UNIVERSIDADE FEDERAL DE PELOTAS
Centro de Ciências Químicas, Farmacêuticas e de Alimentos
Programa de Pós-Graduação em Bioquímica e Bioprospecção

Tese

Os mecanismos fisiopatológicos envolvidos na fibromialgia diferem entre camundongos *Swiss* machos e fêmeas: 4-amino-3-(fenilselanil) benzenosulfonamida como uma estratégia terapêutica

Carolina Cristóvão Martins

Pelotas, 2023

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RESUMO

MARTINS, Carolina Cristóvão**. Os mecanismos fisiopatológicos envolvidos na fibromialgia diferem entre camundongos *Swiss* machos e fêmeas: 4-amino-3-(fenilselanil) benzenosulfonamida como uma estratégia terapêutica.** Orientadora: Ethel Antunes Wilhelm. 2023. Tese (Doutorado em Ciências com ênfase em Bioquímica e Bioprospecção) – Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, 2023.

A fibromialgia é uma condição dolorosa crônica que acomete principalmente mulheres. Essa doença é acompanhada por comorbidades, como a depressão, que afetam a qualidade de vida desses pacientes. Somando-se a isso, os mecanismos envolvidos na fibromialgia ainda permanecem inconclusivos, o que dificulta o desenvolvimento de um tratamento adequado. Diante disso, estudos pré-clínicos buscam elucidar a fisiopatologia, assim como novas estratégias terapêuticas eficazes e seguras para tratar essa patologia. Portanto, a 1ª etapa deste estudo explorou os mecanismos fisiopatológicos envolvidos na fibromialgia por meio de um modelo induzido pelo estresse ao frio intermitente (EFI) em camundongos *Swiss*, considerando as especificidades do sexo. A via de sinalização NMDA/ON/GMPc e os receptores serotoninérgicos podem contribuir para o desenvolvimento dos sinais nociceptivos em camundongos de ambos os sexos expostos ao EFI. Entretanto, esse modelo de fibromialgia alterou alguns marcadores de estresse oxidativo e inibiu a atividade da Na^+, K^+-ATPase em camundongos machos, enquanto que, foi observado um aumento na atividade dessa enzima em camundongos fêmeas. Assim, diferentes vias de sinalização podem contribuir para o desenvolvimento da fibromialgia de acordo com as particularidades do sexo. Na 2ª etapa deste estudo, foi avaliado se a diferença entre os sexos afeta o perfil de expressão de microRNAs e de seus genes alvos em camundongos expostos ao EFI. A diminuição na expressão do miR-338-3p pode ter estimulado a expressão do TRPV1 no plasma de camundongos machos expostos ao EFI. Por outro lado, o aumento na expressão do miR-338-3p pode ter inibido a expressão do TRPV1 no plasma de camundongos fêmeas expostos ao EFI. No córtex cerebral, a expressão aumentada do miR-155-5p pode ter inibido a expressão do BDNF em camundongos de ambos os sexos expostos ao EFI. Portanto, as alterações no miR-155-5p e no miR-338-3p podem participar dos processos fisiopatológicos envolvidos na fibromialgia. Na 3ª etapa deste estudo, foi investigado os efeitos farmacológicos do composto 4-amino-3-(fenilselanil) benzenosulfonamida (4-APSB), um derivado de sulfonamida contendo selênio, no modelo do EFI. Os resultados demonstraram que o tratamento com o composto 4-APSB atenuou os sinais nociceptivos e o fenótipo do tipo-depressivo em camundongos de ambos os sexos expostos ao EFI. Ainda, a ação farmacológica do 4-APSB neste modelo experimental parece estar associado à modulação dos marcadores de estresse oxidativo, da enzima Na^+, K^+-ATPase, e da expressão do Nrf-2 e do NFκB, vias relacionadas ao estresse oxidativo e a inflamação. Os achados dessa tese revelam que o sexo interfere nos aspectos neurobiológicos envolvidos na fibromialgia e, portanto, deve ser considerado para fins de diagnóstico e tratamentos mais eficazes.

Palavras-chave: Fibromialgia, dor, comorbidades, estresse oxidativo e selênio.

ABSTRACT

MARTINS, Carolina Cristóvão**. The pathophysiological mechanisms involved in fibromyalgia differ between male and female mice *Swiss*: 4-amino-3-(phenylselanyl) benzenesulfonamide as a therapeutic strategy.** Adviser: Ethel Antunes Wilhelm. 2023. Thesis (Doctorate in Sciences with an emphasis on Biochemistry and Bioprospecting) – Center for Chemical, Pharmaceutical and Food Sciences, Pelotas, 2023.

Fibromyalgia is a chronic painful condition that affects mainly women. Generally, it is accompanied by the development of comorbidities, including mood disorders, which impaired the quality of life of those patients. In addition, the pathophysiology of this disease remains poorly understood, making it difficult to develop a properly treatment. In view of this, preclinical studies would be expected for elucidate the mechanisms underlying fibromyalgia, as well as new effective and safe therapeutic strategies to treat this pathology. Therefore, the 1st stage of this study explored the mechanisms related to fibromyalgia, using the intermittent cold stress (ICS) model in *Swiss* mice, considering the sex specificities. The results demonstrated that the NMDA/ON/cGMPc signaling pathway and the serotonergic receptors may contribute to the development of nociceptive signs in mice of both sexes exposed to ICS. Nevertheless, this fibromyalgia model altered some markers of oxidative stress and inhibited the Na^+, K^+-ATPase activity in male mice, whereas an increase in the activity of this enzyme was observed in female mice. Thus, different signaling pathways may contribute to the development of fibromyalgia, regarding the particularities of sex. In the 2nd stage of this study, it was evaluated whether the sex differences affect the expression profile of microRNAs and their target genes in mice exposed to ICS, in order to identify possible pathophysiological markers for fibromyalgia. The results suggest that the decrease in miR-338-3p expression may have stimulated the TRPV1 expression in the plasma of male mice exposed to ICS. On the other side, the increase in miR-338-3p expression may have inhibited the TRPV1 expression in the plasma of female mice exposed to ICS. In the cerebral cortex, the increased expression of miR-115-5p may have inhibited the expression of BDNF in mice of both sexes exposed to ICS. Therefore, miR-155-5p and miR-338-3p alterations may participate in the pathophysiological processes involved in fibromyalgia. In the 3rd stage of this study, we investigated the pharmacological effects of the compound 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB), a sulfonamide derivative containing selenium, in the ICS model. The results demonstrated that the 4-APSB treatment attenuated the nociceptive signs and the depressive-like phenotype in mice of both sexes exposed to ICS. Furthermore, the pharmacological action of 4-APSB in this experimental model might be associated to the modulation of the oxidative stress markers, the Na^+, K^+-ATPase activity and the expression of Nrf-2 and NFκB, signaling pathways related to oxidative stress and inflammation. The findings of this thesis reveal that sex impacts the neurobiological aspects related to fibromyalgia and, therefore, it should be considered for purposes of diagnosis and the development of more effective treatments.

Keywords: Fibromyalgia, pain, comorbidities, oxidative stress and selenium.

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LISTA DE ABREVIATURAS

4-APSB: 4-amino-3-(fenilselanil) benzenosulfonamida

ACR: Colégio americano de Reumatologia (do inglês *American College of Reumathology*)

AMPA: Receptor ionotrópico α-amino-3-hidroxi-metil-5-4-isoxazolpropiônico (do inglês α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)

ATP: Adenosina trifosfato (do inglês *Adenosine triphosphate*)

BDNF: Fator neurotrófico derivado do cérebro (do inglês *Brain derived neurotrophic factor*)

CAT: Catalase

CREB: proteína de ligação ao elemento de resposta ao AMPc (do inglês *cAMP response element binding protein*)

CRH: Hormônio liberador de corticotrofina

DNA: Ácido desoxirribonucleico (do inglês *Deoxyribonucleic acid)*

EFI: Estresse ao frio intermitente

ER: Espécies reativas

ER: Receptores de estrogênio (do inglês *Estrogen receptor*)

GABA: Ácido gama-aminobutírico (do inglês *Gamma-aminobutyric acid*)

GMPc: Monofosfato de guanosina cíclico (do inglês *Cyclic guanosine monophosphate*)

GPx: Glutationa peroxidase

GR: Glutationa redutase

GSH: Glutationa

IASP: Associação internacional para o estudo da dor (do inglês *International Association for the Study of Pain*)

IRSN: Inibidores da recaptação da serotonina e norepinefrina

ISRS: Inibidores seletivos da recaptação de serotonina

LCR: Líquido cefalorraquidiano

MAO: Monoamina oxidase

MAPK: Proteína quinase ativada por mitógeno (do inglês *Mitogen activated protein kinase*)

mRNA: Ácido ribonucleico mensageiro (do inglês *Messenger ribonucleic acid*)

MDA: Malonaldeído

NFκB: Fator nuclear kappa B (do inglês *Nuclear factor kappa B*)

NGF: Fator de crescimento nervoso (do inglês *Nerve growth factor*)

NMDA: N-metil-D-aspartato

NPSH: Tiol não proteico (do inglês *Non-protein thiol*)

Nrf-2: Fator nuclear eritroide 2 relacionado ao fator 2 (do inglês *Nuclear factor-erythroid-2 related factor*)

OH-1: Heme oxigenasse-1 (do inglês *Heme oxygenase-1*)

ON: Óxido nítrico

PKA: Proteína quinase A (do inglês *protein kinase A*)

PKC: Proteína quinase C (do inglês *protein kinase C*)

RNA: Ácido ribonucleico (do inglês do inglês *Ribonucleic acid*)

SNC: Sistema nervoso central

SOD: Superóxido dismutase

TBARS: Substâncias reativas ao ácido tiobarbitúrico (do inglês *Thiobarbituric acid reactive substances*)

TLR: Receptor do tipo Toll (do inglês *Toll like receptor*)

TRκ: Receptores de tropomiosina quinase (do inglês *Tropomyosin receptor kinase*)

TRPV: Receptor de potencial transiente vaniloide (do inglês *Transient receptor potential vanilloid*)

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1. INTRODUÇÃO

A fibromialgia é caracterizada pela rigidez, sensibilidade nos músculos e pela dor, que predomina nas regiões cervico-escapular e lombo-pélvica (OFFENBAECHER et al., 2021; KOCYIGIT e AKYOL, 2022). De acordo com *American College of Reumathology* (ACR), essa doença acomete principalmente mulheres jovens ou no meio da idade adulta, e se manifesta como uma condição dolorosa crônica que frequentemente coexiste com uma série de outros sintomas, incluindo ansiedade e/ou depressão (ARNOLD et al., 2019; SARZI-PUTTINI et al., 2020). Portanto, a fibromialgia tem sido considerada como um problema de saúde púbica visto que esses sintomas comprometem as atividades de rotina e a qualidade de vida de uma proporção considerável destes pacientes (NEUMEISTER e NEUMEISTER, 2020).

A fibromialgia faz parte de uma família de distúrbios denominados transtornos do espectro afetivo. Normalmente, esses distúrbios ocorrem em indivíduos e famílias que compartilham anormalidades fisiológicas ou até mesmo, fatores de risco genéticos que podem ser centrais para sua etiologia (McCARTHY, 2016). De fato, diversos fatores etiológicos parecem estar envolvidos no desenvolvimento dos sintomas clínicos desta doença, incluindo a susceptibilidade genética, os estímulos nocivos e não nocivos, os traumas físico e psicológico e a especificidade de gênero (STAUD e RODRIGUEZ, 2006; McBETH e MULVEY, 2012; D'AGNELLI et al., 2019).

Além disso, o quadro fisiopatológico da fibromialgia ainda é considerado complexo e multifatorial (GYORFI et al., 2022). Nesse sentido, alterações nas vias nociceptivas periféricas e centrais foram associadas à uma percepção intensa anormal de dor frente a estímulos não nocivos, em pacientes diagnosticados com essa doença (ARNOLD et al., 2019). Ademais, estudos bioquímicos indicam que a disfunção dos sistemas autonômico, neuroendócrino e imunológico pode levar a manifestação dos sintomas clínicos da fibromialgia (WALLACE et al., 2001; SARCHIELLI et al.,2007; PÉREZ-NERI et al., 2023). Apesar dessas evidências, o diagnóstico da fibromialgia é realizado apenas com base em evidências clínicas, pois não foram identificados marcadores biológicos validados associados à doença (MAFFEI, 2020). No entanto, estudos mais recentes sugerem que os perfis de microRNAs em diferentes fluidos biológicos

podem ajudar no diagnóstico desta doença (BJERSING et al., 2013; MASOTTI et al., 2017; DAVIS et al., 2018; D'AGNELLI et al., 2019).

Além das alterações no eixo hipotálamo-pituitária-adrenal (HPA), as aminas biogênicas, os aminoácidos excitatórios, as citocinas pró-inflamatórias, os canais iônicos e os agentes pró-oxidantes foram descritos como alguns dos mediadores fisiopatológicos envolvidos na fibromialgia (SINGH et al., 2019). Nesse âmbito, a disfunção de múltiplos sistemas orgânicos desencadeada por esta síndrome dificulta tanto o progresso no desenvolvimento de novos medicamentos, como a prescrição de uma terapia farmacológica adequada para os pacientes diagnosticados com fibromialgia. De maneira geral, recomenda-se que esses pacientes realizem ambos tratamentos farmacológico e não-farmacológico, com uma abordagem empírica focada nos sintomas individuais, em particular na dor física e emocional (NAGAKURA, 2015; LAWSON, 2016; NEUMEISTER e NEUMEISTER, 2020).

Em relação aos medicamentos atualmente disponíveis para tratar a fibromialgia, além de serem razoavelmente limitados, muitos pacientes descontinuam o uso dos mesmos devido à baixa eficácia clínica, ao aparecimento dos efeitos adversos e a reduzida capacidade de minimizar os principais sintomas (CLAUW, 2014; KIA e CHOY, 2017; TZADOK e ABLIN, 2020). Nesse sentido, os fármacos prescritos no manejo da fibromialgia incluem os antidepressivos tricíclicos e os inibidores seletivos da recaptação de serotonina (ISRS), os quais podem ser classificados como "medicamentos reposicionados" (ASHBURN e THOR, 2004). Portanto, torna-se emergente o desenvolvimento de novas estratégias terapêuticas, que possam ser mais eficazes e seguras, para tratar a fibromialgia.

Os compostos orgânicos de selênio são considerados potenciais alvos de estudo como uma possível intervenção farmacológica em diversos modelos experimentais que mimetizam condições dolorosas e neuropsiquiátricas crônicas (BRUNING et al., 2015; ROSA et al., 2018; BIRMANN et al., 2019; REIS et al., 2020). A elevada lipofilicidade destes compostos permite que eles atravessem a barreira hematoencefálica e alcancem o sistema nervoso central (SNC), onde podem desempenhar seus efeitos farmacológicos ou toxicológicos (NOGUEIRA et al., 2021). Dados prévios do nosso grupo de pesquisa revelaram que o composto 4-amino-3-(fenilselanil) benzenosulfonamida (4-APSB), um derivado

de sulfonamida contendo selênio, apresentou promissores efeitos antinociceptivo e anti-edematogênico à nível central e periférico em modelos animais de dor aguda. Ainda, os resultados sugerem que tais efeitos farmacológicos do 4-APSB podem ser atribuídos a sua capacidade de modular o estresse oxidativo (SACRAMENTO et al., 2022).

Considerando que os aspectos moleculares e celulares envolvidos na fibromialgia persistem como um problema desafiador, sob uma perspectiva da ciência básica e da clínica, se torna necessário uma melhor compreensão dos mecanismos fisiopatológicos subjacentes à essa patologia. Tendo em vista a crescente demanda pelo desenvolvimento de novas alternativas terapêuticas para a fibromialgia, este estudo também propõe o composto 4-APSB como um promissor candidato para o tratamento da fibromialgia por meio de um modelo pré-clínico.

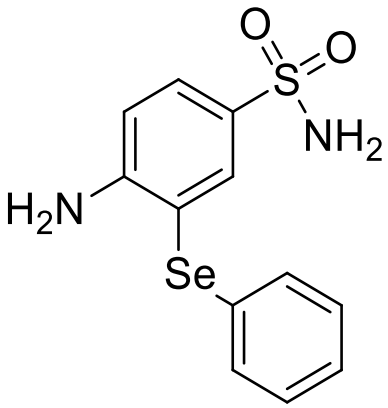

Figura 1. Estrutura química do 4-amino-3- (fenilselanil) benzenosulfonamida (4-APSB)

2. OBJETIVO

2.1 Objetivo geral

Investigar a influência do sexo nos mecanismos fisiopatológicos envolvidos na fibromialgia e elucidar as propriedades farmacológicas do composto sintético 4-APSB por meio de um modelo experimental que mimetiza essa doença em camundongos *Swiss*.

