SO ($P < 0.01$), and 2 mM Met + 0.5 mM Met-SO ($P < 0.05$) when compared to control cells. Yet, after 48 and 72 h, the reduction in the ATP hydrolysis remained in astrocytes exposed to 0.5 mM Met-SO ($P < 0.001$), 1 mM Met + 0.5 mM Met-SO ($P < 0.001$ and $P < 0.05$ respectively), and 2 mM Met + 0.5 mM Met-SO ($P < 0.01$ and $P < 0.05$ respectively) compared with the control cells.

Hydrolysis of ADP increased when astrocytes were incubated with this nucleotide for 48 h in presence of 2 mM Met ($P < 0.001$), 0.5 mM Met-SO ($P < 0.05$), 1 mM Met + 0.5 mM Met-SO ($P < 0.05$), and 2 mm Met + 0.5 mM Met-SO ($P < 0.05$) in comparison to control group. No change in the hydrolysis of this nucleotide was seen in astrocytes, after 24 and 72 h exposure to amino acids.

5'-nucleotidase activity was significantly lower after 48 and 72 h of exposure to amino acids. AMP hydrolysis was significantly reduced in astrocytes exposed for 48 and 72 h to 1 mM Met ($P < 0.001$ and $P < 0.001$ respectively), Met 2 mM ($P < 0.001$ and $P < 0.05$ respectively), 0.5 mM Met-SO ($P < 0.05$ and $P< 0.01$ respectively), 1 mM Met + 0.5 mM Met-SO ($P < 0.001$ and $P < 0.001$ respectively), and 2 mM Met + 0.5 mM Met-SO ($P < 0.001$ and $P < 0.001$ respectively) when compared to the control cells.

3.4 Oxidative stress parameters

The results of the oxidative stress parameters are described in Table 1 and Figure 6. A significant reduction in ROS production by astrocytes was seen after 24 h

exposure under all experimental conditions when compared to control cells (P < 0.001, Table 1). In contrast, no changes were observed in ROS production by astrocytes exposed for 48 h to Met and / or Met-SO exposure (P > 0.05, Table 1). Interestingly, ROS levels were reduced after 72 h of exposure to 0.5 mM Met-SO (P < 0.05), 1 mM Met + 0.5 mM Met-SO (P < 0.01), and 2 mM Met + 0.5 mM Met-SO (P < 0.01) compared to control cells (Table 1). Nitrite levels were increased when the cells were exposed to 0.5 mM Met-SO for 24 and 48 h (P < 0.05, Table 1). On the other hand, after 72 h of exposure, this increase was present in all experimental conditions (Met and/or Met-SO) compared to control cells (P < 0.001, Table 1).

A reduction in SH levels was observed when astrocytes were treated with the combination of 1 or 2 mM Met + 0.5 mM Met-SO for 24 h (P < 0.01, Table 1). This reduction was significant after 48 h in all experimental conditions when compared with control cells (P < 0.01, Table 1). After 72 h, there was a reduction in SH concentration when the astrocytes were exposed to 2 mM Met, 0.5 mM Met-SO, 1 mM Met +0.5 mM Met-SO, and 2 mM Met + 0.5 mM Met-SO in relation to the control cells (P < 0.05, Table 1). TBARS levels were increased in the astrocytes treated for 72 h with 2 mM Met (P < 0.05), 0.5 mM Met-SO (P < 0.001), 1 mM Met + 0.5 mM Met-SO (P < 0.001), and 2 mM Met + 0.5 mM Met-SO (P < 0.001). No change was observed after 24 and 48 h of exposure to 1 or 2 mM Met and/or 0.5 mM Met-SO (P > 0.05, Table 1).

SOD activity was highest in astrocytes exposed for 72 h to 0.5 mM Met-SO (P < 0.01) or 1 and 2 mM Met + 0.5 mM Met-SO (P < 0.05) compared to the control. At exposure times of 24 and 48 h, no changes were observed (P > 0.05) (Figure 6). The CAT activity was reduced 24 h after exposure of the astrocytes to a combination of 1 and, 2 mM Met + 0.5 mM Met-SO (P < 0.05). This increase was present in all experimental conditions at 72 h when compared to control cells (P < 0.001). A reduction in GST activity was seen after 48 h of exposure to Met-SO (P < 0.05), 1 mM Met + 0.5 mM Met-SO (P < 0.05), and 2 mM Met + 0.5 mM Met-SO (P < 0.01) compared to the control cells (Figure 6). Similar results were seen when cells were treated for 72 h with 1, and 2 mM Met + 0.5 mM Met-SO compared to control cells (P < 0.05) (Figure 6). No changes were seen in the CAT and GST activities after 24 h of treatment (Figure 6).

4 Discussion

Several *in vitro* and *in vivo* studies using experimental models of hypermethioninemia showed many neurological changes (Schweinberger and Wyse 2016; Soares et al. 2017). An investigation into changes occurring in the brain is essential because the most prominent clinical symptom in hypermethioninemia is neurological dysfunction, including cognitive deficit, edema, delay in psychomotor development, and facial dysmorphism (Couce et al., 2008; Mudd 2011; Schweinberger and Wyse 2016). Some studies reported that these symptoms may be associated with cerebral dysmyelination attributed to S-adenosylmethionine deficiency in the cerebrospinal fluid, a condition identified as the leading cause for isolated and persistent hypermethioninemia, due to deficiency of the enzyme methionine adenosyltransferase (MAT I/III) (Suertees et al., 1991; Mudd 2011). However, the exact mechanisms, as well as the involvement of specific CNS cells have not yet been elucidated.

The concentrations of Met and Met-SO used in this work are similar to levels seen in the MAT I/III deficiency, in which Met plasma levels increase up to 30-fold higher, and Met-SO levels may reach 460 μM in the plasma (Gahl et al. 1988). Also, previous *in vitro* studies from our group showed that Met and/or Met-SO at concentrations used in this study, induce M1/classical macrophage polarization, modulate oxidative stress, and purinergic signaling (Dos Santos et al. 2017). Considering this, in this study, we aimed to understand the mechanisms involved in the modulation of CNS function in hypermethioninemia and analyzed the effects of Met and Met-SO alone or together in primary astrocyte culture.

Our results showed, no effect on cell viability, proliferation, or induction of necrosis when astrocytes were exposed to Met and/or Met-SO over 24, 48, and 72 h. These findings are in agreement with the study by Dos Santos et al., (2017) wherein Met and / or Met-SO did not alter, *in vitro* viability, and proliferation of primary peritoneal macrophages. In addition, in other models of inborn errors of sulfur amino acid metabolism, it was shown that high levels of homocysteine, as seen in homocystinuria, induces glial reactivity but did not affect membrane integrity or the cell viability of adult rat astrocytes in culture (Longoni et al. 2017).

In this study, we did not evaluate the glial reactivity in astrocyte, as the primary mechanism in reactive astrocytes is changes in the gene expression and cell

hypertrophy (Anderson and Sofroniew 2014). Interestingly, we observed that Met and / or Met-SO at all times and concentrations tested, is capable of inducing different morphological changes. A significant increase in size of astrocytes is seen after Met and / or Met-SO treatment at all times tested, while the basal-cultured astrocytes showed a polygonal to fusiform and flat morphology. This finding supports the idea that although Met and Met-SO do not cause cell death, this amino acid possibly induces astrocyte reactivity.