2.2 Objetivos específicos

- Avaliar se o fator sexo influencia no desenvolvimento dos sinais nociceptivos;
- Avaliar se o envolvimento dos receptores serotoninérgicos na fibromialgia pode ser influenciado pelo fator sexo;
- Explorar se o papel da via de sinalização NMDA/NO/GMPc na fibromialgia pode ser modulada pelo fator sexo;
- Determinar o papel do sistema redox no desenvolvimento da fibromialgia e se este pode ser influenciado pelo fator sexo;
- Investigar as enzimas Na^+, K^+ -ATPase e Mg^{2+} -ATPase como alvos de interesse para o tratamento da fibromialgia;
- Relacionar o eixo do Nrf2-NFkB com as alterações comportamentais que mimetizam a fibromialgia em camundongos;
- Identificar alterações na expressão de microRNAs (miR-155-5p e miR-338-3p) e nos seus genes (BDNF, Nrf2 e TRPV1) alvos que possam ser utilizados como marcadores fisiopatológicos da fibromialgia;
- Elucidar o potencial farmacológico do composto 4-APSB em um modelo experimental de fibromialgia.

3. REFERENCIAL TEÓRICO

3.1 As diferenças sexuais na dor

A fibromialgia, a síndrome da dor regional complexa ou a dor miofascial, condições clínicas que incluem a dor musculoesquelética como um componente, são mais prevalentes em mulheres (QUEME e JANKOWSKI, 2021; ELSAMADICY et al., 2018; WOLFE et al., 2018. Os dimorfismos sexuais na percepção da dor foram estabelecidos em estudos clínicos e pré-clínicos, em que as pacientes do sexo feminino apresentaram maior sensibilidade a estímulos nocivos. De forma semelhante, as fêmeas exibiram limiares de dor mais baixos, assim como uma diminuição na tolerância à dor, quando comparadas aos machos (BULLS et al., 2015; STAIKOU et al., 2017; MOGIL, 2020).

Sob o ponto de vista mecanicista, um estudo relatou que as estruturas cerebrais relacionadas à dor são mais volumosas em homens saudáveis do que em mulheres (ZHANG et al., 2021). Além disso, estudos de neuroimagem da atividade cerebral evocada pela dor revelaram uma desativação específica das regiões de controle cortical pré-frontal no sexo masculino, mas um recrutamento das áreas corticais do cíngulo anterior, relacionadas ao processamento das emoções, específica no sexo feminino (GIRARD-TREMBLAY et al., 2014). Esses indícios sugerem que as diferenças sexuais relacionadas a percepção da dor podem ter uma base anatômica ou fisiológica, especialmente em regiões que envolvem o processamento emocional da dor.

Geralmente, as diferenças sexuais nas respostas da dor são explicadas pela influência dos hormônios gonadais (MAURER et al., 2016), uma vez que eles podem interagir com o sistema nervoso periférico e central, de forma direta via mecanismos baseados na ativação de receptores, ou indiretamente, por meio de interações com os neurotransmissores em caminhos nociceptivos. Ainda, há substanciais evidências de que o dimorfismo sexual pode alterar a expressão de genes relacionados ao sistema imune, modulando assim a fisiologia da dor e o comportamento dos animais, de forma dependente do sexo (SORGE et al., 2015; VILLA et al., 2018; GUNEYKAYA et al., 2018). Portanto, os modelos animais de dor crônica são considerados ferramentas úteis para explorar as bases biológicas para as respostas diferenciais à dor em humanos, pois oferecem uma

visão crítica sobre os mecanismos que podem ser influenciados não apenas por fatores hormonais, mas também genéticos e moleculares.

3.2 Fibromialgia

A dor crônica pode ser considerada como uma das reclamações mais onipresente na medicina clínica. Segundo a *Global Burden of Disease Study*, estima-se que as dores musculoesqueléticas e articulares, as dores de cabeça, no pescoço e nas costas, a dor oncológica e a dor pós-cirúrgica, reconhecidas como condições dolorosas crônicas, afetam cerca de 20% (10-55%) da população adulta em todo o mundo (VOS et al., 2017; MILLS et al., 2019; BARROSO et al., 2021). As consequências físicas e psicológicas para os portadores desses quadros clínicos causam grande impacto social e econômico negativo, não apenas para eles como indivíduos, mas também para a família e a sociedade em geral (RICE et al., 2016).

A fibromialgia, uma síndrome sistêmica complexa, é caracterizada pela dor crônica generalizada, pela rigidez articular e pela presença de vários pontos sensíveis ao logo do corpo, em que esses sintomas se manifestam devido a sensibilização do sistema nervoso central (SNC). Ainda, outros sintomas associados podem estar presentes, incluindo episódios depressivos (WANG et al., 2015; CHINN et al., 2016). Estima-se que essa síndrome contribua significativamente para a alta prevalência da dor crônica (GRODMAN et al., 2011).

De acordo com os dados epidemiológicos, a prevalência da fibromialgia varia entre 2 a 8% da população mundial, sendo que as mulheres apresentam mais riscos de desenvolver essa síndrome do que os homens. Na maioria das vezes, os sintomas aparecem no meio da idade adulta, mas também podem ocorrer durante o período da adolescência e em pessoas idosas (QUEIROZ, 2013; HEIMANN et al., 2017). Um estudo realizado no Brasil revelou que a fibromialgia atinge cerca de 2% da população brasileira, com proporção de 1 homem para 5,5 mulheres (SOUZA e PERISSINOTTI, 2018).

Até o momento, existe uma grande controvérsia em relação a avaliação e ao diagnóstico da fibromialgia. Por esse motivo, acredita-se que aproximadamente 75% das pessoas com essa síndrome permaneçam sem um diagnóstico adequado (ARNOLD et al., 2011). Em 1990, o *American College of*

Reumathology (ACR) publicou alguns critérios para a classificação da fibromialgia. Desde então, vários métodos alternativos de diagnóstico já foram propostos. De forma geral, a maioria dos pesquisadores e dos profissionais da área da saúde concordam em avaliar quatro domínios principais como critérios de diagnóstico para a fibromialgia: (1) intensidade da dor, (2) funcionamento físico, (3) funcionamento emocional e (4) melhora/bem-estar geral (WILLIAMS e SCHILLING, 2009; MAFFEI, 2020).

Em 2010, o ACR introduziu novos critérios de diagnóstico preliminares que consideravam o número de regiões corporais doloridas por meio de uma avaliação sobre a presença e a gravidade da fadiga, a dificuldade cognitiva, o sono não revigorado e a extensão dos sintomas somáticos. Assim, esses critérios de diagnóstico também poderiam ser aplicados por profissionais da atenção primária, uma vez que não exigia o exame de pontos dolorosos, referido por muitos como de difícil interpretação. Nesse contexto, foi estipulado que a Escala da Severidade dos Sintomas acompanhado do Índice de Dor Generalizado poderiam ser utilizados para quantificar a gravidade dos sintomas físicos e somáticos relacionados à fibromialgia (WOLFE et al., 2010). Com especificidade de 96,6% e sensibilidade de 91,8%, os critérios do ACR 2010 foram considerados mais sensíveis do que os 1990, permitindo que pacientes subdiagnosticados com fibromialgia fossem identificados e tratados corretamente (ONCU et al., 2013; MAFFEI, 2020).

As adversidades encontradas para desenvolver um diagnóstico apropriado para a fibromialgia também são evidenciadas em dificuldades para identificar a patogênese dessa síndrome. Estudos indicam que fatores familiares, genéticos, ambientais, endócrinos e neurológicos podem contribuir para o desenvolvimento da fibromialgia (LICHTENSTEIN et al., 2018). Recentemente, uma análise de imunofenotipagem realizada em amostras de sangue de pacientes com fibromialgia demonstrou que a presença do receptor opioide μ nos linfócitos B pode ser um biomarcador específico nessa síndrome (RAFFAELI et al., 2020). Além disso, um método rápido baseado em biomarcadores para o diagnóstico da fibromialgia foi desenvolvido. Nesse método, o uso de espectroscopia vibracional diferencia pacientes com fibromialgia daqueles com outras doenças relacionadas à dor (HACKSHAW et al., 2019). Apesar dessas

evidências, biomarcadores biológicos validados para a fibromialgia ainda não foram identificados.

3.3 Fatores que predispõe a fibromialgia

3.3.1 Contribuição de fatores ambientais no desenvolvimento da fibromialgia

Além da contribuição genética, fatores ambientais também podem estar envolvidos na fibromialgia (D'AGNELLI et al., 2019). Eventos no início da vida, tais como traumas físicos e estressores psicossociais, podem influenciar na expressão gênica e assim, predispor o desenvolvimento da fibromialgia (AL-ALLAF et al., 2002; GUR et al., 2002). Nesse contexto, experiências adversas durante o período neonatal e infantil, como o nascimento prematuro ou abusos físico e sexual, podem provocar mudanças duradouras nos circuitos nociceptivos, resultando na diminuição do limiar de dor na fase adulta, o qual é considerado um dos principais sintomas da fibromialgia (PARAS et al., 2009; HAUSER et al., 2011; LOW e SCHWEINHARDT, 2012).

Há relatos de que estressores físicos repetidos, particularmente atividades relacionadas com levantamento de peso, movimentos repetitivos e agachamentos por longos períodos de tempo, podem contribuir para o desenvolvimento da dor crônica generalizada (HARKNESS et al., 2004). Entretanto, os estressores psicológicos e sociais (estresse crônico, trauma emocional, agressão/abuso físico) representam os principais gatilhos ambientais relacionados ao desenvolvimento da fibromialgia na idade adulta, especialmente em mulheres (BENNETT et al., 2007; HAVILAND et al., 2010). Além desses fatores, a negligência, o abuso emocional e o transtorno do estresse pós-traumático durante a fase da infância e da adolescência também podem desencadear a manifestação dos sintomas físicos e somáticos da fibromialgia (HAUSER et al., 2015; HELLOU et al., 2017).

Os processos fisiológicos que interconectam a experiência do estresse com o desenvolvimento da fibromialgia permanecem inconclusivos. Alguns estudos evidenciaram que tanto os níveis reduzidos do hormônio liberador de corticotrofina (CRH) no hipotálamo, como os níveis aumentados de substância P e de glutamato no fluído cérebro-espinhal podem estar associados com a

diminuição no limiar de dor em pacientes com fibromialgia (BECKER e SCHWEINHARDT, 2012). Ainda, pacientes com fibromialgia exibiram uma diminuição na atividade dos sistemas dopaminérgico, opioidérgico e serotoninérgico sugerindo, portanto, um complexo desequilíbrio nos padrões psicobiológicos (BLEAKMAN et al., 2006).

3.3.2 Contribuição de fatores genéticos no desenvolvimento da fibromialgia

Variantes genéticas e mecanismos de herança genética relacionados à dor contribuem para o desenvolvimento da dor crônica. Um estudo previamente realizado evidenciou uma correlação forte entre variantes genéticas e a resposta à dor (MOGIL, 2012). Até o momento, centenas de genes que regulam a dor já foram identificados, dentre os quais, genes que codificam canais de sódio voltagem dependente, receptores opioides μ, catecol-O-metiltranferase e proteínas da via GABAérgica, dopaminérgica e glutamatérgica (OERTEL e LOTSCH, 2008; D'AGNELLI et al., 2019).

À medida que o papel dos fatores genéticos nos mecanismos da dor se tornou mais evidente, foi possível estabelecer uma melhor compreensão entre os fatores genéticos e o desenvolvimento da fibromialgia. Assim, PELEGRINO et al. (1989) analisaram 17 famílias de pacientes com fibromialgia a fim de elucidar a hereditariedade nessa síndrome. Os dados demonstraram que 52% dos pais e irmãos, inscritos nesse estudo clínico, exibiam os sintomas clínicos característicos da fibromialgia, enquanto que 22% daqueles sem sintomas aparentes, apresentavam consistência muscular anormal. Portanto, os autores sugeriram que o modo de herança genética da fibromialgia seja autossômico dominante, apesar do tamanho amostral ser considerado relativamente pequeno.

Um outro estudo analisou 58 filhos de 20 famílias nucleares completas de mães com fibromialgia. Embora 28% desses filhos também receberam o diagnóstico dessa síndrome, a exposição da prole a fatores externos (ambiental, psicológico e familiar) não interferiu na susceptibilidade à fibromialgia (BUSKILA e NEUMANN, 1996). Ainda, BUSKILA e NEUMANN (1997) avaliaram mulheres com fibromialgia e seus familiares próximos, incluindo parentes consanguíneos

(pais, irmão e filhos) e membros não aparentados (maridos). Os resultados indicam que a prevalência da fibromialgia entre parentes consanguíneos é maior do que em membros não aparentados de pacientes com diagnóstico de fibromialgia. Além disso, a sensibilidade à dor dos parentes, em ambos os sexos, foi significativamente maior quando comparado ao grupo controle. Esses estudos reforçam que tanto a sensibilidade à dor, como a ocorrência familiar dessa síndrome podem ser atribuídas a determinados fatores genéticos (PARK e LEE, 2018).

Contribuindo com a hipótese genética, um estudo de varredura de ligação de todo o genoma revelou que parentes de primeiro grau são, cerca de 14 vezes, mais susceptíveis a desenvolver fibromialgia. Nesse estudo, dois genes foram mapeados como possíveis marcadores dessa doença, os genes que codificam o transportador de serotonina (SLC4A4) e o receptor de potencial transiente vaniloide-2 (TRPV2) (ARNOLD et al., 2013). De fato, alterações no gene TRPV2 têm sido relacionadas com a diminuição no limiar de dor em pacientes com fibromialgia (MICKLE et al., 2015). Portanto, sendo uma condição crônica, é possível que os mediadores neurobiológicos envolvidos na fibromialgia juntamente com os aspectos ambientais desencadeiem mudanças na expressão gênica à longo prazo (DENK e McMAHON, 2012). No entanto, poucos estudos descrevem o envolvimento dos processos epigenéticos na fibromialgia.

3.4 O perfil de microRNAs na fibromialgia

Estudos anteriores evidenciaram que as experiências no início da vida e os fatores ambientais podem modular a função do genoma e o fenótipo por meio de mecanismos epigenéticos (SZYF e BICK, 2013). Em outras palavras, a epigenética estuda as mudanças hereditárias na expressão gênica ou no fenótipo celular de acordo com o ambiente ou com o estilo de vida de cada indivíduo, sem modificar a sequência de nucleotídeos do ácido desoxirribonucleico (DNA). Para isso, os mecanismos epigenéticos englobam a metilação do DNA, a variação da cromatina e os RNAs não codificantes, particularmente os microRNAs (BIRD, 2007; MEYDAN et al., 2016). Considerando que o desenvolvimento da fibromialgia pode estar relacionado com a interação entre o gene e o ambiente, é plausível que os mecanismos

epigenéticos desempenhem um papel importante como mediadores de alterações no sistema nervoso central e periférico a longo prazo (DENK e McMAHON, 2012).

Nos últimos anos, os microRNAs foram identificados como importantes moduladores da expressão gênica em condições fisiológicas ou patológicas. Os microRNAs são moléculas curtas de ácido ribonucleico (RNA) não codificantes altamente conservadas evolutivamente, com cerca de 20 a 22 nucleotídeos de comprimento. Eles inibem a expressão gênica pós-transcricional, seja pela inibição da tradução ou pela degradação do RNA mensageiro (RNAm) alvo (Figura 2). Entretanto, essas moléculas curtas de RNA também podem mediar a regulação positiva da tradução. Em qualquer cenário, um único microRNA pode modular a expressão de centenas de genes e, por outro lado, o RNAm individual pode ser alvo de vários microRNAs (BAEK et al., 2008; VASUDEVAN, 2012; CERDÁ-OLMEDO et al., 2015).