In addition to the morphological alterations, our results also showed that Met and Met-SO induced biochemical alterations in astrocytes, including an increase in AChE activity. AChE hydrolyses acetylcholine, a classical neurotransmitter involved in behavioral processes, cognition, learning, memory, and attention, also acts as a modulator of immune and inflammatory responses. In this sense, changes in AChE activity becomes an important target of studies, since it modulates the levels of acetylcholine in the CNS (Picciotto et al. 2012; Beckmann and Lips 2013). Increased AChE activity has been reported in an experimental rat brain model (Stefanello et al. 2007) and lymphocytes of rats (Soares et al. 2018). In addition, Chacón et al. (2003) showed that injecting AChE intrahippocampally induces neuronal cell loss, behavioral deficits, and astrocyte hypertrophy. Thus, our results suggest that increase in AChE activity positively correlates with astrocyte hypertrophy after exposure to Met and Met-SO. In addition, an increase in AChE activity may lead to a decrease in the levels of acetylcholine, an important molecule with anti-inflammatory potential, thus contributing to a pro-inflammatory environment that is strongly associated with reactive astrogliosis.

Conversely, we showed that Na^+, K^+-ATPase activity was reduced in astrocytes exposed to Met (1 or 2 mM) and/or Met-SO for 48 and 72 h. Four α-subunit isoforms of the enzyme Na^+/K^+-ATPase have been described in mammals designated as α1-α4. The α2 isoform of Na, K-ATPase is expressed in astrocytes. A reduction in the Na^+, K^+-ATPase activity in the brain corresponds with high concentrations of Met (Stefanello et al. 2007). In addition, Schweinberger et al. (2016) showed that high Met levels inhibited Na^+, K^+-ATPase activity; however, the enzyme content increased in the brain of the offspring. Considering that the primary neurological symptom reported in hypermethioninemia is brain edema, it is suggested that dysfunction in Na^+, K^+-ATPase can be associated with this pathological alteration (Arnaiz and Ordieres 2014).

Purinergic signaling is emerging as a means of integrating functional activity between neurons, glial, and vascular cells in the CNS (Burnstock 2007, 2013; Franke and Illes 2014). In this study, we observed changes in the NTPDase activity in astrocyte culture using ATP and ADP as substrates, after incubation with Met (1 or 2 mM) and/or Met-SO. For the first time, we showed that ATP hydrolysis was reduced by amino acid treatment, leading increased ATP levels in the extracellular environment. ATP binds and activates the P2X7, PY1, and PY2 receptors in astrocytes leading to the onset of astrogliosis (Burnstock 2007, 2013; Franke and Illes 2014). In this context, considering the hypertrophy observed in this study, we can infer that an increase in ATP in the extracellular environment caused by a decrease in the NTPDase activity may be contributing to the morphological and functional changes of the astrocytes in hypermethioninemia.

In contrast, there was an increase in NTPDase activity when ADP was used as a substrate. An increase in ADP hydrolysis leads to increased AMP levels, and combined with a reduction in 5'-nucleotidase activity by Met (1 or 2 mM) and / or Met-SO, favors a further accumulation of AMP in the extracellular medium. Ecto-5'nucleotidase, the enzyme responsible for extracellular dephosphorylation of AMP to adenosine, is highly abundant in astrocytes than in neurons (Zamzow et al. 2008). Although AMP does not play a direct role in astrocytes, hydrolysis of AMP by the 5'-nucleotidase is extremely important for the maintenance of extracellular adenosine levels. Considering a significant reduction of 5'-nucleotidase activity by Met and / or MeSO, the levels of adenosine in the extracellular medium are likely reduced. Adenosine acts as an anti-inflammatory and immunosuppressant by interacting with P1 receptors that play an important role in presynaptic neuromodulation. Thus, a reduction in adenosine levels may be detrimental to the neuroglial interaction.

Oxidative stress is the leading cause of cell damage in such pathological conditions. However, we report that Met and Met-SO induce oxidative stress but did not cause astrocyte cell death. The increased SOD activity may contribute to reduction in ROS levels in astrocytes exposed to Met (1 or 2 mM) and / or Met-SO. Similar results were reported by Dos Santos et al. (2017), showing a reduction in ROS production, and an increase in SOD activity in peritoneal macrophages exposed to Met and Met-SO. Costa et al. (2013) also observed that Met and Met-SO reduced ROS production and increased SOD activity in rat liver. In contrast, the decrease in the activity of CAT and GST enzymes may be in response to reduced ROS levels.

Previous studies reported that ATP protects hippocampal astrocytes from hydrogen peroxide-induced oxidative injury in astrocyte monocultures (Fujita et al. 2009), which is in agreement with reduced NTPDase activity.

Another hypothesis for decrease in ROS is, ROS are being used for peroxynitrite formation since nitrite levels increased significantly in Met and Met-SO treated astrocytes. Thus, an increase in peroxynitrite induces lipid peroxidation, and a decrease in total thiols levels, also shown in this work. Another interesting point to be discussed is that some authors have reported that adenosine may protect astrocytes from peroxynitrite-induced mitochondrial dysfunction (Choi et al. 2005). This finding corroborates with the decrease in the activity of 5'-nucleotidase, which is responsible for hydrolyzing AMP to adenosine. Soares et al. (2018) reported that in chronic hypermethioninemia, there is a reduction in serum adenosine levels in young rats treated with Met-SO.

Finally, astrocytes do not lose their neuroprotective ability after surviving intense oxidative stress (Bhatia et al. 2019). Thus, astrocytes may have evolved to tolerate the oxidative stress caused by Met and Met-SO to continue to fulfill their protective role in the damaged brain. These findings have important implications for hypermethioninemia, since they suggest that the loss of neurons, as previously observed by Schweinberger et al. (2017), after oxidative stress caused by hypermethioninemia would progress rapidly, if not for the stress resistance of astrocytes.

5 Conclusion

Exposure of astrocytes to Met and/or Met-SO induces alterations in astrocyte homeostasis through oxidative stress, increases AChE activity, reduces Na^+, K^+-ATPase activity, and induces changes in the activity of ecto-nucleotidases (Figure 7), which modulate purinergic signaling, resulting in significant hypertrophy of astrocytes. These data help understand the neurological changes found in patients with hypermethioninemia. In addition, these results provide new perspectives for future studies that seek therapeutic targets for this pathology.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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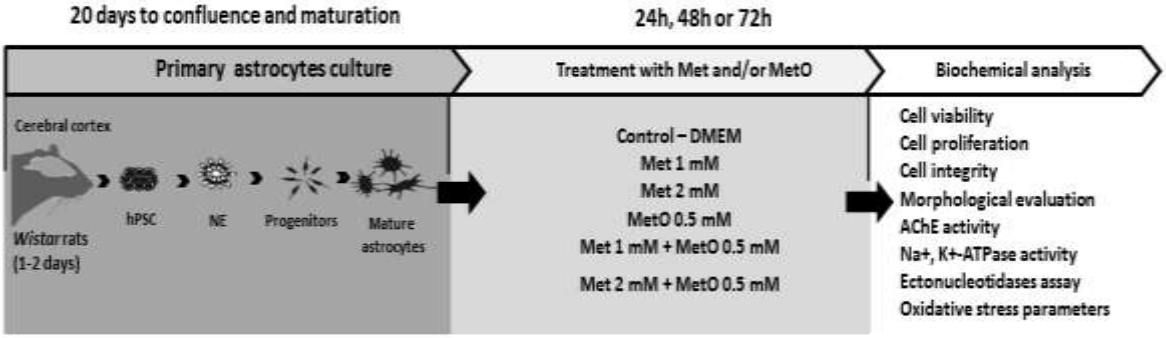

Figure 1: Experimental design of the protocols used in the study. Astrocytes were obtained from primary cultures and maintained under standard conditions for 20 days. After that, the astrocytes were treated with 0.5 mM Met-SO and 1 or 2 mM Met in combination or alone at 24, 48 or 72 h.

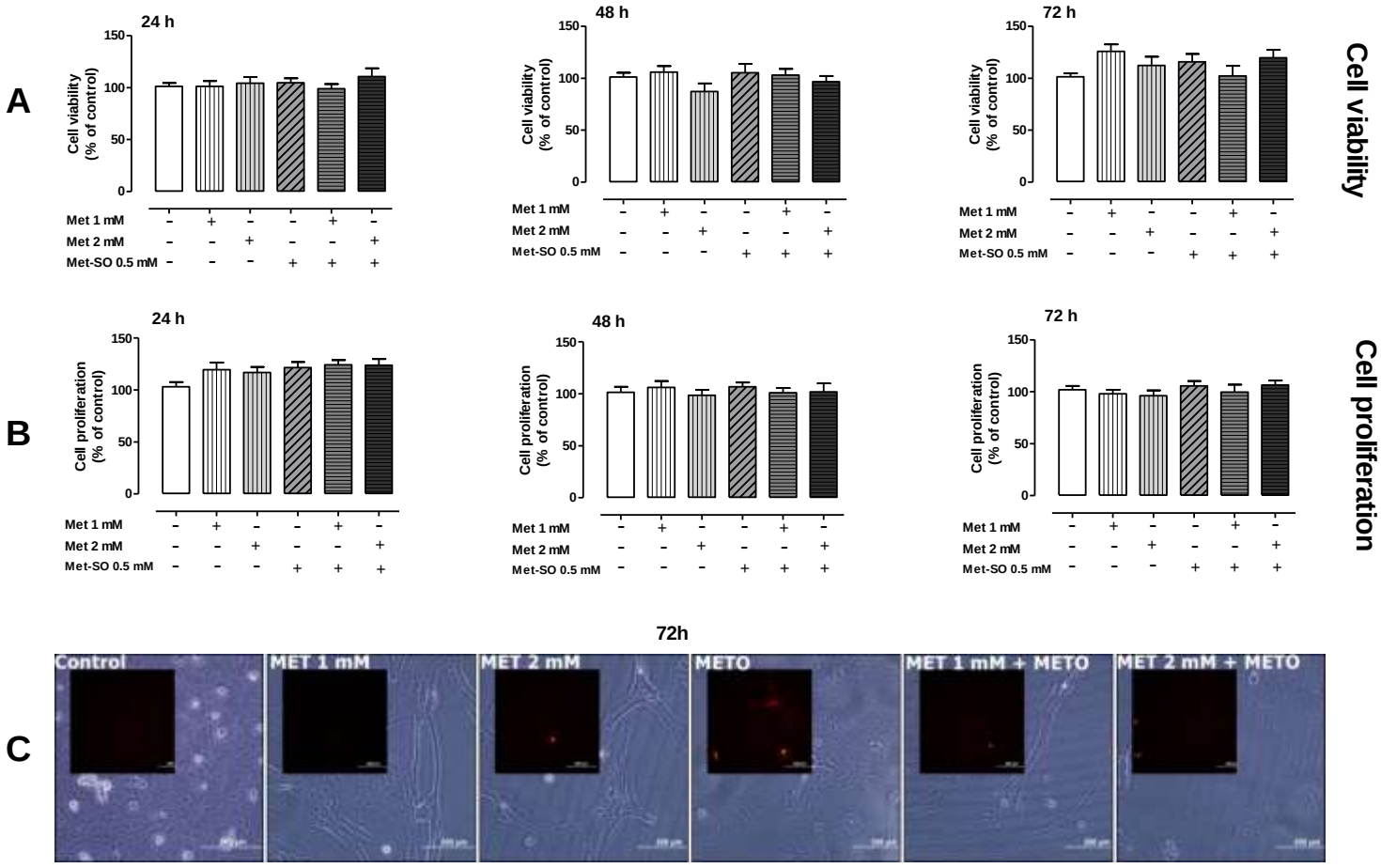

Figure 2: Evaluation of cytotoxic effects on primary culture of astrocytes exposed 0.5 mM Met-SO and 1 or 2 mM Met in combination or alone for 24, 48 and 72 h. (**A**) Astrocyte viability was assessed by the MTT test. (**B**) Astrocyte proliferation was measured using the SRB test. (**C**) Necrosis was evaluated by PI incorporation in astrocytes after 72 h treatment. Viability and proliferation were expressed as percentages of control. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Bars represent mean ± SEM.

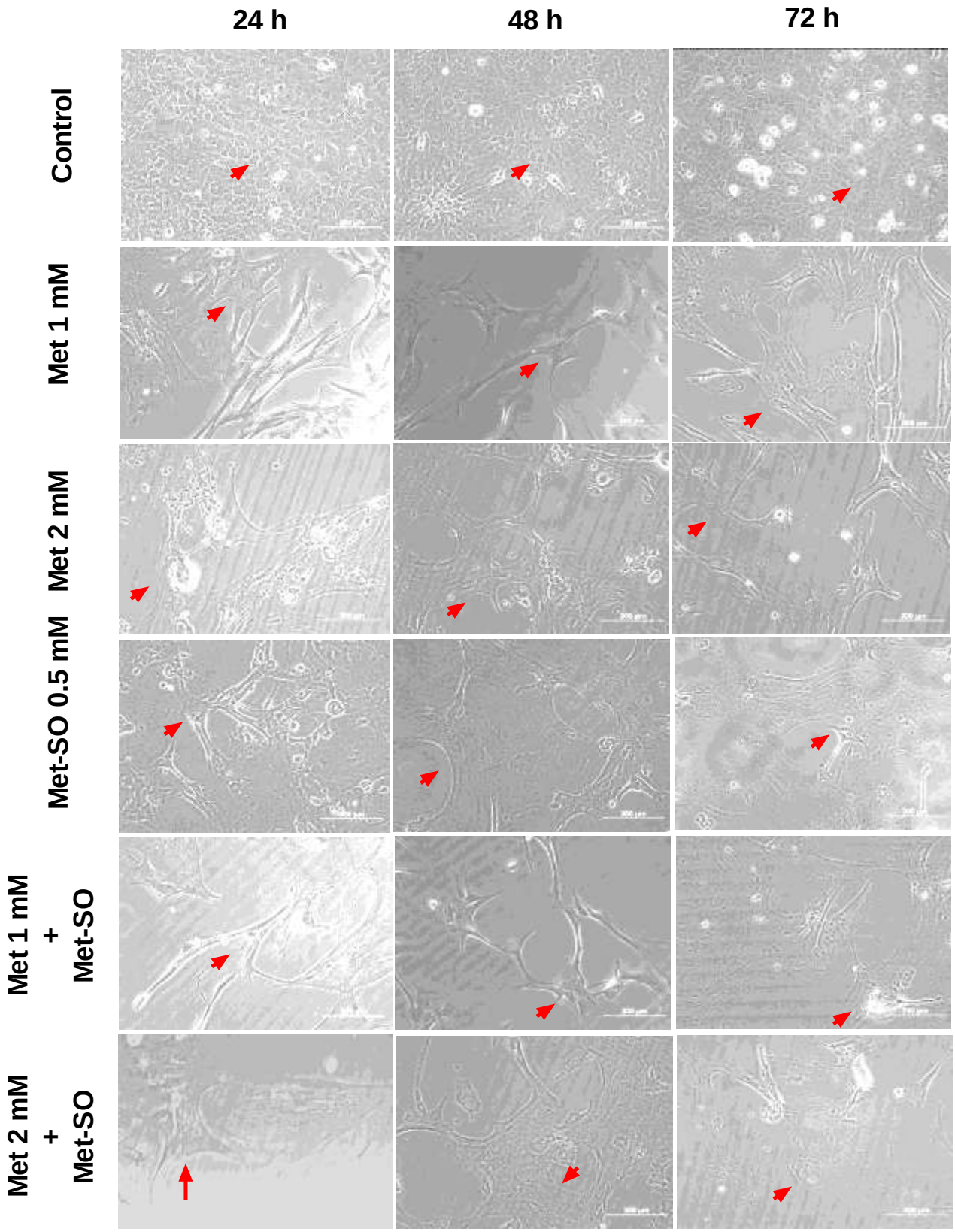

Figure 3: Representative phase-contrast microphotographs of astrocytes exposed to 0.5 mM Met-SO and 1 or 2 mM Met in combination or alone after 24, 48 and 72 h (images were taken using an Olympus inverted microscope; magnification 40×). Arrows indicate astrocytes with different morphologies among different treatment groups.