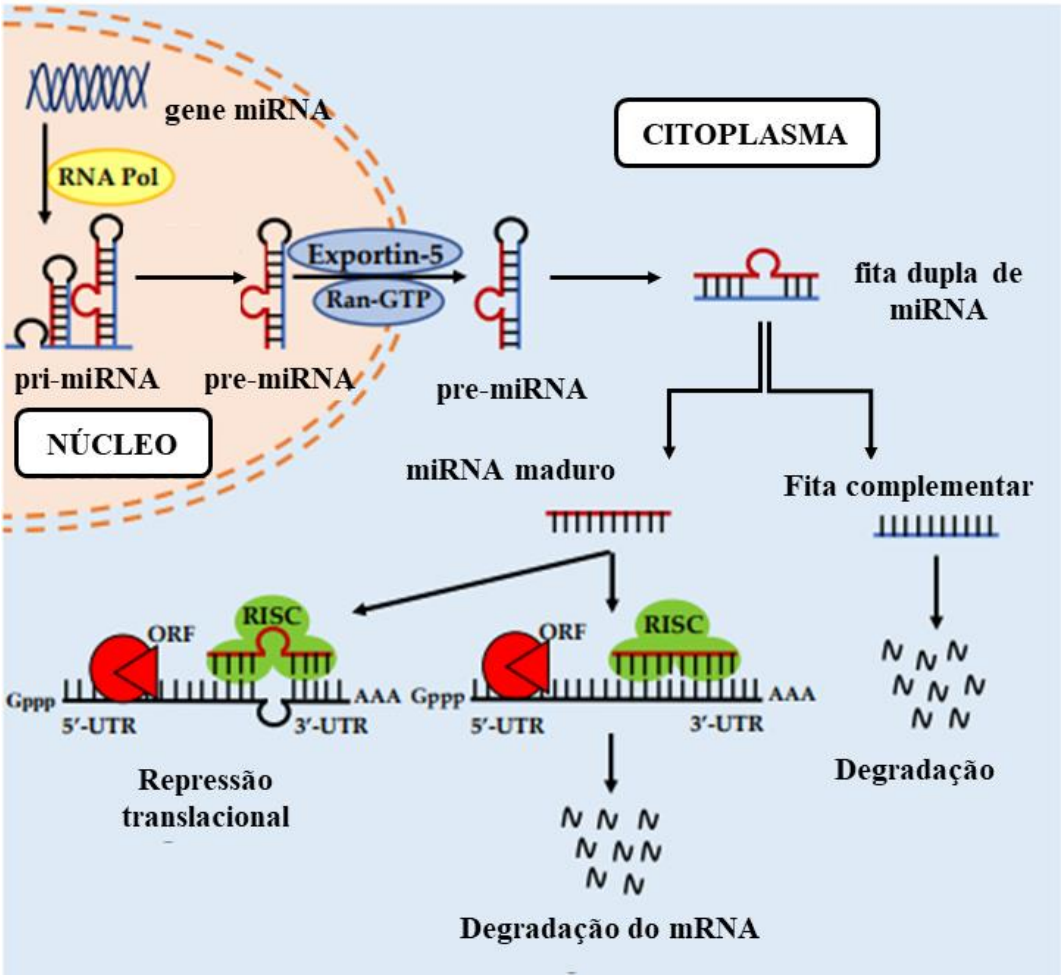

Figura 2. Representação esquemática da regulação da expressão gênica mediada pelos microRNAs. Os microRNAs podem degradar o RNA mensageiro (mRNA) ou bloquear a

progressão do ribossomo ao longo da fita de mRNA, interferindo na tradução de proteínas (Adaptado a partir de MEYDAN et al., 2016).

Sob o ponto de vista clínico, os microRNAs apresentam alta estabilidade em amostras biológicas. Eles podem ser liberados para os fluidos biológicos (microRNAs circulantes) por meio dos exossomos ou embalados em complexos associados às proteínas argonautas (RAYNER e HENNESSY, 2013). Dentro desse contexto, a presença dessas moléculas curtas de RNA tanto em compartimentos celulares, como no ambiente extracelular, favorece e intensifica o seu uso como promissores candidatos a biomarcadores para a melhor compreensão da etiologia de doenças crônicas, como a fibromialgia (RAYNER e HENNESSY, 2013). Nos últimos anos, alguns estudos apontaram um perfil diferente na expressão de microRNA entre controles saudáveis e pacientes com fibromialgia (BJERSING et al., 2015; CERDÁ-OLMEDO et al., 2015; MASOTTI et al., 2017; QIU et al., 2021).

BJERSING et al. (2015) analisou a expressão genômica de microRNAs no líquido cefalorraquidiano (LCR) em mulheres com fibromialgia e correlacionou com os sintomas característicos dessa doença, incluindo o limiar e os níveis de dor, bem como a fadiga. De acordo com os resultados obtidos, dentre os 742 microRNAs humanos analisados, 9 desses foram expressos de forma significativamente diferente no LCR quando comparados controles saudáveis e pacientes com fibromialgia, sendo que apenas o miR-145-5p apresentou uma correlação positiva com os sintomas de dor e fadiga em pacientes com fibromialgia. Os autores também identificaram um padrão diferente de microRNAs no soro e no LCR de mulheres com fibromialgia. Dos 374 microRNAs totais avaliados, a expressão do miR-320a foi maior, enquanto que a expressão dos miR-103a-3p, miR-107, let-7a-5p, miR-30b-5p, miR-151a-5p, miR-142-3p e miR-374b-5p foram menores no soro de pacientes com fibromialgia em relação aos controles saudáveis. Em relações aos sintomas clínicos, o miR-374b-5p exibiu uma correlação negativa com o limiar de dor, já o miR-103a-3p e o let-7a-5p tendem a ser associados com a quantidade de sono e a dor. Por outro lado, o miR-320a, mais expresso na fibromialgia, foi inversamente correlacionado com a dor (BJERSING et al., 2015).

Em um outro estudo, foi analisada a expressão de microRNAs nas células mononucleares do sangue periférico em pacientes com fibromialgia. Os principais achados demonstraram a inibição na expressão dos miR-451a, do miR-338-3p, do miR-143-3p, do miR-145-5p e do miR-223-3p em pacientes com fibromialgia quando comparados aos controles. Embora nenhuma correlação com os critérios clínicos tenha sido encontrada, o miR-223-3p e o miR-145-5p também estavam inibidos no LCR de pacientes com fibromialgia e em virtude disso, foram propostos como possíveis biomarcadores desta doença (CERDÁ-OLMEDO et al., 2015).

Em um estudo mais recente, MASOTTI et al. (2017) evidenciou que 6 microRNAs (miR-23a-3p, miR-1, miR-133a, miR-346, miR-139-5p e miR-320b) foram regulados negativamente no soro de pacientes com fibromialgia em comparação aos controles (MASOTTI et al., 2017). Os autores destacaram que a expressão do miR-23a-3p foi encontrada inibida em mais de um fluido biológico (soro e LCF), o qual eles atribuíram ao papel desse microRNA em modular os genes responsáveis pela manutenção da integridade do músculo esquelético (WADA et al., 2015). De forma geral, os microRNAs encontrados desregulados possuem não somente conexões com a dor, com o estresse e com os sintomas depressivos, mas também parecem estar envolvidos no funcionamento inadequado do SNC e do músculo esquelético (MASOTTI et al., 2017).

3.5 Mecanismos fisiopatológicos envolvidos na fibromialgia

3.5.1 Sensibilização central

A *International Association for the Study of Pain* (IASP) define o processo de sensibilização central como um fenômeno responsável pela estimulação exacerbada dos neurônios nociceptores presentes no SNC diante de um estímulo normal ou sublimiar (LOESER e TREEDE, 2008). Resumidamente, a sensibilização central é resultante de uma série de processos adaptativos nos neurônios de segunda ordem, bem como nas regiões supra espinhal e subcortical, resultando na transição de um estado de dor aguda para a crônica (FIELDS et al., 1998; APKARIAN et al., 2004; MEACHAM et al., 2017). Em pacientes com fibromialgia, assim como nas demais condições dolorosas crônicas, os mecanismos que desencadeiam a sensibilização central envolvem:

(1) o aumento dos mecanismos excitatórios, (2) a inativação dos mecanismos inibitórios e (3) a interação entre os neurônios e as células da glia (IWATA et al., 2017).

A liberação excessiva de glutamato ativa o receptor ionotrópico α-amino-3-hidroxi-metil-5-4-isoxazolpropiônico (AMPA), gerando potencias pós-sinápticos excitatórios nos neurônios de ordem superior (JI et al., 2018). De forma geral, os canais do receptor N-metil-D-aspartato (NMDA) encontram-se bloqueados pelos íons magnésio (Mg^{+2}). Entretanto, a ativação das fibras nociceptoras aferentes (A-δ e C) promove a liberação de diversos mediadores, como o glutamato, a substância P, o fator neurotrófico derivado do cérebro (BDNF) e a adenosina trifosfato (ATP), favorecendo assim a despolarização da membrana. Como consequência, ocorre a abertura dos canais do receptor NMDA por meio da remoção dos íons Mg^{+2}. Cabe ressaltar que esses receptores exercem um papel essencial nas fases iniciais e na manutenção da sensibilização central (WOOLF, 2004; BASBAUM et al., 2009).

Os receptores NMDA podem estimular o influxo de cálcio (Ca^{+2}), as vias de sinalização dependentes de Ca^{+2} e a síntese de óxido nítrico (ON). Por sua vez, o ON estimula a produção de monofosfato de guanosina cíclico (GMPc) o qual pode ativar as proteínas quinases ativadas por mitógenos (MAPK) e as proteínas quinases (PKA e PKC), de forma direta ou indireta. Essas últimas podem fosforilar os canais iônicos favorecendo o disparo dos potenciais de ação e resultando na geração da dor. De fato, o desenvolvimento da sensibilização central permite que os estímulos de baixa intensidade aplicados na pele ou no tecido muscular possam gerar altos níveis de impulsos nociceptivos para o cérebro, culminando em alterações na percepção da dor (JI et al., 2003; LATREMOLIERE e WOOLF, 2009).

Em condições normais, os interneurônios inibitórios liberam GABA e/ou glicina para diminuir a excitabilidade dos neurônios nociceptivos e modular a transmissão nociceptiva. Entretanto, a perda da função desses interneurônios também pode desencadear o aumento da dor. Dentro desse contexto, a disfunção desses interneurônios inibitórios, em conjunto com hiperexcitabilidade mediada pelos receptores do tipo NMDA, pode alterar a estrutura, o fenótipo e a função das fibras A-β não nociceptivas, fazendo que com que elas participem do

circuito da transmissão nociceptiva, de forma que estímulos não nocivos sejam percebidos como dolorosos (KELLER et al., 2007; BASBAUM et al., 2009).

As células gliais também podem contribuir no processamento disfuncional da dor (WATKINS et al., 2001). No SNC, as sinapses são envoltas pela glia que, normalmente, não responde a entrada dos impulsos nociceptivos no sítio local. No entanto, após o início da sensibilização central, as células da glia podem ser estimuladas. Na micróglia, a ativação de receptores purinérgicos e dos receptores do tipo Toll (TLR) desencadeia a liberação do BDNF que, por meio dos receptores de tropomiosina quinase B (TRkB), promove o aumento da excitabilidade dos neurônios nociceptivos e consequentemente, o aumento da resposta à dor frente a estímulos não nocivos (alodinia) e nocivos (hiperalgesia) (WATKINS et al., 2001; 2003).

A micróglia ativada também libera diversas citocinas pró-inflamatórias, incluindo o fator de necrose tumoral, as interleucinas 1 e 6, as prostaglandinas, dentre outros fatores que contribuem para a sensibilização central. Por outro lado, as células astrogliais também podem se tornar hiperativas e liberar glutamina, captada nos terminais pré-sinápticos, favorecendo ainda mais a liberação de glutamato a partir das fibras nociceptoras aferentes (A-δ e C), causando um aumento na hiperexcitação dos neurônios no corno dorsal da medula espinhal (Figura 3) (OKADA-OGAWA et al., 2009).

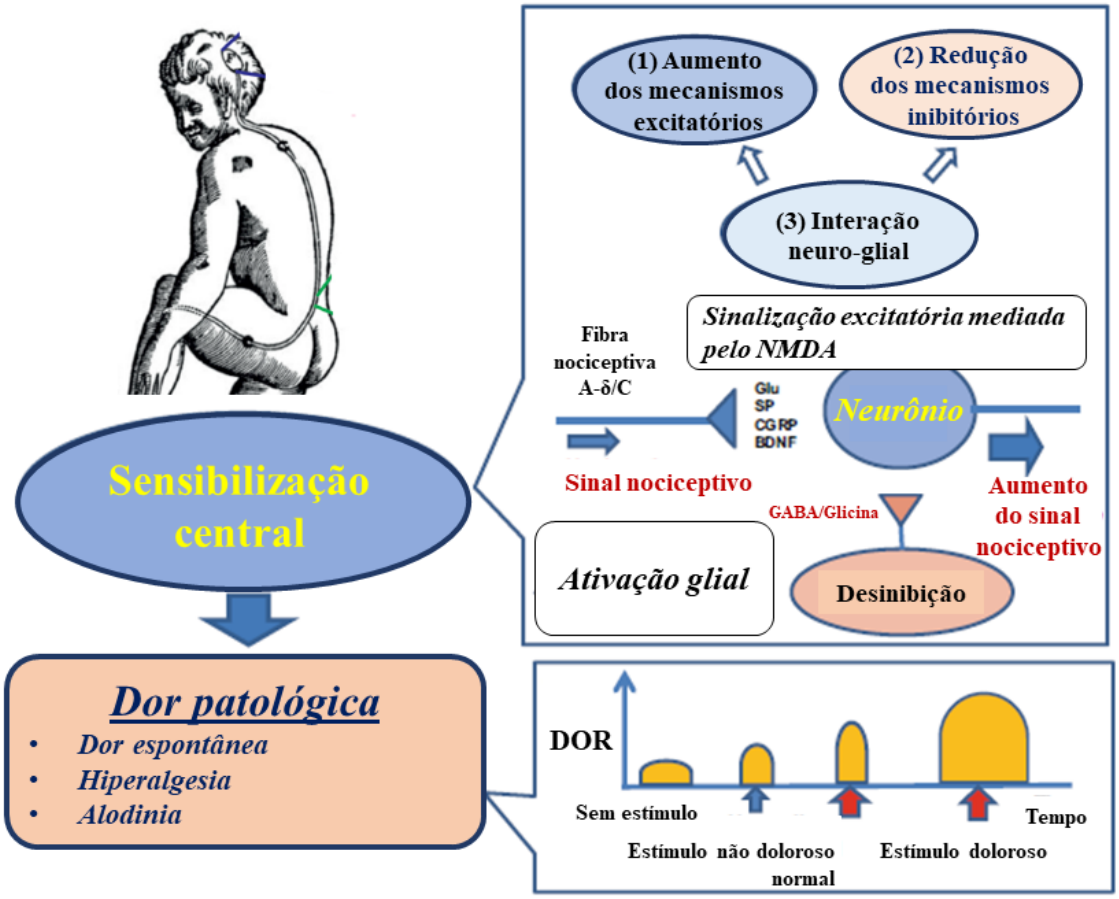

Figura 3. Representação esquemática dos mecanismos envolvidos na sensibilização central e na dor patológica. As fibras Aδ-/C conduzem os disparos de potencias de ação, que estão aumentados, para os terminais centrais. Esses impulsos de alta frequência que transportam o sinal nociceptivo, por sua vez, estimulam o mecanismo excitatório e inativam o mecanismo de inibição, via interação glial-neuronal. Esses sinais nociceptivos aumentados estão associados à dor patológica. (Adaptado a partir de IWATA et al., 2017).

3.5.2 Sinalização das aminas biogênicas

Evidências cumulativas sinalizam a depleção dos níveis cerebrais de aminas biogênicas como o principal mecanismo responsável pelo desenvolvimento dos sintomas característicos da fibromialgia. Níveis reduzidos de neurotransmissores aminérgicos, como a serotonina, a dopamina e a norepinefrina foram observados no LCR em pacientes com fibromialgia (RUSSEL et al., 1992).