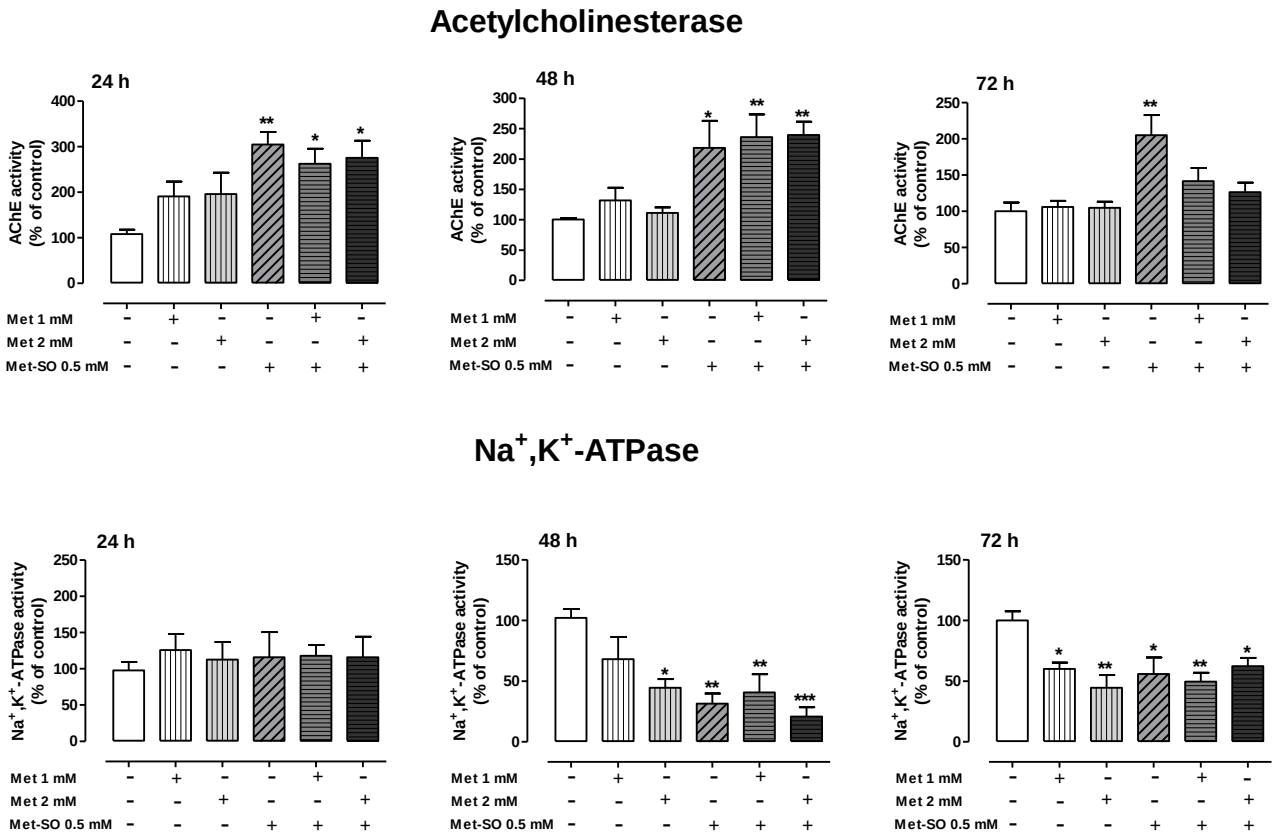

Figure 4: Activity of acetylcholinesterase (AChE) and Na^+, K^+- ATPase in astrocytes exposed to 0.5 mM Met-SO and 1 or 2 mM Met in combination or alone for 24, 48 and 72 h. AChE and Na^+, K^+- ATPase activities were expressed as percentages of control. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Bars represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with the control cells.

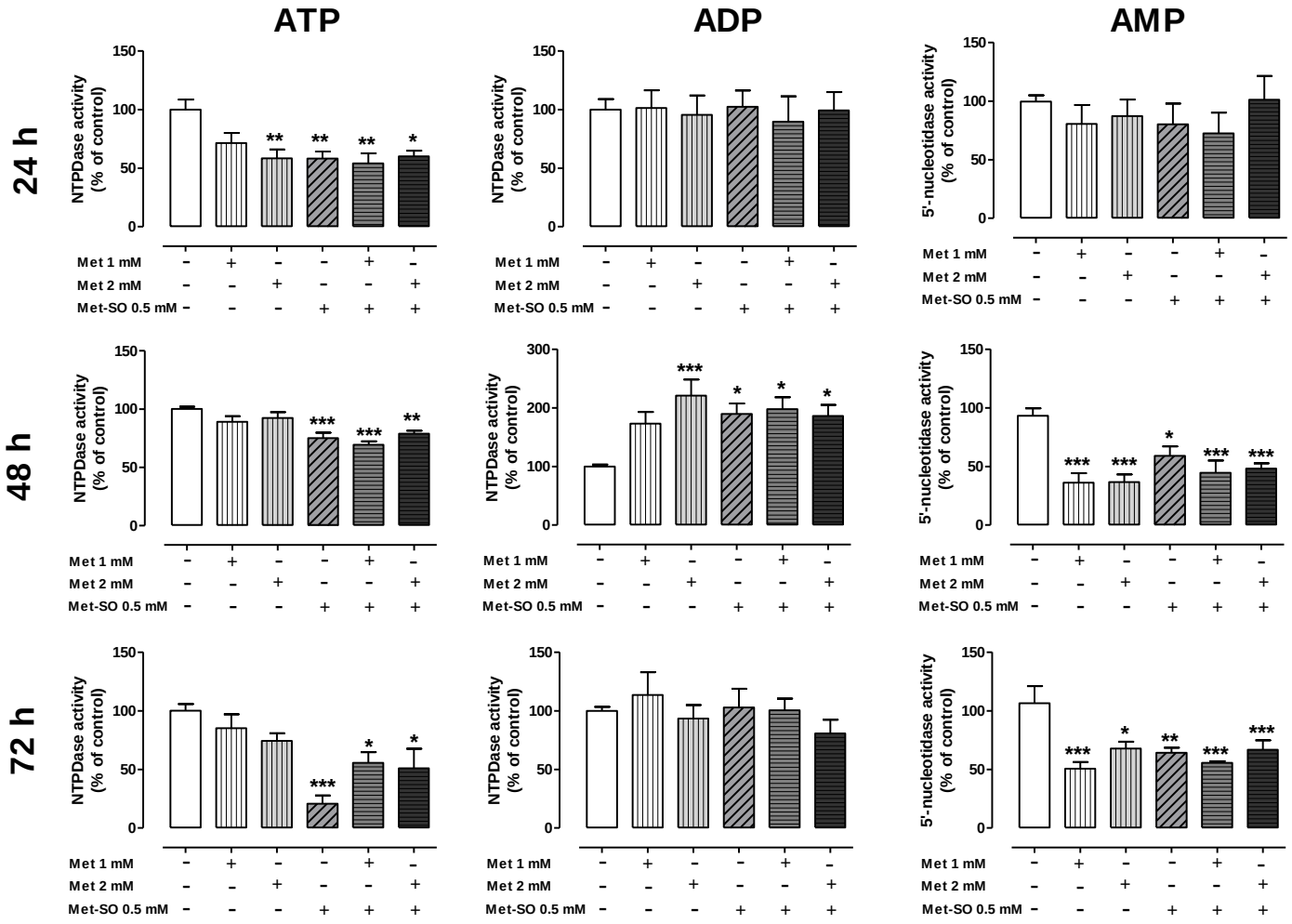

Figure 5: Activity of NTPDase using ATP and ADP as substrate and 5'-nucleotidase activity using AMP as substrate in astrocytes exposed to 0.5 mM Met-SO and 1 or 2 mM Met in combination or alone during 24, 48 and 72 h. NTPDase and 5'-nucleotidases activities were expressed as percentages of control. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Bars represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with the control cells.

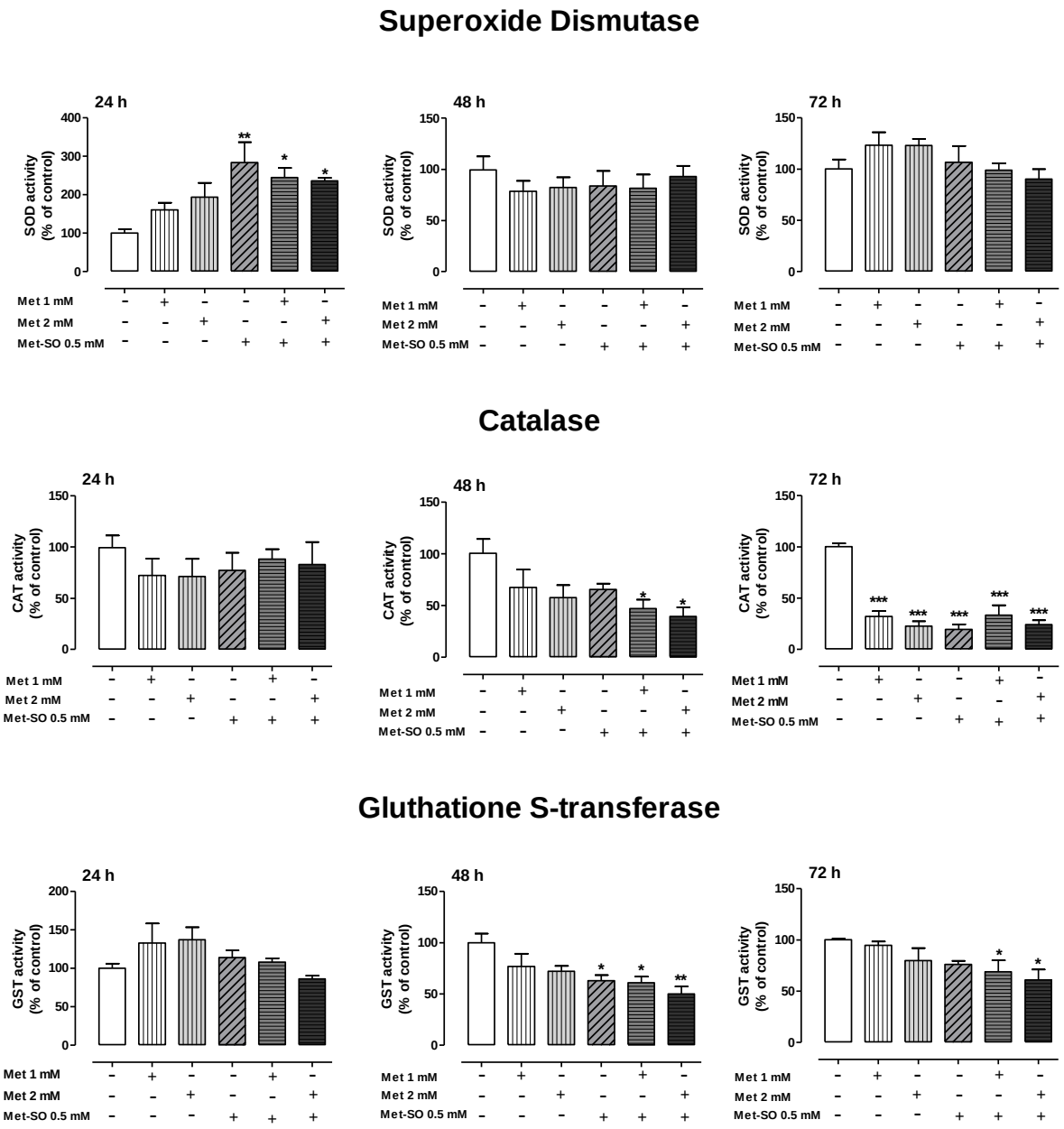

Figure 6: Superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities in astrocytes exposed to 0.5 mM Met-SO and 1 or 2 mM Met in combination or alone during 24, 48 and 72 h on. SOD, CAT, and GST activities were expressed as percentages of control. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Bars represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with the control cells.

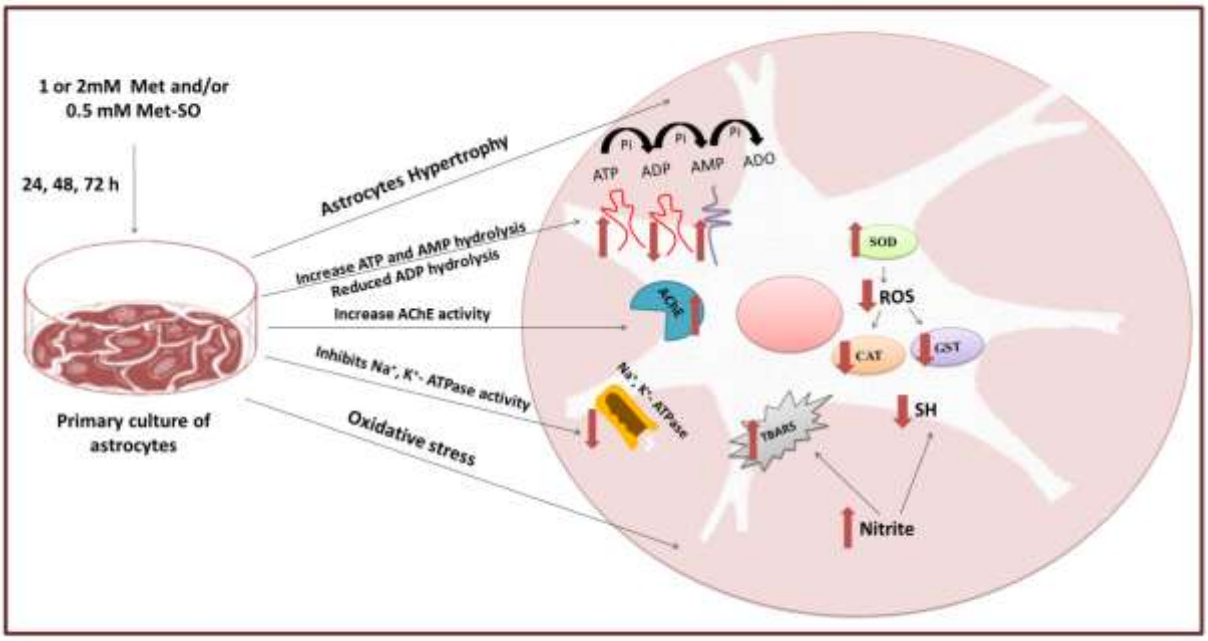

Figure 7: Effect of high concentrations of Met and Met-SO on the morphology and neurochemical parameters in primary culture of cortical astrocytes.