A serotonina e a norepinefrina estão envolvidas na regulação dos mecanismos endógenos para o alívio da dor, por meio da via descendente da dor (WOOD, 2008). Em particular, a depleção de serotonina na via descendente da dor contribui com as alterações na percepção da dor em pacientes

diagnosticados com fibromialgia. Ainda, estudos prévios demonstraram uma redução na taxa do transporte de serotonina e dos seus metabólitos, assim como uma diminuição no seu precursor, o triptofano em pacientes que sofrem de fibromialgia (RUSSEL et al., 1989, RUSSEL et al., 1992, STRATZ et al., 1993). Tais indícios sugerem que a redução da atividade endógena serotoninérgica pode estar envolvida na funcionalidade inadequada da via analgésica descendente em pacientes com fibromialgia (SLUKA e CLAUW, 2016).

No sistema monoaminérgico, a principal função da enzima monoamina oxidase (MAO) consiste em regular a disponibilidade das aminas biogênicas nas sinapses, ou seja, ela é responsável pela catálise oxidativa da serotonina, da norepinefrina e da dopamina (KUMAR et al., 2018). Nesse contexto, o aumento na expressão ou atividade da MAO é atribuído como uma das principais causas que levam à depleção de aminas biogênicas (VIANELLO et al., 2012). De fato, uma alteração funcional no gene que codifica a enzima MAO foi identificada em indivíduos que apresentam os sintomas mais severos da fibromialgia (GÜRSOY et al., 2008). Confirmando o papel dessa enzima na fibromialgia, um estudo conduzido por TORT et al. (2012) demonstraram a eficácia do tratamento com pirlindol, um inibidor da MAO-A, em pacientes com fibromialgia, ao diminuir os pontos dolorosos e a sensação da dor de forma geral (TORT et al., 2012). Coletivamente, estes indícios sugerem que a inibição da MAO pode promover o alívio da dor e das comorbidades associadas à fibromialgia, incluindo a depressão.

Semelhante às evidências clínicas, estudos realizados em animais também demonstraram que a disfunção das vias descendentes da dor pode estar relacionada com uma redução na atividade da via serotoninérgica (SLUKA e CLAUW, 2016). Em um modelo animal de fibromialgia induzido pela reserpina, os animais exibiram uma diminuição na força muscular, o qual foi atribuído a uma depleção nos níveis de serotonina detectado nas análises de microdiálise (OGINO et al., 2013). Em um estudo mais recente, SINGH et al. (2020) também reportaram que as alterações comportamentais induzidas pela reserpina podem ter relação com o aumento da atividade da enzima MAO-A (SINGH et al., 2020). Por outro lado, a administração sistêmica da locarserina, da vabicaserina e do YM348, agonistas do receptor 5-HT_{2C}, diminuiu a sensibilidade muscular

induzida pela reserpina, indicando que esse receptor pode modular a percepção da dor na fibromialgia (OGINO et al., 2013). Somando-se a isso, há evidências de que a produção de anticorpos contra os receptores serotoninérgicos pode desencadear os sintomas da fibromialgia (SAMBORSKI et al., 1998).

Portanto, a partir das evidências mencionadas acima, foi proposto que a depleção nos níveis de serotonina devido à redução nos níveis de triptofano, juntamente com a formação de anticorpos direcionados para os receptores serotoninérgicos podem mediar o aparecimento dos sintomas e as comorbidades relacionadas à fibromialgia. Assim, os fármacos antidepressivos, principalmente aqueles pertencentes a classe dos inibidores seletivos da recaptação de serotonina, são usualmente prescritos para o tratamento da fibromialgia (LI et al., 2016). Estudos pré-clínicos e clínicos demonstraram que eles atenuam os sintomas e as comorbidades associadas à fibromialgia (NISHIYORI e UEDA, 2011; CLAW, 2014; TZADOK e ABLIN, 2020).

Além dos neurotransmissores serotonina e norepinefrina, a dopamina também desempenha um papel fundamental na regulação da sensação de dor e na analgesia natural, em diferentes regiões do cérebro e da medula espinhal (WOOD, 2008). Nesse sentido, os pacientes com fibromialgia exibiram uma diminuição na síntese de dopamina em diversas regiões do cérebro relacionadas à dor, como o mesencéfalo, o hipocampo, o tálamo e a ínsula (WOOD et al. 2007a). Um outro estudo desenvolvido por WOOD et al. (2007) mostrou uma diminuição na atividade dopaminérgica cerebral mediante um estímulo doloroso em indivíduas diagnosticados com essa doença com fibromialgia em comparação com indivíduos saudáveis (WOOD et al. 2007b). Ainda, foi documentada uma correlação negativa entre a disponibilidade do receptor dopaminérgico D2/D3 e o limiar de dor em pacientes com fibromialgia, com ou sem depressão (LEDERMANN et al., 2016). Apesar desses indícios, o papel do sistema dopaminérgico na fibromialgia ainda não está totalmente elucidado.

3.5.3 Estresse oxidativo

O oxigênio e o nitrogênio molecular são descritos como os elementos vitais para todos os organismos vivos, incluindo para as células neuronais. No

entanto, ambos podem exercer efeitos deletérios às biomoléculas na forma de espécies reativas (ER) (SINGH et al, 2019).

Em condições fisiológicas, a geração de ER nas células encontram-se em equilíbrio com uma ampla variedade de defesas antioxidantes, as quais podem ser subdivididas em enzimáticas como as peroxirredoxinas, a superóxido dismutase (SOD), a glutationa peroxidase (GPx) e a catalase (CAT), ou não enzimáticas, representadas pelas vitaminas C e E, o ácido lipoico, a glutationa (GSH) e os carotenoides. Portanto, o sistema antioxidante é responsável por neutralizar e até mesmo combater os danos oxidativos deletérios provocados pelas ER dentro das células (REDZA-DUTORDOIR e AVERILL-BATES, 2016).

Em contrapartida, o funcionamento inadequado do sistema de detoxificação antioxidante, em conjunto com níveis exacerbados de ER podem desencadear o estresse oxidativo nas células (HALLIWELL, 2011). Diante disso, o estresse oxidativo tem sido associado ao desenvolvimento de diversas condições dolorosas crônicas, incluindo a fibromialgia (SALVEMINI et al, 2011; BRIEGER et al; 2012). CORDERO et al. (2009) demonstraram uma redução nos níveis da coenzima Q-10, acompanhada por um aumento na produção de ER nas células mononucleares sanguíneas de pacientes com fibromialgia, sugerindo que um déficit no metabolismo da coenzima Q-10 pode favorecer o desenvolvimento do estresse oxidativo (CORDERO et al., 2009).

Cabe ressaltar que a gravidade dos sintomas característicos da fibromialgia, como a dor e o sofrimento psíquico, foram negativamente associados com a atividade das enzimas CAT, GPx e GR (SHUKLA et al., 2020). Além disso, níveis elevados de malonaldeído (MDA) e de ON, juntamente com a inibição na atividade das enzimas antioxidantes (SOD, CAT, GPx e GR) foram observados no lisado de glóbulos vermelhos de pacientes diagnosticados com essa doença (SHUKLA et al., 2020). Nesse sentido, há dados concretos na literatura de que o desequilíbrio entre marcadores pró oxidantes e antioxidantes está associado ao aparecimento dos sintomas dolorosos e emocionais em indivíduos com fibromialgia, conforme ilustrado na Figura 4 (RUS et al., 2021; ASSAVARITTIRONG et al, 2022). Corroborando com essas evidências, a fluoxetina, um dos medicamentos recomendados para o tratamento da fibromialgia, reduz o estresse oxidativo. Esse fármaco modula indiretamente a

cadeia transportadora de elétrons, diminuindo a fosforilação oxidativa no cérebro de ratos (CURTI et al., 1999).

Os estudos pré-clínicos também sugerem o estresse oxidativo como um importante modulador da dor em diferentes modelos experimentais de fibromialgia (KAUR et al., 2019; HEIMFARTH et al., 2020). Diante dessas evidências, compostos derivados de produtos naturais ou sintéticas exibiram promissores efeitos farmacológicos em modelos animais de fibromialgia (OLIVEIRA et al., 2016; SIQUEIRA-LIMA et al., 2017; KAUR et al., 2019; KAUR et al., 2021). Esses compostos, ao modular o estresse oxidativo, alteram os níveis de neurotransmissores, de citocinas pró-inflamatórias e de outras cascatas de sinalização envolvidas na fibromialgia, atenuando, assim, os sinais característicos dessa doença em roedores (KAUR et al., 2019; KAUR et al., 2021). Portanto, compostos com potencial efeito antioxidante podem ser considerados ferramentas farmacológicas promissoras para o tratamento da fibromialgia.

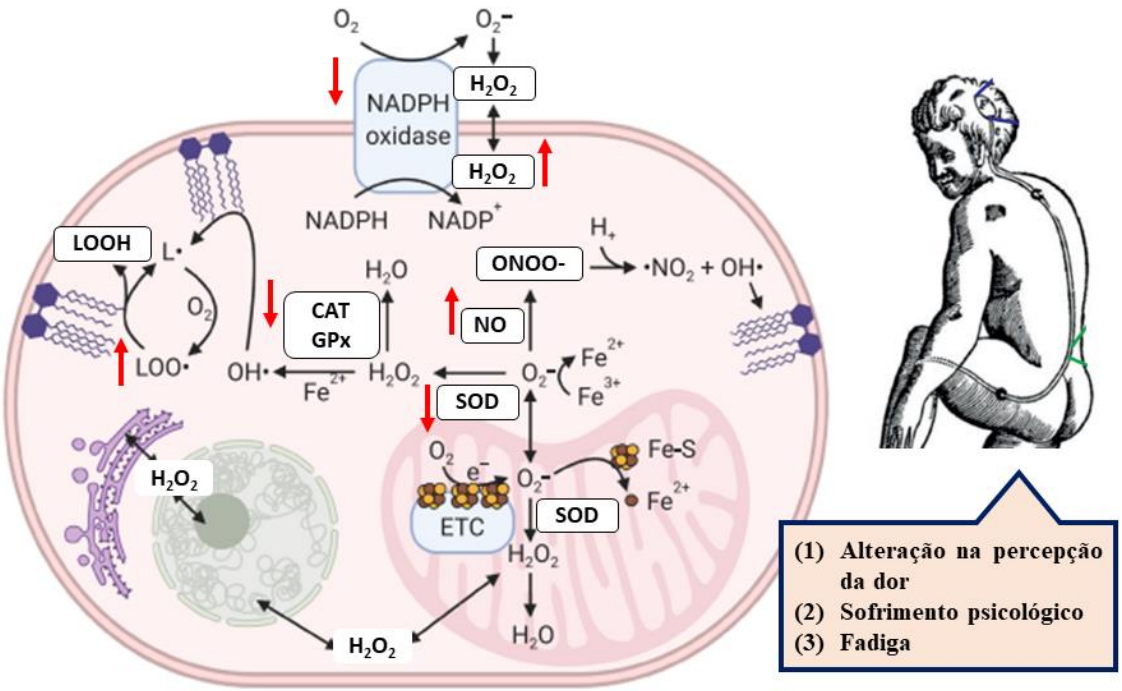

Figura 4. Representação esquemática dos processos moleculares e oxidativos envolvidos na fibromialgia. (Adaptado a partir de ASSAVARITTIRONG et al., 2022).

3.6 Tratamentos farmacológicos e não-farmacológicos disponíveis para a fibromialgia

Até agora, os ensaios clínicos não forneceram, de forma conclusiva, os benefícios gerais de terapias específicas para tratar a fibromialgia. Portanto, os tratamentos farmacológicos para esses pacientes são direcionados apenas para o alívio dos sintomas, com benefícios clínicos relevantes experimentados apenas por uma minoria dos indivíduos. Naqueles que fazem uso de terapia medicamentosa, uma redução de 50% na intensidade da dor geralmente é alcançada em apenas 10% a 25% dos pacientes (MOORE et al., 2013). No entanto, alguns tratamentos parecem melhorar significativamente a qualidade de vida dos pacientes com fibromialgia (ESPEJO et al., 2018).

Enquanto nenhum medicamento foi aprovado para esta indicação pela *European Medicinal Agency*, apenas três fármacos foram aprovados para o tratamento da fibromialgia pela *Food and Drug Administration*: dois inibidores da recaptação da serotonina e norepinefrina (IRSN), a duloxetina e a milnaciprano, e um anticonvulsivante, a pregabalina (TZADOK e ABLIN, 2020). Entretanto, os medicamentos devem ser iniciados em doses baixas e aumentados com cautela porque alguns pacientes não toleram ou se beneficiam da terapia medicamentosa. Como os distúrbios do sono, a dor e o sofrimento psicológico são relatados como os sintomas que mais afetam a qualidade de vida dos pacientes com fibromialgia, os medicamentos devem ser escolhidos para controlar os sintomas predominantes individualmente (KWIATEK, 2017). Além disso, outras classes de medicamentos estão em fase de estudo para o tratamento da fibromialgia, com vários níveis de evidência sobre sua eficácia (Tabela 1) (MAFFEI, 2020; TZADOK e ABLIN, 2020).

Recentemente, ensaios clínicos do composto ((2S, 3R)-3-hidroxi-2-((R)-5-isobutiril-1-oxo-2,5-diazaspiro[3,4]octan-2-il)butanamida (NYX-2925) estão em desenvolvimento com a perspectiva de se tornar um novo agente para o tratamento de condições dolorosas crônicas, incluindo a fibromialgia. Para isso, KHAN et al. (2018) demonstraram que o NYX-2925 afeta a plasticidade sináptica do receptor NMDA e, portanto, foi classificado como um modulador desse receptor. GHOREISHI-HAACK et al. (2018) evidenciaram que esse composto exibe promissor efeito antinociceptivo em modelos animais de dor neuropática,

sugerindo a sua eficácia em distúrbios do SNC associados ao receptor NMDA. Ainda, os dados obtidos a partir do primeiro estudo clínico de fase I sugerem que o NYX-2925, além de ser seguro e bem tolerável em voluntários saudáveis, é capaz de atravessar a barreira-hematoencefálica (HOUCK et al., 2019).

Por outro lado, as diretrizes atuais publicadas sobre o tratamento da fibromialgia defendem unanimemente uma abordagem multidisciplinar, combinando o tratamento farmacológico com modalidades complementares, incluindo a terapia cognitivo-comportamental, o treinamento físico aeróbio e de fortalecimento e até mesmo, terapias de movimento meditativo (HÄUSER et al., 2017; MACFARLANE et al., 2017; SOSA-REINA et al., 2017; BERNARDY et al., 2018). Muitas vezes, os pacientes procuram ajuda em terapias alternativas devido à limitada eficácia das opções terapêuticas. A Tabela 1 resume as principais evidências clínicas sobre as terapias farmacológicas preconizadas para o tratamento da fibromialgia.

Tabela 1. Visão geral sobre as evidências clínicas dos agentes terapêuticos prescritos para a fibromialgia.

Fármaco	***Mecanismo***	***Evidências clínicas***
Amitriptilina	Antidepressivo tricíclico	Melhorias na dor, na fadiga e nas anormalidades do sono
Duloxetina	Inibidores da recaptação de serotonina e noradrenalina	Melhorias na dor e na depressão
Milnacipram	Inibidores da recaptação de serotonina e noradrenalina	Melhorias na dor e na fadiga
Paroxetina	Inibidores seletivos da recaptação de serotonina	Melhorias na dor e na depressão
Pregabalina	Gabapentinoide	Melhorias na dor, na fadiga e nas anormalidades do sono
Naltrexona	Antagonista dos receptores opioides	Melhorias na dor e na depressão
Dronabinol	Canabinoide	Melhorias na dor e na depressão
Cetamina	Antagonista NMDA	Melhorias na dor

Adaptado a partir de TZADOK e ABLIN (2020).

3.7 Compostos orgânicos de selênio

Os compostos orgânicos de selênio, com potencial propriedade antioxidante, exercem efeitos promissores sobre a dor, assim como em

processos inflamatórios em modelos experimentais (WILHELM et al., 2009; 2019; NOGUEIRA e ROCHA, 2011; BRUNING et al., 2014; SOUZA et al., 2017; VOGT et al., 2018; PERIN et al., 2019; REIS et al., 2019; 2020; JARDIM et al., 2020). Dentre as vantagens desses compostos, se destacam a síntese simples e as atividades farmacológicas relevantes, incluindo propriedades antioxidante, antinociceptiva, anti-inflamatória e antidepressiva (PINZ et al., 2016; SILVA et al., 2017; WILHELM et al., 2019).