Table 1: Reactive oxygen species (ROS), nitrite, total thiol content (SH), and thiobarbituric acid reactive species (TBARS) levels in astrocytes exposed to 0.5 mM Met-SO and 1 or 2 mM Met in combination or alone during 24, 48 and 72 h. The results were expressed as percentages of control. One-way ANOVA and post-hoc Tukey's multiple comparisons test were performed. Data are represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with the control cells.

	Control	Met 1 mM	Met 2 mM	Met-SO 0.5 mM	Met 1 mM + Met-SO 0.5 mM	Met 2 mM + Met-SO 0.5 mM
				24 h		
ROS	100 ± 10.9	48.28 ± 6.9 ***	37 ± 5.52 ***	28.67 ± 3.14 ***	37.08 ± 2.22 ***	41.86 ± 8.54 ***
Nitrite	100 ± 2.8	114.2 ± 16.1	124 ± 16.2	162.9 ± 9.4 *	115.4 ± 15.2	98.78 ± 15.5
SH	100 ± 6.1	92.61 ± 6.2	103.1 ± 5	96.78 ± 10	50.48 ± 11.5 **	42.88 ± 17.3 **
TBARS	100 ± 15.4	125.6 ± 9.8	115.6 ± 10.4	122.5 ± 13	131.7 ± 9.6	14.16 ± 6.2
				48 h		
ROS	100 ± 5.15	80.67 ± 4.6	77.3 ± 10.6	96.95 ± 8.86	77.93 ± 9.53	69.45 ± 6.4
Nitrite	100 ± 7.4	150.5 ± 19.7	140.1 ± 14.5	197.5 ± 31.09 *	147.4 ± 16.9	152.9 ± 25.36
SH	100 ± 5.5	77.06 ± 5.7**	67.47 ± 5**	62.21 ± 8.2**	61.27 ± 4.8 **	58.38 ± 9.3 ***
TBARS	100 ± 5.4	75.51 ± 12.74	79.67 ± 19.6	98.28 ± 10.8	116 ± 15.7	110.5 ± 8.9
				72 h		
ROS	100 ± 5.1	77.09 ± 14.5	75.47 ± 13.4	61.52 ± 8.1 *	59. 45 ± 7.9 **	40.41 ± 12.7 **
Nitrite	100 ± 11.1	248.9 ± 21.7 **	357.5 ± 40.0 ***	304.2 ± 15.9 ***	254.8 ± 16.5 **	333.1 ± 34.6 ***
SH	100 ± 13.4	73.77 ± 5.1	50.69 ± 16.2 *	55.22 ± 7.8*	55.04 ± 5.4*	60.45 ± 0.6*
TBARS	100 ± 16.1	206.2 ± 1.6	291.2 ± 19.1 *	569.5 ± 56.1 ***	420.03 ± 50***	515.8 ± 13.6***

5. DISCUSSÃO

Estudos prévios têm demonstrado que a exposição *in vitro* e *in vivo* a Met e/ou MetO pode ser extremamente tóxica causando danos cerebrais e hepáticos, além de alterar a homeostase de linfócitos e macrófagos favorecendo um ambiente pró-inflamatório. Entretanto os exatos mecanismos fisiopatológicos da hipermetioninemia ainda não são completamente elucidados. Sendo assim, o objetivo do presente trabalho foi contribuir para complementar os achados obtidos anteriormente pelo nosso grupo de pesquisa e, consequentemente, auxiliar no entendimento dos mecanismos celulares e moleculares associados a patogênese da hipermetioninemia.

Primeiramente, os resultados do presente trabalho demonstraram algumas alterações periféricas através de ambos os protocolos, agudo e crônico. No protocolo crônico observou-se que a administração crônica de Met e/ou MetO induz dano oxidativo hepático e renal e altera marcadores metabólicos no soro de ratos jovens. Os níveis de glicose foram maiores nos animais tratados com Met e/ou MetO, o que pode ser devido ao aumento da produção de glicose a partir de aminoácidos na gliconeogênese. No entanto, sabe-se que altos níveis de glicose podem causar um aumento na produção de ERO pelas mitocôndrias (YU et al. 2011). Além disso, houve uma diminuição do colesterol total nos animais tratados com MetO e Met+MetO e um aumento nos níveis de TAG no grupo MetO. Além disso, observou-se também um aumento nos níveis de ureia no sangue em todos os grupos, o que era esperado já que a ureia é uma substância produzida no fígado como resultado do metabolismo de aminoácidos tais como Met e/ou MetO.

No tecido hepático observou-se uma redução na atividade da SOD e da CAT seguido de um aumento na atividade da GPx. Esse resultado pode explicar os níveis normais de ERO encontrados no fígado e consequentemente a homeostase no TBARS e no teor total de tios totais, já que a GPx é responsável por detoxificar H_2O_2. Cabe salientar, que esses resultados estão de acordo com trabalho previamente publicado no qual demonstrou-se que a administração crônica de Met diminui a atividade da CAT, aumenta a tividade da GPx e não induz peroxidação lipídica (STEFANELLO et al., 2009). Além disso, uma vez que a MetO é formada a partir da oxidação da Met por ERRO, essas moléculas podem estar sendo desviadas nesta

via de metabolização, o que poderia contribuir para a manutenção dos níveis normais no fígado.

Por outro lado, no tecido renal, foi possível observar um dano oxidativo bastante caracterizado pelo aumento dos níveis de ERO e de TBARS, redução no conteúdo tiólico total, seguido da diminuição na atividade das enzimas SOD e CAT nos animais tratados com Met e/ou MetO. Embora pouco se conhece sobre os efeitos da hipermetioninemia sobre o tecido renal, sabe-se que esse tecido participa como um dos principais reguladores das concentrações plasmáticas dos aminoácidos, removendo produtos como ureia e amônia. Dessa forma, elevadas concentrações de Met e MetO podem aumentar a taxa de filtração glomerular progressivamente levando a uma disfunção da função renal.

Nesse mesmo artigo, também foi demonstrado um aumento na atividade da enzima ALA-D em fígado e rim dos animais tratados com Met e/ou MetO. Um aumento da atividade da ALA-D já foi demonstrado em outras patologias e foi relacionado ao aumento da síntese de heme estimulado pela anemia ou pela presença de processos inflamatórios crônicos, uma vez que citocinas pró-inflamatórias reduzem a eritropoietina (POLACHINI et al., 2016; ZANINI et al., 2014; FRANÇA et al., 2011). Corroborando com essa hipótese, Stefanello et al. (2009) demonstraram que a hipermetioninemia crônica aumenta o número de células inflamatórias e altera a morfologia normal dos lóbulos hepáticos (STEFANELLO et al. 2009). Nesse sentido, pode-se concluir que os altos níveis de Met e MetO, observados na hipermetioninemia, podem sobrecarregar esses órgãos levando à lesão tecidual.

Posteriormente, em plaquetas e soro também foi encontrado um desbalanço entre a capacidade antioxidante e pró-oxidante, favorecendo o estresse oxidativo, além de alterações importantes nos componentes do sistema purinérgico. Neste estudo, demonstrou-se pela primeira vez que a hidrólise de ATP, ADP e AMP diminui nas plaquetas após tratamento agudo (3 horas) e crônico com Met e/ou MetO. Essa inibição da atividade das ectonucleotidases nas plaquetas pode ser explicada, pelo menos em parte, pelo metabolismo da Met. O ATP transfere o grupo adenosil a Met para formar SAM em uma reação catalisada pelo MAT. Assim, um aumento na concentração de Met exige maior consumo de ATP para sua metabolização, o que pode estar associado a baixos níveis de ATP no soro e reduzida atividade NTPDase em plaquetas. Aliado a isso, é importante considerar

que elevados níveis de ERO, como encontrado nesse trabalho, também podem contribuir para a inativação das ectonucleotidases nas membranas das plaquetas.

Por outro lado, o ADP liberado durante a ativação das plaquetas interage com os receptores purinérgicos na membrana plaquetária, levando a alterações morfológicas, liberação do conteúdo granular e agregação plaquetária (BURNSTOCK, 2015; HEIJNEN e VAN DER SLUIJS, 2015; IDZKO et al., 2014). O tratamento com Met e MetO reduziu a atividade da NTPDase nas plaquetas e soro, sugerindo que esses aminoácidos interferem na ativação e agregação plaquetária à medida que o ADP se acumula no meio extracelular. Corroborando com esses resultados, demonstramos recentemente que os níveis de ADP aumentam no soro de animais após o tratamento crônico (SOARES et al., 2018).

Além disso, as enzimas 5′-nucleotidase e ADA são fundamentais para a regulação dos níveis de ADO extracelular. Aqui, um ponto importante a ser discutido é que durante a metabolização da Met, moléculas de ADO são formadas (SCHWEINBERGER e WYSE, 2016). Assim, a via de produção de ADO pode estar associada a alterações na atividade da ADA 21 dias após o tratamento. Entretanto, nosso grupo de pesquisa mostrou que a administração de MetO diminui os níveis de ADO no soro no tratamento crônico (SOARES et al., 208). Em conjunto, esses achados sugerem que tanto o tratamento agudo quanto o crônico com Met e seu metabólito MetO podem alterar a função plaquetária ao interferir nos níveis de ADO.

Ainda nesse trabalho, foi demonstrada a relação intrínseca de altas concentrações de Met e MetO e estresse oxidativo nas plaquetas e no soro, em ambos os protocolos, agudo e crônico. Sabe-se que a oxidação de resíduos de Met para produzir MetO pode causar alterações estruturais e funcionais com potenciais papeis regulatórios na patogênese da vascularização ou doenças trombóticas (SUZUKI et al., 2016, GU et al., 2015, TARRAGO et al., 2015). Os resultados do presente trabalho demonstraram um aumento na produção de ERO em plaquetas e soro após tratamento agudo e crônico com Met e/ou MetO. Associado a isso, houve uma redução na atividade das enzimas antioxidantes, o que poderia ter favorecido o acúmulo de H_2O_2. Nesse sentido, pode-se concluir que a hipermetioninemia pode induzir agregação plaquetária através da modulação do sistema purinérgico com contribuição da indução de estresse oxidativo por essa condição patológica.

Além disso, objetivou-se investigar alterações neurológicas causadas pela hipermetioninemia. No primeiro artigo referente a esse objetivo, demonstramos que

a administração aguda de Met e/ou MetO causa estresse oxidativo em córtex cerebral, demonstrado através do aumento dos níveis de ERO e TBARS, seguido do aumento da atividade da SOD pela Met, redução da atividade da CAT e alterações na atividade da GPx. Além de causar lesão celular, o estresse oxidativo é um mecanismo modulador de vários processos cruciais do corpo, incluindo metabolismo, crescimento, diferenciação e morte celular, agindo dessa forma em todos os estágios fisiológicos ou patológicos do procedimento (HALLIWELL e GUTTERIDGE, 2007).

Portanto, investigou-se se o desequilíbrio entre os níveis de ERO e as defesas antioxidantes enzimáticas comprometeriam a viabilidade celular, já que é bem relatado na literatura que o estresse oxidativo pode levar a danos celulares e teciduais por peroxidação lipídica, de proteínas e danos no DNA. Quando foi analisada a viabilidade celular, os resultados indicaram uma redução na sobrevivência celular por Met, MetO e Met + MetO 3 h após o tratamento. Também observou-se um aumento nas células em processo de apoptose, juntamente com um aumento das caspase-9 e caspase-3 após 3 h do tratamento com Met e/ou MetO. O mecanismo de morte celular dependente de caspase é crucial para manter a homeostase celular e esse sistema é mediado principalmente pela atividade de caspases (D'AMELIO et al., 2010).

Ao analisar a integridade do DNA observou-se um aumento significativo nos efeitos genotóxicos 3 h após o tratamento nos grupos Met, MetO e Met + MetO. Esses resultados suportam a hipótese de que o aumento dos níveis de ERO e diminuição das defesas antioxidantes levam a danos no DNA e consequente processo apoptótico.

Para complementar os efeitos do aumento de ERO, avaliou-se o potencial de membrana mitocondrial (PMM). Os resultados obtidos demostraram uma redução de PMM nos grupos MetO e Met + MetO após o tratamento agudo, o que reforça a ideia de que há uma produção intracelular aumentada de ERO. A integridade da PMM está diretamente associada à produção de energia e é essencial para a função fisiológica da cadeia respiratória gerar ATP (CHEN, 1988). Uma disfunção na PMM pode levar à depleção de energia com subsequente morte celular. É importante ressaltar que as alterações observadas no PMM parecem estar relacionadas à presença do metabólito MetO.

Para dar continuidade as investigações dos efeitos da hipermetioninemia no SNC, o manuscrito I teve como objetivo entender os mecanismos pelos quais a hipermetioninemia crônica atua na deteriorização cognitiva, avaliando parâmetros bioquímicos, moleculares e comportamentais *in vivo*. Os resultados obtidos demonstraram que essa condição altera a homeostase redox em córtex cerebral, hipocampo e estriado, além de alterar a atividade das enzimas AChE e Na^+,K^+-ATPase, reduzir os níveis de BDNF e o número de neurônios em hipocampo.

Primeiramente, foi relatado que a Met e/ou MetO não foram capazes de alterar a atividade locomotora espontânea dos animais. No entanto, os animais dos grupos Met, MetO e Met+MetO apresentaram o número de rearings reduzido, indicando uma menor atividade exploratória. Portanto, é plausível que uma diminuição nesse parâmetro possa explicar os achados do labirinto em Y e dos testes de reconhecimento de objetos (PAWLAK e SCHWARTING, 2002). A diminuição no comportamento de autolimpeza pode estar associado ao comportamento ansiolítico induzido por Met e MetO. Em consonância, Hrncic et al. (2016) demonstraram que a dieta rica em Met reduz esse comportamento e promove o comportamento semelhante à ansiedade. Sendo assim, os resultados desta investigação mostraram que a administração de Met e/ou MetO, prejudicou a memória de reconhecimento de objetos a curto prazo. Além disso, o comprometimento no teste do labirinto em Y também foi observado.

Neste trabalho também foi demonstrado que Met, MetO e Met + MetO induziram dano oxidativo no cérebro, principalmente em hipocampo e estriado. Além disso, os níveis de nitrito foram reduzidos no hipocampo e estriado. Níveis diminuídos de nitrito podem ser uma medida indireta de níveis reduzidos de óxido nítrico que está associado à neurodegeneração, prejuízo cognitiva e perda de memória, semelhante ao comportamento encontrado na presente investigação (TODA e OKAMURA, 2016).