A química fisiológica do selênio em animais é desempenhada quase que exclusivamente pelos resíduos de selenocisteína, um análogo da cisteína e da serina, encontrados em alguns tipos de selenoproteínas. Diante disso, alguns compostos orgânicos de selênio podem exibir forte atividade eletrofílica, formando ligações de selenenilsulfeto com os resíduos de cisteinila de tióis proteicos e não proteicos. Assim, a atividade de várias famílias de proteínas, incluindo enzimas antioxidantes, bem como os níveis de GSH celulares, podem ser afetados e representam um dos mecanismos principais pelos quais tais compostos modulam um amplo espectro de processos biológicos. Ainda, os compostos orgânicos de selênio podem complementar as defesas endógenas celulares contra os agentes oxidantes (ARTEEL e SIES, 2001; NOGUEIRA e ROCHA, 2011; NOGUEIRA et al., 2021). Portanto, em doenças nas quais o estresse oxidativo esteja envolvido, incluindo a fibromialgia, os compostos orgânicos de selênio podem ser considerados como uma nova alternativa terapêutica.

Evidências indicam que a modulação redox é fundamental para a sensibilização de nociceptores periféricos (BHAVE e GEREAU, 2004; MEOTTI et al. 2009), sugerindo que moléculas capazes de restaurar a homeostase redox, de fato, podem aliviar a dor. Portanto, os compostos de organosselênio foram avaliados quanto à atividade antinociceptiva, em que tanto o disseleneto de difenila, quanto o ebselen, administrados sistemicamente ou localmente, foram eficazes em modelos de nocicepção em camundongos (NOGUEIRA et al., 2003; ZASSO et al., 2005; ROSA et al., 2015). Na última década, novos compostos orgânicos de selênio foram sintetizados a fim de se obter uma atividade antinociceptiva potente e eficaz, com menos efeitos adversos. Portanto, derivados de dipiridil disseleneto (REIS et al. 2019), de selenosteroide (SARI et al. 2014), de quinolina (PINZ et al. 2016), de indol (BIRMANN et al. 2018) e

derivados de seleneto de pirazol (OLIVEIRA et al. 2020) exibiram efeito nociceptivo em modelos experimentais com relativo sucesso.

Recentemente, nosso grupo de pesquisa vem buscando elucidar as propriedades farmacológicas do 4-amino-3-(fenilselanil) benzenosulfonamida (4-APSB), um composto orgânico de selênio derivado da sulfonamida. Um estudo preliminar demonstrou que esse composto, em doses relativamente baixas, exerce efeito antinociceptivo à nível central e periférico em modelos experimentais de nocicepção aguda. Ainda, o 4-APSB exibiu propriedade anti-edematogênica ao modular alguns marcadores de estresse oxidativo em um modelo de dor aguda inflamatória (dados não publicados). Entretanto, mais estudos são necessários para uma melhor compreensão sobre os efeitos farmacológicos ou toxicológicos do 4-APSB, bem como os mecanismos pelo qual ele age.

Tendo em vista que os compostos orgânicos de selênio podem mimetizar a química fisiológica redox do selênio em sistemas biológicos, bem como o promissor efeito antinociceptivo agudo do composto 4-APSB, esse projeto tem o intuito de expandir o conhecimento em relação às propriedades farmacológicas desse composto em um modelo animal de fibromialgia, uma condição dolorosa crônica, assim como elucidar o seu mecanismo de ação.

4. CAPÍTULOS

Os resultados que fazem parte dessa tese encontram-se divididos em capítulos e apresentados sob a forma de artigo e de manuscrito. O item 4.1 aborda o artigo publicado na revista *Brain Research Bulletin*, volume 187, p. 11-23, DOI: 10.1016/j.brainresbull.2022.06.005, intitulado: *Mechanistic pathways of fibromyalgia induced by inttermitent cold stress in mice is sex-dependently*. O item 4.2 se refere aos resultados preliminares obtidos a partir do estudo intitulado: *MicroRNA expression profiles and pathological responses in fibromyalgia induced by inttermitent cold stress in mice: the role of sex differences*. Por fim, o item 4.3 se refere ao manuscrito intitulado: *Insights of the imbalance redox signaling in fibromyalgia induced by inttermitent cold stress in mice: 4-amino-3-(phenylselanyl) benzenesulfonamide a promising approach to treat fibromyalgia*. Esse manuscrito está submetido à revista *Neurochemical Research*, respeitando as diretrizes de formatação para a submissão. O artigo e o manuscrito estão organizados em seções de introdução, materiais e métodos, resultados, discussão e referências.

4.1 CAPÍTULO 1

Os resultados deste capítulo da tese estão apresentados sob o formato de artigo científico. As seções Introdução, Materiais e métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprio artigo.

A Tabela 2 resume as principais alterações comportamentais e bioquímicas, assim como algumas vias de sinalização que podem contribuir para o desenvolvimento dos sinais nociceptivos observados em camundongos machos e fêmeas após exposição ao EFI.

Tabela 2. Resumo dos principais resultados obtidos no Capítulo 1.

Testes Comportamentais		**Machos**	**Fêmeas**
		EFI	EFI
Sensibilidade mecânica		↑	↑
Sensibilidade térmica		↑	↑
Força muscular		↓	↓
Análises Farmacológicas		**Machos**	**Fêmeas**
		EFI	EFI
Antagonista do receptor NMDA		✓	✓
Precursor do ON		✕	✓
Inibidor da guanilato ciclase		✓	✓
Antagonista do receptor 5-$HT_{1A/1B}$		✓	✓
Antagonista do receptor 5-$HT_{2A/2C}$		✓	✓
Antagonista do receptor 5-HT_3		✓	✓
Análises *ex vivo*		**Machos**	**Fêmeas**
		EFI	EFI
Níveis de ER	Córtex cerebral	↑	✕
	Medula espinhal	✕	✕
Níveis de NPSH	Córtex cerebral	✕	✕
	Medula espinhal	✕	✕
Atividade da GPx	Córtex cerebral	↓	✕
	Medula espinhal	✕	✕
Atividade da Na^+,K^+-ATPase	Córtex cerebral	↓	↑
	Medula espinhal	✕	↑

(↑) Aumento induzido pela exposição ao EFI; (↓) diminuição induzida pela exposição ao EFI; (✓) contribuição para o desenvolvimento dos sinais nociceptivos induzidos pelo EFI; (✕) nenhuma alteração induzida pela exposição ao EFI.

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Mechanistic pathways of fibromyalgia induced by intermittent cold stress in mice is sex-dependently

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ABSTRACT

Fibromyalgia results from a complex interplay of biochemical and neurobiological elements mediated sensitization of nociceptive pathways. Despite the symptoms of fibromyalgia negatively affect the quality of life of patients, the pathophysiology of this disease remains inconclusive, which difficult the development of an appropriate treatment. The present study investigated the involvement of the serotonergic receptors, the N-methyl-D-aspartate (NMDA)/ nitric oxide (NO)/ cyclic guanosine monophosphate (cGMP) pathway and the oxidative stress in an animal model of fibromyalgia induced by intermittent cold stress (ICS), considering the specificities of male and female Swiss mice. The ICS exposure increased mechanical and thermal sensitivities, and decreased muscle strength in mice of both sexes. Female mice exhibited a longer-lasting mechanical sensitivity than male mice exposed to ICS along with an enhancement of the Na^+, K^+-ATPase activity in the spinal cord and cerebral cortex. Conversely, an inhibition in the Na^+, K^+-ATPase and glutathione peroxidase activities accompanied by an increase in the reactive species levels in the cerebral cortex of male mice were observed. The treatment with different serotonergic antagonists (pindolol, ketanserin and ondasetron) reversed the mechanical sensitivity in mice of both sexes, after the ICS exposure. The administration of MK-801, L-arginine and methylene blue also blocked the mechanical sensitivity in female mice exposed to ICS. Except L-arginine, MK-801 and methylene blue also attenuated this nociceptive signal in male mice, after ICS exposure. In conclusion, the modulation of serotonergic receptors, the NMDA/NO/cGMP pathway, and the oxidative stress seems contribute to nociceptive behaviors induced by ICS exposure sex-dependent.

1. Introduction

Fibromyalgia, a common and complex chronic pain syndrome, affects around 2–8 % of the world population, with a higher prevalence over women than men. Epidemiological data indicate that Turkey and Greece have the highest and the lowest prevalence rates of this disease, respectively (Sarzi-Puttini et al., 2020). Fibromyalgia is recognized as the third most prevalent musculoskeletal condition, with more than 10 tender points persisting for more than 3 months, without any organic lesions. Generally, it is accompanied by the development of comorbidities, including fatigue, sleep disturbances and depression (Clauw, 2014; Neumeister and Neumeister, 2020).

In this sense, fibromyalgia requires the high use of pharmacological and non-pharmacological resources because these patients exhibit both physical and mental disabilities. As a result, this clinical painful condition severely impaired the quality of life of patients (Arnold et al., 2016; Kim et al., 2013). Despite the socioeconomic impact of fibromyalgia is difficult to determine at global levels, the annual costs for its management are estimated to reach approximately 12 billion euros for a population of 80 million, assuming a 3 % prevalence rate (Spaeth, 2009).

In 2010, the American College of Rheumatology (ACR) proposed a new diagnosis criterion for fibromyalgia, replacing the tender point

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count for two scoring systems, which they are used to quantify fibromyalgia-type symptoms severity. The number of painful body regions, the severity fatigue, the cognitive difficult and the extent of somatic symptoms are now evaluated as a fibromyalgia diagnose (Maffei, 2020). However, a proper diagnosis and treatment for fibromyalgia remain delayed due to limited information about the pathophysiology of this syndrome. Some studies indicate dysfunctions in the peripheral, central and autonomic nervous systems, including abnormalities in the descending inhibitory pain pathways mediate by the biogenic amines depletion, a dysregulation in the hypothalamic pituitary adrenal (HPA) axis, as well as, changes in the number and function of small nerve fibers (Choy, 2015; Clauw, 2014; Singh et al., 2019).

Besides, previous studies demonstrated that an overproduction of reactive species (RS) associated to an abnormal antioxidant status might represent one of the mechanistic bases of fibromyalgia (Ozgocmen et al., 2006a; Singh et al., 2019). Notably, the establishment of oxidative stress within the cells could lead to a dysfunctional activity of the integral membrane enzymes, including ATPases (Barcelos et al., 2020). In particular, the transmembrane Na^+, K^+-ATPase pump, under physiological conditions, generates Na^+ and K^+ gradients to support the excitability of neurons, the regulation of intracellular calcium levels, as well as, the signaling transduction (Bogdanova et al., 2016; Liu et al., 2018). In this sense, a failure or a reduction in Na^+, K^+-ATPase activity in the central nervous system promotes adaptations in the neuronal excitability which may be responsible for the generation of pain and mood disorders, the major symptoms complained by fibromyalgia patients (Kinoshita et al., 2016). Indeed, chronic painful conditions and neuropsychiatry disorders have been linked to Na^+, K^+-ATPase dysfunction (Shrivastava et al., 2020). However, the role of ATPases on the development of fibromyalgia is poorly understood. A recent study has evidenced that the gene ATPase secretory pathway Ca^{2+} transporting 1 (SPCA1) might be implicated in the chronic widespread musculoskeletal pain, a prevalent symptom of this disease (Rahman et al., 2021).

Regarding the pathophysiology, some studies reported abnormalities of sensory processing within the central nervous system in fibromyalgia patients, as evidenced by a lowered nociceptive threshold at the dorsal horn of spinal cord and the brain (Choy, 2015). In view of this, central sensitization results from a dysfunction in the neurotransmitters (glutamate, serotonin and dopamine) and the neuroplasticity, thereby, leading to an augmented sensory processing in chronic painful conditions, including fibromyalgia (Clauw, 2014). In general, when the nociceptors are activated, the stimulation of glutamate N-methyl--D-aspartate (NMDA) receptors triggers a cascade of events that include an increase in nitric oxide (NO) production and subsequently, an enhancement in the synthesis of second messenger cyclic guanosine monophosphate (cGMP). This pathway is described for been involved in peripheral and central modulation of nociception (Vale et al., 2007).

Besides, a dysregulation of serotonergic transmission is considered one of the primary neurobiological aspects of fibromyalgia in preclinical and clinical studies. Indeed, reduced levels of serotonin (5-HT) in the descending pain pathway may trigger the development of fibromyalgia symptoms (Brusco et al., 2019; Mease et al., 2009). In addition to a reduced 5-HT availability, some studies proposed that occur a production of antibodies against 5-HT receptors, suggesting a down-regulation of these receptors in patients with fibromyalgia (Klein et al., 1992; Singh et al., 2019). Despite the evidences that this disease involves several factors, basic studies using animal models would be expected for advancing and better understanding the mechanisms underlying fibromyalgia.

The intermittent cold stress (ICS) animal model mimics the chronic widespread pain of fibromyalgia patients, since it led to the development of mechanical and thermal sensorial changes in rodents, as well as, muscle weakness. Some studies also reported that mice and rats exposed to ICS have presented a depressive-like phenotype, sleep disturbances and fatigue, comorbidities frequently related to fibromyalgia (Brum et al., 2022). Moreover, at the spinal and supraspinal levels, it was demonstrated that adaptations in excitatory and inhibitory systems related to pain processing might mediate the development and maintenance of nociceptive behavior in this model (DeSantana et al., 2013). The ICS experimental model shares some neurobiological aspects, the clinical features and associated comorbidities of fibromyalgia (Nishiyori and Ueda, 2008). However, the ICS model may respond to drugs that have not been found to be efficacious for fibromyalgia treatment, as nonsteroidal anti-inflammatory drugs and opioids (Brum et al., 2022; Montserrat-De La Paz et al., 2014; Nishiyori et al., 2011; Nishiyori and Ueda, 2008).

Considering that ICS mimics the symptoms and the pathophysiology of fibromyalgia, the purpose of the present study was evaluated the sex influence in the effect of ICS in the nociceptive behaviors in mice. The contribution of the NMDA/NO/cGMP pathway, the serotonergic receptors, and the oxidative stress also was investigated in attempt to better understand and extend the mechanisms underlying ICS induced pain-like fibromyalgia in male and female mice.

2. Material and methods

2.1. Animals

The experimental protocols were authorized by the Committee on Care and Use of Experimental Resources of the Federal University of Pelotas (Brazil), affiliated to the National Council for the Control of Animal Experimentation, and registered under the number CEEA 28142–2019. All animal handling and experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 823, revised 1978) and International Guiding Principles for Biomedicals Research Involving Animals which every effort was made to reduce both the number of animals used and their discomfort. Behavioral and biochemical evaluations were blinded to the treatment when performing tests. All experiments were performed between 8:00 a.m. and 5:00 p.m.

The experiments were performed using 210 male and 210 female Swiss mice (25–35 g, bred in house, two months old). The animals were housed in individually cages (3–4 animals per cage) at an acclimatized (22 ± 2 °C) animal room, with wood shaving bedding and nesting material. Mice were maintained under a 12 h light/12 h dark cycle (the lights were turned on at 07:00 a.m.) and a commercial rodent pellet diet and filtered water were provided ad libitum. The animals were acclimatized to the housing environment for at least 7 days before the commencement of the experiments and to the behavioral room for 1 h prior to behavior tests. No previous procedure was performed on these mice.

2.2. Drugs

Drugs used were administered through intraperitoneal (ip) route. MK-801, L-arginine, pindolol, ketanserin and ondansetron, were obtained from Sigma Aldrich Chemical Company (St. Louis, MO, USA). Methylene blue was purchased from Synth (São Paulo, Brazil). The reagents were dissolved in a saline solution (0.9 %), except pindolol which was dissolved in Tween 80 (10 %). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.3. Experimental design

2.3.1. Intermittent cold stress (ICS)-induced fibromyalgia model

ICS was developed and validated as an experimental mouse model of fibromyalgia by Nishiyori and Ueda (Nishiyori and Ueda, 2008). Briefly, the animals (3–4 mice in each cage) were placed in a cold environment (4 ± 2 °C) at 4:30 p.m. on the first day (day 1), with feeding and filtered water ad libitum. In the next morning (10:00 a.m.), mice were transferred to a temperature room (22 ± 2 °C) during 30 min and then, they

were put in the cold room again for 30 min. This process was repeated until 4:30 p.m. (day 2). Finally, the animals were allocated in the cold room overnight. The same procedure was performed on the next day of the experimental protocol (day 3). In the morning (10:00 a.m.) of the fourth day, following the completion of the ICS exposure, mice were returned and adapted to a normal room temperature (22 ± 2 °C) for at least 2 h prior to behavioral tests. Mice from the control group were kept at a constant temperature of 22 ± 2 °C during all four days of the ICS model (from 4:30 p.m. on day 1–10:00 a.m. on day 4).

Two hours after the adaptation period, the animals were evaluated on pain-related behavioral tests to confirm the development of mechanical and thermal sensitivities, as well as muscle hyperalgesia. Thereafter, the possible effect of ICS on locomotor and exploratory activities of male and female mice were evaluated in the open field test.

Immediately after the behavioral tests, the animals were deeply anesthetized by adding isoflurane-soaked cotton balls into a clear glass cage until loss of consciousness and euthanized through cardiac puncture for blood collection from the heart ventricle. Also, the entire cerebral cortex as well as the cervical and thoracic spinal segments were rapidly dissected, weighed and frozen at − 20 °C to further *ex vivo* assays (Fig. 1).

2.4. Behavioral tests

2.4.1. Measurement of mechanical sensitivity

As described by Alamri et al., (2018), mechanical sensitivity in mice was estimated using an electronic aesthesiometer (Insight, Ribeirão Preto, SP, Brazil). This apparatus consists in a device connected to a specific paw pressure transducer. First, the animals were acclimatized during 30 min in an individual clear plastic chambers on an elevated wire mesh platform which allowed to access the plantar surface of the paws. The investigator, who was blinded to the treatment conditions, was trained to apply constant progressive pressure on the central area of the hind paw using a blunt-tipped probe connected to a transducer until paw withdrawal followed by clear flinching movements. After this response, the pressure intensity was automatically recorded. The paw withdrawal threshold (g), defined as a measure of mechanical sensitivity, was evaluated after the ICS exposure, on the fourth day of the experimental protocol. In another set of experiments, we evaluated the time-course of mechanical sensitivity after the ICS exposure, on days 1, 6, 11 and 16 for male and on days 1, 6, 11, 16, 21 and 26 for female mice.

2.4.2. Measurement of thermal sensitivity

Pain reflexes in response to thermal stimulus in the hot plate test were assessed as previously described (Macdonald and Woolfe, 1946). Surrounded by a clear acrylic cage, the animals were individually kept on a heated metallic surface plate (52 ± 1 °C). The latency period was recorded from point zero using a stopwatch, when the mouse was gently placed on the hot surface until the time that it jumped off the surface, shake or licked the hind paws. These kinds of movement were considered as a positive nociceptive reflex. A cut-off time of 45 s was established to avoid any injury on animal paws. The paw withdrawal latency (s), defined as a measure of thermal sensitivity, was evaluated after the ICS exposure on the fourth day of the experimental protocol by an observer trained and blinded to the experimental groups.

2.4.3. Measurement of muscular strength

The grip strength test, a simple non-invasive method, was design to assess mouse muscle strength in vivo. The experiment was conducted by a condition-blind observer using a digital force-gauging apparatus (Insight, Ribeirão Preto, SP, Brazil) after the ICS exposure on the fourth day of the experimental protocol. Mice were gently pulled parallel away from the bar by the tail until the forelimbs released the bar. The maximum force prior to release of the mouse's paw from the bar was recorded. The test was repeated 3 times and an average was reported as the muscle strength (N) (Burnes et al., 2008).

2.4.4. Assessment of locomotor and exploratory performance

The open field test (OFT) evaluates the exploratory behavior and the general locomotor activity of mice. The apparatus was made of plywood (30 H x 45 L x 45 W) in which the floor was divided into 9 squares (3 rows of 3) by masking tape markers. Two observers were trained for blindly placed the mouse at the center of each apparatus and recorded the locomotor (number of segments crossed with the four paws) and exploratory (number of rearing on the hind limbs) activities for 4 min on the fourth day of the experimental protocol, after the ICS exposure

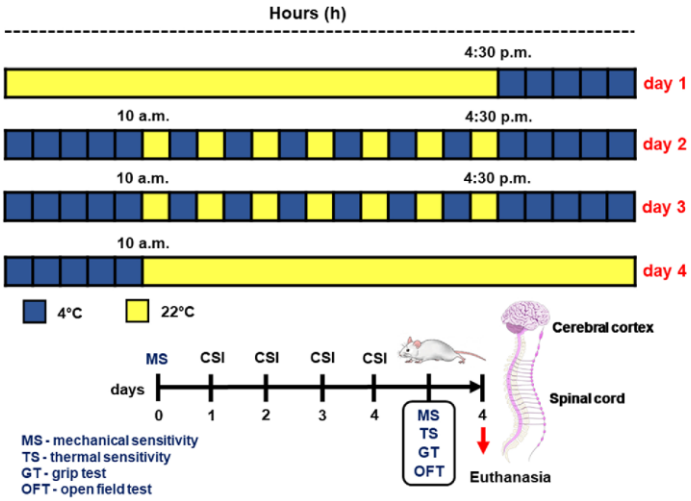

Fig. 1. Schematic representation of the experimental design of this study.

(Walsh and Cummins, 1976). The preference of each animal to walk in the central or peripheral areas was not registered in this behavioral experiment. The arena was cleaned with 30 % ethanol after each session.

2.5. Ex vivo assays

2.5.1. Tissues processing for biochemical analyses

Samples of cerebral cortex and spinal cord were homogenized in cold 50 mM Tris-HCl at pH 7.4 (1/5 wt/volume). The homogenates were centrifugated at 3000 rpm for 10 min at 4°C and the supernatant fraction (S_1) was used to biochemical analysis, including reactive species (RS), non-protein thiol (NPSH) and nitrite and nitrate (NOx) levels, as well as gluthatione peroxidase (GPx) and Na^+/K^+-ATPase activities. Protein concentration in S_1 was estimated by the Bradford method (Bradford, 1976), using bovine serum albumin (1 mg ^{1}mL ^{1}) as a standard. Briefly, an aliquot of S_1 was mixed with 50 mM Tris-HCl buffer at pH 7.4 and Comassie blue for 10 min. The color was measured spectrophotometrically at 595 nm.

2.5.2. RS levels

The RS levels were determined as reactive species to 2′,7′-dichlorofluorescein diacetate (DCHF-DA). An aliquot of 1 mM DCHF-DA in ethanol was incubated with the supernatant S_1 and 10 mM Tris-HCl buffer at pH 7.4, for 1 h at room temperature, protected from light. DCHF-DA, easily oxidized to DCF, is used as a fluorescent probe to detect intracellular RS levels (Loetchutinat et al., 2005). Thus, the oxidation of DCHF-DA into DCF was determined at 488 nm for excitation and 525 nm for emission. DCF fluorescence intensity was expressed as arbitrary fluorescence units.

2.5.3. NPSH content

NPSH content, a non-enzymatic antioxidant defense, was determined by Ellman's method (Ellman, 1959). Briefly, S_1 was mixed (1:1) with 10 % trichloroacetic acid (TCA). After centrifugation (3000 rpm for 10 min), an aliquot of supernatant S_1 containing free SH-groups was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB). The color reaction was measured at 412 nm and NPSH levels were expressed as nmol of NPSH/g tissue.

2.5.4. GPx activity

GPx activity was estimated spectrophotometrically, as described by Wendel, (1981). This assay involves monitoring the dismutation of H_2O_2 in the presence of S_1 at 340 nm. An aliquot of the supernatant S_1 was added in a system composed by reduced glutathione (GSH)/NADPH/-glutathione reductase (GR). The enzymatic reaction was initiated by the addition of H_2O_2. GPx activity is indirectly measured by NADPH decay. Briefly, H_2O_2 is reduced and generates oxidized glutathione (GSSG) from GSH. In turn, GSSG is regenerated back to GSH by the GR present in the analysis medium at the expense of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.5.5. Na^+, K^+ ATPase activity

In this assay, a reaction mixture containing an aliquot of S_1, 3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris-HCl at pH 7.4, in a final volume of 500 μL, was preincubated at 37 °C for 10 min. Control samples were prepared under the same conditions with the addition of 0.1 mM of ouabain. The reaction initiated with the addition of ATP to a final concentration of 3.0 mM. All samples were incubated at 37 °C for 30 min and the reaction was stopped by adding TCA solution (10 %) with 10 mM $HgCl_2$. Na^+, K^+ ATPase activity was calculated through the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow, (1925). Enzyme activity was expressed as nmol Pi/mg protein/min.

2.5.6. NOx content

Samples of cerebral cortex were homogenized in $ZnSO_4$ (200 mM) and acetonitrile (96 %). The homogenates were then centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatant was collected for the NOx assay. The accumulation of nitrite in the supernatant, an indicator of NO oxidation, was assessed by Griess reaction (Green et al., 1982). Briefly, the NOx content was estimated in a medium containing 2 % vanadium chloride (in 5 % HCl), 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride and 2 % sulfanilamide (in 5 % HCl). After incubation at 37°C for 1 h, the color reaction was measured spectrophotometrically at 540 nm. The concentration of nitrite/nitrate in the supernatant was determined from a sodium nitrite standard curve and expressed as nmol NOx/g of tissue.

2.5.7. Lactic dehydrogenase (LDH) activity

Blood samples were centrifugated at 2500 rpm for 10 min to collect the serum portion that was quickly frozen at −20 °C. The LDH activity was measured according to commercial kit instructions (Bioclin, Minas Gerais, Brazil).

2.6. Contribution of the serotonergic receptors and the NMDA/NO/GMPc pathway in the mechanical sensitivity induced by ICS

In order to elucidate the possible mechanisms underlying ICS induced nociceptive signs, male and female mice were randomly divided into four experimental groups for each agonism or antagonism drug investigated in this study. In this line, the animals of both sexes from control or ICS groups received an injection of vehicle (saline solution, 10 mL/Kg) or the respectives drugs MK-801 (0.2 mg/kg, i.p. route), an uncompetitive antagonist for NMDA receptor, L-arginine (600 mg/kg, i. p. route), a nitric oxide precursor, and methylene blue (10 mg/kg, i.p. route), a guanylate cyclase (GC) inhibitor. Thirty minutes after the treatments, the mechanical sensitivity of those mice was estimated using an electronic aesthesiometer (Fig. 4A). The dose of each drug used for mechanism analyses was selected according to the available literature data, as well as, to previous studies developed by our research group (Jesse et al., 2009; Naserzadeh et al., 2019; Silva et al., 2017).

In another set of experiments, male and female mice were again randomly divided into four experimental groups for each serotonergic antagonism drug. Mice of both sexes from control or ICS groups were treated with vehicle (saline solution for ketanserin and ondansetron or 10 % Tween 80 for pindolol, 10 mL/Kg) or the respective drugs pindolol (1 mg/kg, i.p. route), a nonselective antagonist of 5-$HT_{1A/1B}$ receptors and a β-adreno adrenoceptors blocker/ putative 5-HT_{1A} serotonin receptor agonist, ketanserin (0.3 mg/kg, i.p. route), an antagonist for 5-$HT_{2A/2C}$ receptor, and ondansetron (0.5 mg/kg, i.p. route), a selective antagonist for 5-HT_3 receptor. Thirty minutes later, the mechanical sensitivity of those animals was evaluated using an electronic aesthesiometer (Fig. 5A). The preparation and the doses of the drugs used for mechanism analyses were based on previous reports and studies from our laboratory (Jesse et al., 2009; Naserzadeh et al., 2019; Silva et al., 2017).

For these experiments, the mechanical sensitivity was assessed because the previously nociceptive behavioral tests carried out have shown that the animals were more sensitive and responsive to the electronic aesthesiometer test than to the hot plate test. In addition, this evaluation was done only using electronic aesthesiometer test to reduce the number of animals used in experiments, based on humanitarian principles of animal experimentation.

2.7. Statistical analysis

All experimental results are presented as the mean ± standard error of the mean (SEM). The statistical analyses were performed using GraphPad Prism Software version 6.0 (San Diego, CA, USA). A Gaussian distribution was tested using D'Agostino and Pearson omnibus

normality test. The ROUT test ($Q = 1.0$ %) was applied for the detection of outliers. Data were analyzed by two-way analysis of variance (ANOVA), followed by Tukey post hoc test when appropriate. Time course on mechanical sensitivity were analyzed by Student's t-test. Probability values less than 0.05 ($P < 0.05$) were considered statistically significant.

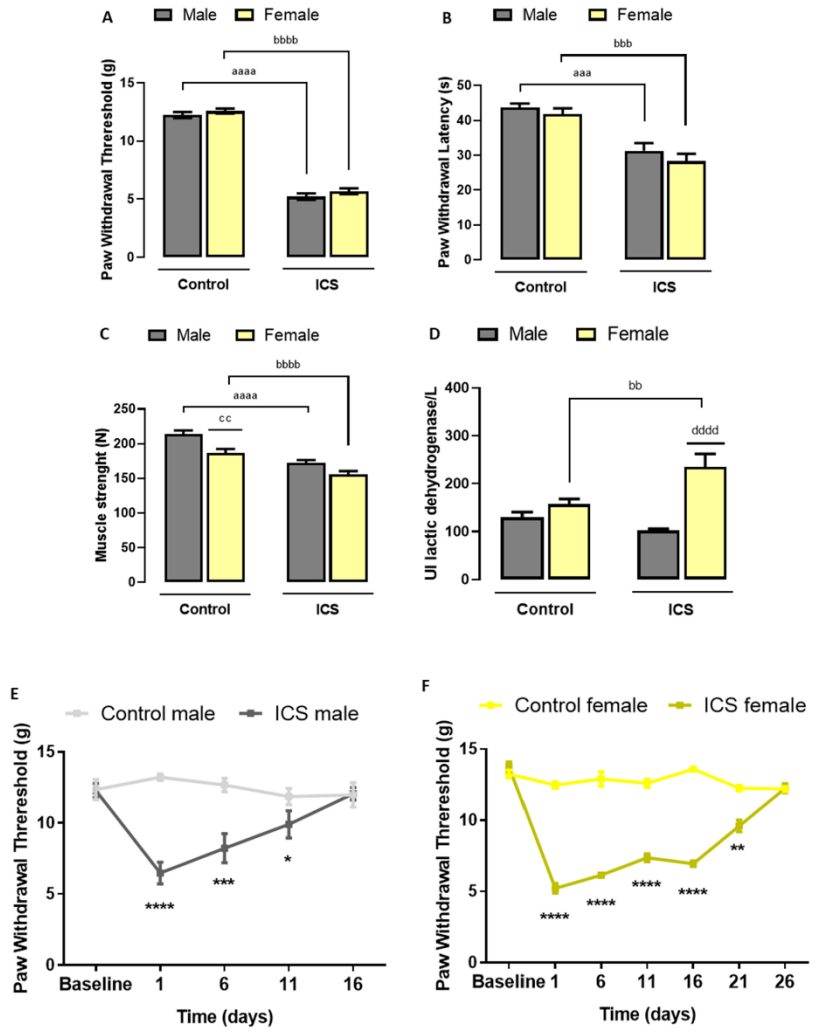

Fig. 2. The exposure to intermittent cold stress (ICS) affects nociceptive behavioral and muscular parameters in male and female mice. (A) Mechanical sensitivity measured at the hind paws with an electronic von Frey aesthesiometer. (B) Thermal sensitivity assessed at the hind paws with the hot plate apparatus. (C) Muscular strength evaluated with a digital force device. (D) Plasmatic LDH activity was measured in male and female mice of control and ICS groups after the behavioral tests. The time-course of mechanical sensitivity was evaluated at different points in male (E) and female (F) mice with an electronic von Frey aesthesiometer. Each column represents mean ± SEM of 7 animals per group. The letter a and b denote significance levels when compared with the ICS group in male and female mice, respectively (a) $p < 0.05$, (aaa) $p < 0.001$, (aaaa) $p < 0.0001$, (bb) $p < 0.01$, (bbb) $p < 0.001$ and (bbbb) $p < 0.0001$; the letter c denotes significance levels between male and female mice of control group (cc) $p < 0.01$; the letter d denotes significance levels between male and female mice of ICS group (dddd) $p < 0.0001$ (two-way ANOVA followed by Tukey post hoc test or Student's t-test).