Met e/ou MetO aumentam a atividade da AChE em todas as estruturas cerebrais avaliadas o que pode reduzir os níveis de ACh, levando aos déficits de memória observados nesse estudo. Os ratos tratados com MetO e Met + MetO também mostraram uma diminuição na atividade da Na^+,K^+,-ATPase no hipocampo. Essa alteração pode estar associada a altos níveis de ERO ou com reduzido número de neurônios observados nos mesmos grupos. Schweinberger et al. (2016) mostraram que embora altos níveis de Met inibam a atividade da Na^+,K^+-ATPase, a

expressão e o conteúdo dessa enzima é aumentado no cérebro da prole (SCHWEINBERGER et al., 2016). Esse resultado indica que altos níveis de ERO podem influenciar diretamente a atividade da Na^+K^+-ATPase.

No hipocampo, também foi observado que o tratamento com MetO e Met+MetO reduziu os níveis de BDNF o qual é essencial para o processo de aprendizado e memória (LEAL et al. 2015, 2016). Além disso, o BDNF pode controlar a diferenciação e a sobrevivência neuronal. Nesse sentido, a diminuição da concentração de BDNF no hipocampo corrobora com a redução do número de neurônios na região CA3 e giro denteado do hipocampo (LEAL et al. 2015, 2016).

Finalmente, investigamos os efeitos *in vitro* da hipermetioninemia sobre os astrócitos. Observou-se que Met e/ou MetO causam um desbalanço no *status* redox nas células glias, bem como reduzem a atividade da Na^+K^+-ATPase e aumentam a atividade da AChE. Interessantemente, também observou-se uma alteração significativa na atividade das ectonucleotidases, além de uma hipertrofia dos astrócitos tratados com Met e/ou MetO. Por outro lado, embora diversas alterações neuroquímicas tenham sido relatadas, essas mudanças causadas pela hipermetioninemia, *in vitro*, não foram capazes de alterar a viabilidade ou a proliferação celular dos astrócitos. Esses achados têm implicações importantes para a hipermetioninemia, uma vez que sugerem que a perda de neurônios, como previamente observado por Schweinberger et al. (2017), após estresse oxidativo causado por hipermetioninemia, progrediria rapidamente, se não fosse pela resistência ao estresse dos astrócitos.

Embora não tenha sido observada alteração na viabilidade dos astrócitos, a hipertrofia encontrada, associada a todas as alterações neuroquímicas, sustentam a ideia de que apesar de Met e MetO não causarem morte celular, possivelmente induzem a reatividade dos astrócitos expostos a essas condições experimentais que mimetizam a principal característica clínica da hipermetioninemia.

Os resultados obtidos nesse último estudo mostraram que a exposição de astrócitos a Met e /ou MetO, como encontrado na hipermetioninemia, induziu alterações na homeostase astrocitária. Esses dados auxiliam na compreensão das alterações neurológicas encontradas nos pacientes com hipermetioninemia. Além disso, esses resultados fornecem novas perspectivas para futuros estudos que buscam alvos terapêuticos para essa patologia.

Com base em todos os resultados obtidos, tanto *in vitro* quanto *in vivo*, e em todos os tecidos e células avaliados, pode-se observar que o metabólito MetO parece ter um efeito tóxico mais proeminente, uma vez que as principais alterações foram observadas na presença de MetO, sozinho ou em combinação com a Met. Além disso, é possível verificar que a presença de estresse oxidativo é encontrado tanto no protocolo *in vitro*, quanto *in vivo*, entretanto, a produção de ERO é discordante em ambos os protocolos. Isso possivelvem está associado ao processo de metabolização de Met e MetO.

As alterações na atividade das ectonucleotidases, da AChE e da $Na^{+,}K^{+}$-ATPase também são alteradas de maneira similiar tanto nos astrócitos *in vitro*, quanto nas estruturas cerebrais *ex vivo*, bem como nas plaquetas nos protocolos agudos e crônicos. Esses resultados em conjunto demonstram que a hipermetioninemia pode prejudicar diversos sistemas essenciais para manutenção da homeostase tecidual e celular e que esta alteração parece estar intimamente relacionada com o favorecimento do processo inflamatório e agregação plaquetária.

Sendo assim, considerando a consistência dos resultados obtidos tanto no SNC quanto nos tecidos e células periféricas, a busca por possíveis alvos terapêuticos com capacidade antioxidante e anti-inflamatória se torna de extrema importância para auxiliar no tratamento das alterações relacionadas a hipermetioninemia.

6. CONCLUSÕES

Com base nos resultados obtidos pode-se observar que a hipermetioninemia altera o *status* redox em fígado, rim, plaquetas e soro, bem como favorece a ativação da agregação plaquetária em ratos jovens. Também causa severas alterações neuroquímicas, astrocitárias e comportamentais. Com isso, concluiu-se que a hipermetioninemia é capaz de modificar a homeostase celular em tecidos periféricos e no SNC. Essas descobertas são extremamente relevantes para o entendimento dos mecanismos fisiopatológicos da hipermetioninemia, e para auxiliar no estudo de possíveis alvos terapêuticos, pricipalmente compostos com capacidade antioxidante, para pacientes portadores dessa patologia.

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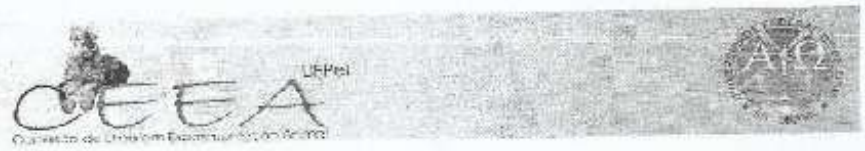
Anexos

1. Carta de parecer do Cômite de Ética em Experimentação Animal



Pelotas, 08 de agosto de 2017

Certificado

Certificamos que a solicitação de adendo à proposta intitulada "**Avaliação de parâmetros inflamatórios e neuroquímicos em ratos tratados com metionina e metionina sulfóxido**" registrada com o nº 23110.003527/2014-58, sob a responsabilidade de **Francieli Moro Stefanelo** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 10/07/2017.

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	Prorrogado até 30 de dezembro de 2019
Espécie/linhagem/raça	*Rattus norvegicus*/wistar
Nº de animais	210
Idade	146-30 dias e 64 -06 dias
Sexo	Ambos
Origem	Biotério Central - UFPel

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao *COBALTO* para posterior registro no *COCEPE* (código para cadastro nº **CEEA 3527-2014**).

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Ciente em: 16/08/2017

Assinatura do Professor Responsável: Francieli Moro Stefanello

2. Carta de parecer do Cômite de Ética em Experimentação Animal

UFPel
CEEA
Comissão de Ética em Experimentação Animal

Pelotas, 16 de agosto de 2017

Certificado

Certificamos que a proposta intitulada **"Investigação dos efeitos do tratamento com metionina e metionina sulfóxido em cultivo primário de astrócitos"**, registrada com o nº **23110.006210/2017-16,** sob a responsabilidade de **Rosélia Maria Spanevello** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 14/08/2017.

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	Início: 10/2017 Término: 10/2019
Espécie/linhagem/raça	Rattus norvergicus / Wistar
Nº de animais	24
Idade	1-3 dias
Sexo	Masculino
Origem	Biotério Central da UFPel

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao *COBALTO* para posterior registro no *COCEPE* (código para cadastro nº **CEEA 6210-2017**).

M.V. Dra. Anelize de Oliveira Campello Felix
Presidente da CEEA

Ciente em: 12/09/2017
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