3. Results

3.1. Characterization of ICS induced pain-like fibromyalgia in male and female mice

The tests assayed demonstrated that ICS affects nociceptive-related behaviors in mice of both sexes (Fig. 2). The groups exposed to ICS exhibited a significant reduction in both paw withdrawal threshold (57 % and 55 % in male and female mice, respectively) and latency to thermal stimulus (28 % and 32 % in male and female mice, respectively) when compared with those of the control groups, suggesting that ICS induces the development of mechanical and thermal sensitivities. The results were similar in male and female mice (Fig. 2A and 2B).

In comparison with the control groups, ICS exposed mice of both sexes also led to a significant deficit in grip strength (20 % and 17 % in male and female mice, respectively), which might reflect muscle weakness or muscle hyperalgesia in both sexes. In contrast, male mice exhibited a higher muscular strength (13 %) than female mice in the control group (Fig. 2C). Notably, the LDH activity in the serum of female mice was significantly increased in the ICS group (149 %), whereas this parameter was similar between the experimental groups in male mice (Fig. 2D).

Moreover, the exposure to ICS induces a longer-lasting mechanical sensitivity in female than male mice (Fig. 2E and 2F). The paw withdrawal threshold in female mice was significantly decreased (58 %) after the ICS exposure when compared with the control group, which persisted for 21 days thereafter. On day 26 of the experimental protocol, the paw withdrawal threshold was similar between control and ICS groups. In male mice, a reduction in the paw withdrawal threshold (51 %) was observed after the ICS exposure compared to control group that only lasted up to 11 days thereafter. There was no statistically significant difference between control and ICS groups on 16th day of the experimental protocol.

The number of rearing and crossings in the open field test is presented in Table 1. The exposure or not to ICS did not cause any significant change in the number of crossings or rearing in male and female mice.

3.2. Involvement of oxidative stress and the Na^+, K^+-ATPase activity in ICS induced pain-like fibromyalgia in male and female mice

To confirm the role of oxidative stress on ICS induced pain-like fibromyalgia, RS and NOx levels, as well as enzymatic and non-enzymatic antioxidant systems (GPx activity and NPSH levels) were evaluated in the spinal cord and/or cerebral cortex of male and female mice (Fig. 3A – 3G). In general, the ICS group did not change the levels of RS in the spinal cord of mice in both sexes in comparison to the control groups, although female mice exposed to ICS exhibited higher levels of RS (157 %) in the spinal cord than male (Fig. 3A). On the other hand, the ICS model promoted an enhancement of RS levels (147 %) in the cerebral cortex of male, but not in female mice, when compared with those of the respective control groups (Fig. 3B). Moreover, male mice submitted to ICS exhibited a reduction (49 %) of the NOx levels in the cerebral cortex when compared with the respectively control group, whereas the cortical levels of NOx were similar between ICS and control groups in female mice (Fig. 3C).

Regarding the antioxidant system, the results demonstrate that GPx activity in the spinal cord of male and female mice remained unchanged after ICS in comparison to control groups (Fig. 3D). On the other hand, the ICS exposure significantly inhibited the GPx activity (70 %) in the cerebral cortex of male mice, but not in females, when compared with those of the respective control groups (Fig. 3E). Curiously, a markedly reduction in the GPx activity (80 %) was observed in the cerebral cortex of female compared with male mice of the control group. Complementarily, the levels of NPSH in the spinal cord and cerebral cortex were similar between ICS and control groups in male and female mice (Fig. 3F and 3G). However, female mice of the control group exhibited lower levels of NPSH (15 %) in the spinal cord than male, similarly to the results obtained from the GPx activity in the cerebral cortex. The biochemical analyses demonstrate that ICS exposure led to the development of oxidative stress in the cerebral cortex of male mice, but not in female mice.

The role of Na^+, K^+-ATPase activity in ICS induced pain-like fibromyalgia in male and female mice was also elucidated in this study. The exposure to ICS did not promote any alteration in the Na^+, K^+-ATPase activity in the spinal cord of male mice, whereas it was observed an impairment in the activity of this enzyme (48 %) in the cerebral cortex when compared with male control group. On the other hand, the enzymatic activity of Na^+, K^+-ATPase was significantly increased in both spinal cord (193 %) and cerebral cortex (187 %) of female mice, when compared ICS with control groups (Fig. 3H and 3I). Interestingly, female mice exposed to ICS showed an exacerbate Na^+, K^+-ATPase activity in these central nervous structures in comparison to male. The results suggest that ICS model differently modulated the activity of Na^+, K^+-ATPase in central nervous structures of male and female mice.

Table 1
Spontaneous locomotor and exploratory activities of male and female mice exposed to ICS-induced fibromyalgia.

Open Field Test				
Groups	*Crossing*[a]		*Rearing*[b]	
	Male	*Female*	*Male*	*Female*
Control	112.7 ± 5.47	118.1 ± 6.73	48.3 ± 2.97	45.7 ± 2.11
ICS	112.3 ± 5.37	120.3 ± 9.19	48.7 ± 3.34	50.0 ± 2.34

Data are reported as means ± SEM for 7 animals per group. Statistical analyses were performed by two-way ANOVA, followed Tukey multiple comparison test when appropriate.
[a] Data are expressed as number of crossings.
[b] Data are expressed as number of rearing.

3.3. Contribution of NMDA/NO/cGMP pathway in ICS induced pain-like fibromyalgia in male and female mice

To investigate the involvement of NMDA/NO/cGMP pathway in the nociceptive process induced by ICS, MK-801 (0.2 mg/kg), L-arginine (600 mg/kg) and methylene blue (10 mg/kg) were administered by the i.p. route, 30 min before the electronic aesthesiometer test, in male and female mice (Fig. 4A).

In male mice, the results demonstrated that MK-801 completely reversed the paw withdrawal threshold induced by ICS, whereas the administration of L-arginine did not alter this parameter when compared with the ICS group (Fig. 4B and 4C). In addition, the treatment with methylene blue partially restored the paw withdrawal threshold induced by ICS (Fig. 4D). Our data suggest that NMDA/NO/cGMP pathway partially contributes to the development of mechanical sensitivity in male mice, after ICS exposure.

Differently, the treatments with MK-801, L-arginine and methylene blue reversed the paw withdrawal threshold in comparison to ICS exposure in female mice (Fig. 4E – 4G). In another words, the mechanical sensitivity triggered by ICS in female mice can involve the NMDA/NO/cGMP pathway. Together, the results indicate that ICS exposure differently modulated the NMDA/NO/cGMP pathway to induce nociceptive behaviors in male and female mice.

3.4. Contribution of serotonergic receptors in ICS induced pain-like fibromyalgia in male and female mice

The results presented in the Fig. 5 demonstrate the role of serotonergic receptors on mechanical sensitivity in male and female mice exposed to ICS. As mentioned above, the ICS model induced a significant reduction in the paw withdrawal threshold in both sexes when

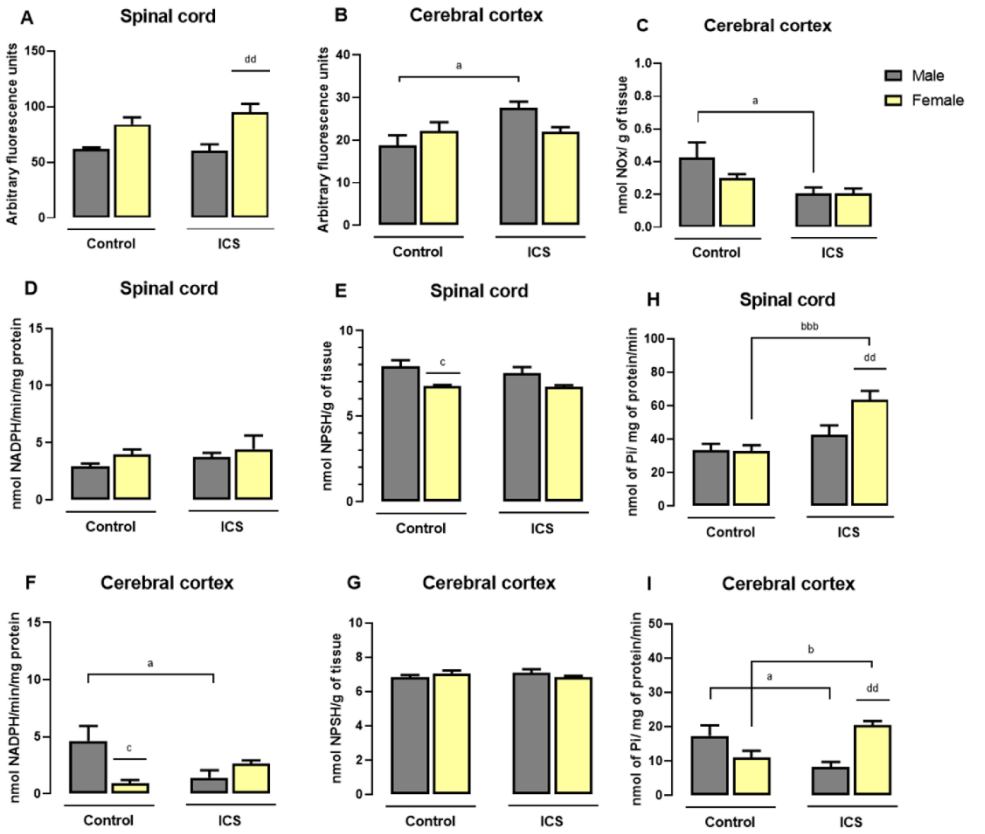

Fig. 3. The role of oxidative stress and the Na^+, K^+-ATPase activity in ICS induced pain-like fibromyalgia in male and female mice. The development of oxidative damage is characterized by the RS levels in the spinal cord (A) and cerebral cortex (B), as well as, the NOx levels in the cerebral cortex (C) of male and female mice. The antioxidant defense system profile is represented by the glutathione peroxidase (GPx) activity (D) and non-protein thiols (NPSH) levels in the spinal cord of mice (E). GPx activity (F) and NPSH levels (G) in the cerebral cortex of male and female mice. Changes in neuronal excitability were investigated trough the Na^+, K^+-ATPase activity in the spinal cord (H) and cerebral cortex (I) of male and female mice. Each point represents mean ± SEM of 6–7 animals per group. The letter a and b denote significance levels when compared with the ICS group in male and female mice, respectively (a) $p < 0.05$, (b) $p < 0.05$ and (bbb) $p < 0.001$; the letter c denotes significance levels between male and female mice of control group (c) $p < 0.05$; the letter d denotes significance levels between male and female mice of ICS group (dd) $p < 0.01$ (two-way ANOVA followed by Tukey post hoc test).

compared with the control groups. In contrast, the treatment with different serotoninergic antagonists (pindolol, ketanserin and ondansetron), administered 30 min before the electronic aesthesiometer test by the intraperitoneal (ip) route, altered the paw withdrawal threshold in male and female mice exposed to ICS (Fig. 5A). In another words, pindolol (1 mg/kg), ketanserin (0.3 mg/kg) and ondansetron (0.5 mg/kg) increased the paw withdrawal threshold in male and female mice exposed to ICS, indicating that these antagonists completely reversed the mechanical sensitivity induced by ICS in both sexes (Fig. 5B - 5G).

4. Discussion

In the present study, we demonstrate that ICS exposure caused not only characteristic nociceptive signs related to fibromyalgia, as previously evidenced in preclinical and clinical studies, but also biochemical adaptations in male and female mice. Moreover, female exposed to ICS exhibited a longer-lasting mechanical sensitivity than male mice. Despite serotonergic 5-$HT_{1A/2B}$, 5-$HT_{2A/2C}$ and 5-HT_3 receptors antagonism reduced the mechanical sensitivity in mice of both sexes exposed to ICS, the agonist and antagonists of NMDA/NO/cGMP pathway partially decreased this nociceptive sign in male mice. Also, ICS led to the development of oxidative stress in the cerebral cortex of male mice, as well as deregulated the Na^+, K^+-ATPase activity in the spinal cord and cerebral cortex of male and female mice. Those data indicate that ICS exposure produced different biochemical adaptations in the central nervous system (CNS) of male and female mice.

Fibromyalgia, one of the major painful conditions, is characterized by the development of a chronic widespread pain associated with other debilitating secondary symptoms, including muscular pain, stiffness, sleep and mood disorders (Clauw, 2014; Häuser et al., 2017; Neumeister

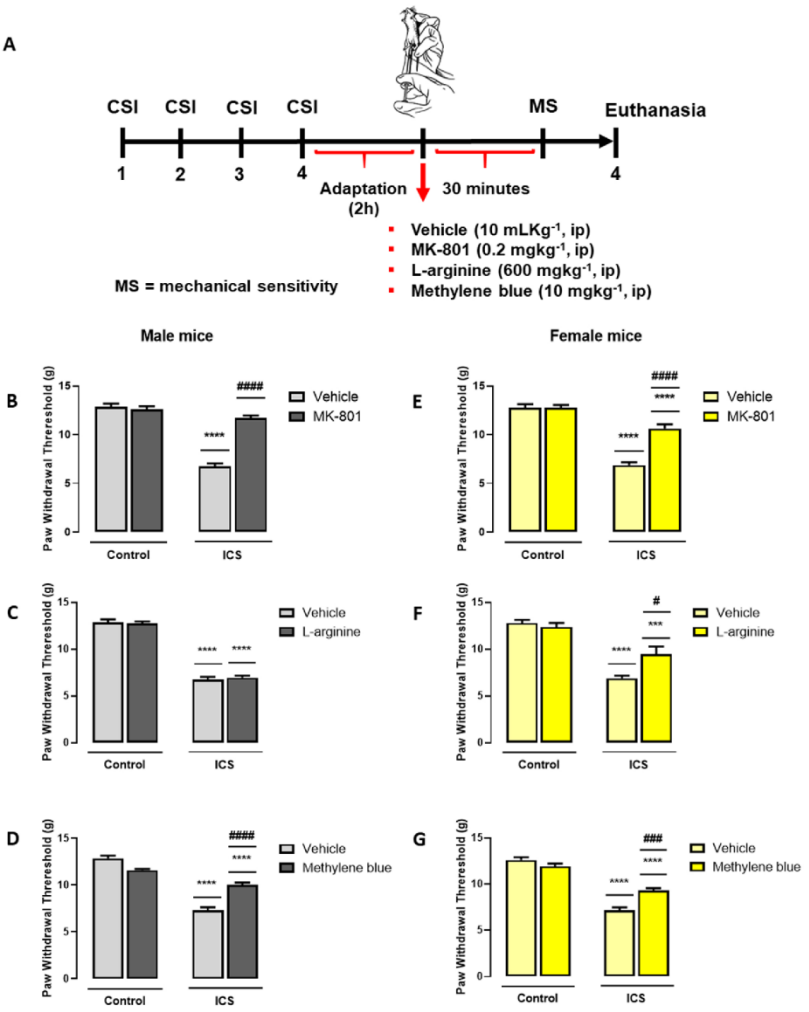

Fig. 4. Involvement of NMDA/NO/cGMP pathway on ICS induced pain-like fibromyalgia in male (B, C and D) and female mice (E, F and G). (A) Scheme of the experimental protocol showing the timeline of ICS exposure, intraperitoneal (i.p.) treatments and behavioral assessment. (B and E) Effect of treatment with MK-801 (0.2 mg/kg) in mechanical sensitivity after ICS exposure in male and female mice, respectively. (C and F) Effect of treatment with L-arginine (600 mg/kg) in mechanical sensitivity in male and female mice exposure to ICS, respectively. (D and G) Effect of treatment with methylene blue (10 mg/kg) in mechanical sensitivity after ICS exposure in male and female mice, respectively. Data are expressed as the mean ± SEM of 7 animals per group). Asterisk denotes significance levels when compared with the control group in male and female mice (****) $p < 0.0001$; hashtag denotes significance levels when compared with the ICS group in male and female mice (#) $p < 0.05$, (###) $p < 0.001$ and (####) $p < 0.0001$ (two-way ANOVA followed by Tukey post hoc test).

and Neumeister, 2020). The low temperature generated from cold stress and a high periodicity of temperature alterations produced a long-lasting and stable allodynia in mouse. Indeed, the authors confirmed that ICS exposure induces an intense thermal and mechanical sensitivities in both hind paw of mice for long periods with a female prevalence, proving the face validity of this experimental model (Itomi et al., 2016; Montserrat-De La Paz et al., 2014; Nishiyori and Ueda, 2008). Besides, mice exposed to ICS have exhibited a depressive-like phenotype and sleep disturbances, suggesting that a scarce sleep may contribute to the hypersensitivity detected in animals subjected to this experimental model of fibromyalgia. Thus, the ICS also shares the main comorbidities associated with fibromyalgia (Brum et al., 2022; Miyamoto et al., 2017; Nasu et al., 2019).

In agreement with the study mentioned above, our results

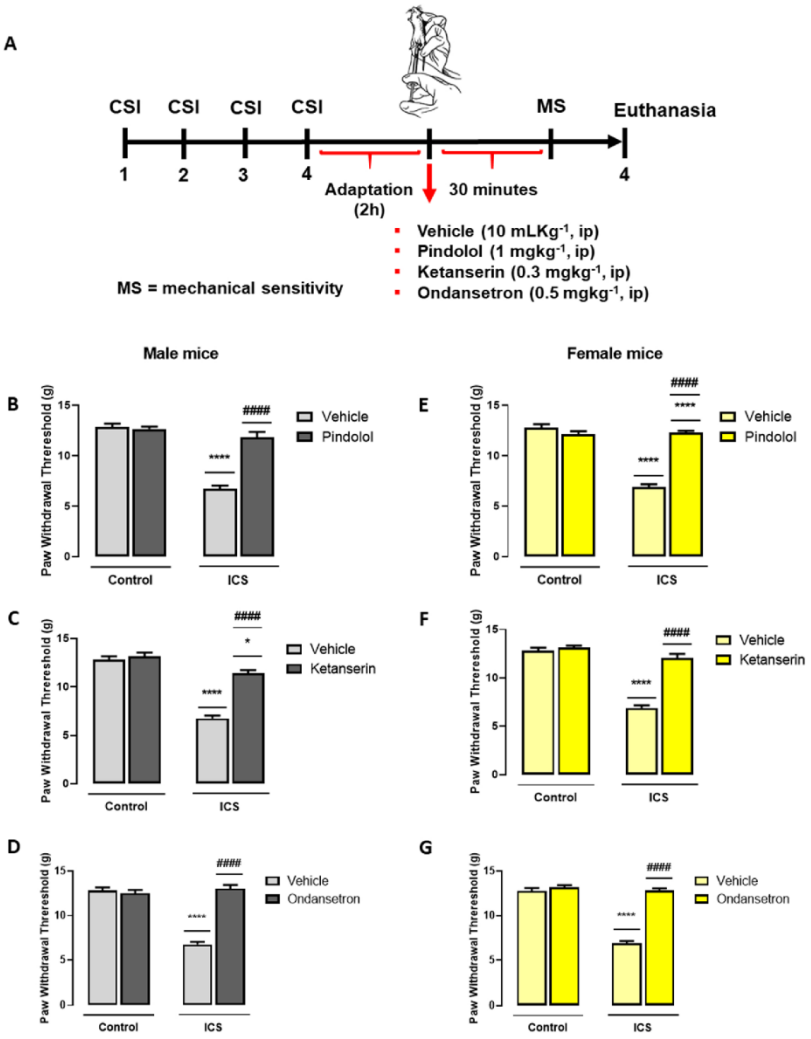

Fig. 5. Involvement of serotonergic receptors on ICS induced pain-like fibromyalgia in male (B, C and D) and female mice (E, F and G). (A) Scheme of the experimental protocol showing the timeline of ICS exposure, intraperitoneal (ip) treatments and behavioral assessment. (B and E) Effect of treatment with pindolol (1 mg/kg) in mechanical sensitivity after ICS exposure in male and female mice, respectively. (C and F) Effect of treatment with ketanserin (0.3 mg/kg) in mechanical sensitivity in male and female mice exposure to ICS, respectively. (D and G) Effect of treatment with ondansetron (0.5 mg/kg) in mechanical sensitivity after ICS exposure in male and female mice, respectively. Data are expressed as the mean ± SEM of 7 animals per group. Asterisk denotes significance levels when compared with the control group in male and female mice (****) $p < 0.0001$; hashtag denotes significance levels when compared with the ICS group in male and female mice (####) $p < 0.0001$ (two-way ANOVA followed by Tukey post hoc test).

demonstrated that both male and female mice exhibited an abnormal mechanical and thermal sensitivities after ICS exposure. Complementary, the animals of both sexes exposed to ICS also showed a muscle weakness or muscle hyperalgesia in the limbs, as evidenced by a deficit in the grip strength. The enzyme LDH catalysis the reversible conversion of pyruvate to lactate. It has been proposed that increased LDH and anaerobic glycolysis produced a large amount of lactate in muscles and blood that lead to muscle fatigue. In turn, an enhancement in the activity of this enzyme in serum could reflect the degree of muscle damage (Li et al., 2018; Zhang et al., 2019). In line with this, patients diagnosed

with fibromyalgia have been shown biochemical abnormalities regarding glucose metabolism (Eisinger et al., 1994). Our findings demonstrated that female exposed to ICS exhibited an enhancement in the serum LDH activity, whereas the activity of this enzyme was similar between control and ICS groups in male mice.

Taken together, our data reinforces that the ICS animal model reproduced the feature symptoms of fibromyalgia, including mechanical and thermal sensitivities, as well as, muscle hyperalgesia. A distinct profile in serum LDH activity between male and female mice after ICS exposure was also evidenced. Moreover, we also showed that ICS exposure caused a persistent mechanical sensitivity for almost two or four weeks in male and female mice, respectively. In this sense, our data suggest that female might be more susceptible than male mice to developed an abnormal nociceptive behavior induced by ICS. Corroborating with our findings, studies evidenced that the risk of clinical pain conditions development is higher in women than in men, including fibromyalgia, which it is considered the major cause of generalized musculoskeletal pain in women (Mogil, 2012).

Many alterations, at central and peripheral levels, have been implicated in the manifestations of painful symptoms of fibromyalgia, such as a reduction in the biogenic amines levels, oxidative stress or even, sex hormones (Hernandez-Leon et al., 2018; Neyal et al., 2013; Singh et al., 2019). Similar to the clinic, the ICS model lead to the development of the cardinal and the less prevalent mechanisms underlying fibromyalgia, such as peripheral and central sensitization, mitochondrial dysfunction and oxidative damage, supporting the construct validity of this experimental model (Meeus et al., 2013; Sarzi-Puttini et al., 2020; Singh et al., 2019). In particular, some studies have reported that an imbalance in redox homeostasis, characterized by an aberrant production of oxidant markers associated with a decrease in the endogenous antioxidant defenses, including catalase, superoxide dismutase (SOD) and GPx, might contribute to the development and the appearance of fibromyalgia symptoms (Bagis et al., 2005; Cordero et al., 2012; Meeus et al., 2013; Ozgocmen et al., 2006a).

Recently, Yao et al. evidenced that a disruption in redox signaling could be involved in reserpine induced fibromyalgia like-pain model. Although the authors used a different fibromyalgia animal model, the nociceptive behaviors were related to an enhancement in the oxidative products (malondialdehyde and NO) accompanied by a decrease in antioxidant defenses (glutathione and SOD) in the thalamus of rats (Yao et al., 2019). In the current study, we also provided evidence that ICS exposure led to the development of oxidative stress only in the cerebral cortex of male mice, as evidenced by an increase in the RS levels, a reduction in the NOx levels and the GPx activity.

Consistent with our findings, Ozgocmen et al. reported that patients with fibromyalgia showed high levels of thiobarbituric acid reactive substances (TBARS) and low levels of NO in the serum in comparison to healthy controls. According to these results, the authors proposed that a decrease in NO availability promoted abnormalities in the blood flow and vascular relaxion, resulting in poor oxygen diffusion, decreased oxidative phosphorylation and ATP synthesis. Such alterations could lead to an excessive production of free radicals which attacked the membrane lipids and proteins (Ozgocmen et al., 2006b). In turn, lipid peroxidation could modify the fluidity of membranes, the membrane potential or even disrupt the Na^+, K^+-ATPase due to its susceptible to membrane damage (Mariani et al., 2005; Scavone et al., 2000).

The enzyme Na^+, K^+-ATPase is responsible for maintaining the ionic homeostasis and the electrochemical gradient of sodium and potassium within the cells. Therefore, the correct function of this enzyme is vital for neuronal excitability and cellular signaling (Cui and Xie, 2017). Our results demonstrated that ICS exposure inhibited the Na^+, K^+-ATPase activity in the cerebral cortex whereas none biochemical alterations were observed in the spinal cord of male mice. Interestingly, our findings showed an increase in the Na^+, K^+-ATPase activity in CNS of female mice, after ICS exposure.

Previous studies demonstrated that an increase in the glutamatergic transmission also contributes to an increase in pain perception in patients diagnosed with fibromyalgia (Harris et al., 2009). Since the NMDA/NO/cGMP pathway has been implicated in physiological and pathological processes of pain, as well as, in the regulation of Na^+, K^+-ATPase activity, we explore whether this intracellular signaling pathway might be a mechanism underlying ICS induced pain-like fibromyalgia (Vale et al., 2007). Our results demonstrated that the antagonists MK-801 and methylene blue reversed the mechanical sensitivity in mice of both sexes, after the ICS exposure. In turn, L-arginine, the NO precursor, was able to decrease the mechanical sensitivity in female, but not in male, exposed to ICS.

Scavone et al. evidenced that glutamate, via NMDA receptor, stimulates the NO synthase which led to an enhancement in the Na^+, K^+-ATPase activity, probably due to an increased cGMP synthesis (Scavone et al., 2005). Although the results of the present study evidenced that ICS induced pain-like fibromyalgia involves, at least in part, the NMDA/NO/cGMP pathway in mice of both sexes, the modulation of Na^+, K^+-ATPase activity seems to be independent of cGMP production. In fact, the exposure to ICS in male and female mice could decrease directly or indirectly cGMP concentration by inhibiting GC and, actually, acting by similar mechanisms to the methylene blue.

A recent study using serum metabolome analysis revealed that the metabolic pathways of glutamate and NO were altered in samples of fibromyalgia patients (Clos-Garcia et al., 2019). In female rats, the steroid 17β-estradiol suppresses the pain GABAergic inhibition trough estrogen receptor (ERα), endocannabinoid- and glutamate-dependent mechanism (Tabatadze et al., 2015). In this scenario, the authors suggest that the increased prevalence of fibromyalgia in the female population might partly be explained by the 17β-estradiol specific regulation, that in the presence of glutamate excess detected in fibromyalgia patients, could be responsible for the suppression of pain inhibition by GABA (Huang and Woolley, 2012). However, the researches have not yet reached a consensus about the role of NO in the pathogenesis of fibromyalgia (Pernambuco et al., 2016). It is noteworthy that the relationship between the NMDA/NO/cGMP pathway and the specificity of sex in fibromyalgia has been poor explored so far in clinical studies. In order to extent the knowledge, our pre-clinical study is one of the pioneers in investigated whether the sex affected the mechanisms underlying fibromyalgia, specially the NMDA/NO/cGMP signaling pathway.

Of particular importance, our data suggest that the nociceptive behaviors induced by ICS in female mice involves the L-arginine/NO pathway which might be favoring the Na^+, K^+-ATPase activity through mechanisms apart from cGMP biosynthesis. On the other side, it has been reported that oxidative stress indirectly affects Na^+, K^+ - ATPase activity. In this line, our findings also showed the imbalance between the oxidant products (RS levels) and the antioxidant system (GPx activity) in the cerebral cortex of male mice, after ICS exposure. In this scenario, the cysteine residues, containing a thiol side chain in the Na^+, K^+-ATPase subunits, can be easily suffer from oxidative or nitrosative modifications by RS, that might promote an inhibition in the activity of this enzyme (Bogdanova et al., 2016).

Therefore, those data reinforce that the mechanisms underlying ICS induced nociceptive behaviors could be different in male and female mice. As a consequence, female might become more susceptible to long-lasting mechanical sensitivity then male mice. Notably, regardless of the direction, a dysfunction in the Na^+, K^+-ATPase activity induced by ICS in mice of both sexes might be affecting the neuronal excitability and the firing of action potentials that contribute to the generation of pain (Andrade Próspero et al., 2018). However, further studies are needed to clarify the role of NO/cGMP/ Na^+, K^+-ATPase pathway and sex influence in ICS induced pain-like fibromyalgia.

Reduced levels of aminergic neurotransmitters, such as serotonin, dopamine and norepinephrine, has been evidenced in the cerebrospinal fluid of fibromyalgia patients (Russell et al., 1992). In particular, a depletion of serotonin in descending pain pathways might be involved in the major painful symptoms of fibromyalgia (Clauw, 2014; Singh et al.,

2019). Previous studies also reported that the etiology of fibromyalgia might involve the polymorphism of 5-HT receptor, transporter and metabolic enzyme (Hardy, 1996; Tander et al., 2008). Regarding our results obtained from von-Frey test, pindolol, ketanserin and ondansetron reversed the mechanical sensitivity in male and female mice exposed to ICS, indicating that the modulation of serotonergic receptors ($5\text{-HT}_{1A/1B}$, $5\text{-HT}_{2A/2C}$ and 5-HT_3) might contribute to ICS induced pain-like fibromyalgia in mice.

The ICS has been described and validated as an animal model for generalized pain-like fibromyalgia. Indeed, some reports have been evidenced that gabapentinoids and SNRIs treatment, the pharmacological approach approved to managing patients with fibromyalgia, ameliorated the nociceptive signs induced by ICS, contributing to the predictive validity of this experimental model (Brum et al., 2022; Montserrat-De La Paz et al., 2014; Nishiyori et al., 2011; Nishiyori and Ueda, 2008). Despite there are consistent clinical and pre-clinical studies that correlated the major symptoms of fibromyalgia with a depletion in the serotonergic neurotransmission, we highlighted that different serotonergic receptors may also be involved in ICS induced pain-like fibromyalgia in mice of both sexes.

In this context, the present study reinforces that ICS may be a useful model for studying fibromyalgia because it mimicked the cardinal signs and the mechanisms involved in this disease in mice of both sexes. It should be noted that few studies evaluated the sex influence in the ICS model. Particularly, we provided strong evidences that some mechanisms underlying ICS induced pain-like fibromyalgia could suffer influence from sex, specially the Na^+, K^+-ATPase activity. Therefore, the main findings of this study suggest that the serotonergic receptors, the Na^+, K^+-ATPase activity, as well as, the sex factor should be considerer for design new drugs to management fibromyalgia.

5. Conclusions

In conclusion, the current study demonstrates that ICS exposure mimics the main nociceptive signs of fibromyalgia in male and female mice, including muscle weakness, mechanical and thermal sensitivities. Both pharmacological and biochemical analyses provided evidences that the serotonergic receptors, the NMDA/NO/cGMP pathway and the oxidative stress may contribute to the development of nociceptive behavior in mice exposed to ICS. However, male and female mice differently modulated the oxidative stress and the NMDA/NO/cGMP pathway to mediated nociceptive signs. Of particular importance, we demonstrated that ICS exposure in male mice promoted an imbalance in the redox homeostasis accompanied by an inhibition of Na^+, K^+-ATPase activity in the cerebral cortex which subsequently deregulated the NMDA/NO/cGMP pathway. Conversely, the involvement of NMDA/NO/cGMP signaling pathway might contribute to an enhancement in the Na^+, K^+-ATPase activity, maintaining the redox homeostasis in the cerebral cortex and spinal cord of female mice exposed to ICS. The mechanistic pathways induced by ICS seems to be sex-dependently which help to better understand the differences in nociceptive signs between male and female mice, or even the prevalence of female in this model of fibromyalgia. In this line, the development of an appropriate treatment for fibromyalgia must considered the biological sex divergences.

Ethics approval and consent to participate

Animal care and all experimental procedures were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80–23, revised in 1996) and in accordance with the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil (CEEA 28142–2019). All efforts were made to minimize the number of animals used and their suffering.

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CRediT authorship contribution statement

C.C.M., A.S.R., K.P.M, C.L. and E.A.W. conceived and designed the study. C.C.M, A.S.R and K.P.M conducted all behavioral tests and biochemical analyses. C.C.M. and E.A.W. wrote and reviewed the manuscript. E.A.W. supervised the study. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.2 CAPÍTULO 2

Os resultados deste capítulo da tese estão apresentados sob a forma de manuscrito científico. Os itens Introdução, Materiais e métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprio manuscrito.

Os dados na Tabela 3 indicam que o tratamento com o composto 4-APSB foi capaz de modular as principais alterações comportamentais e bioquímicas induzidas pela exposição ao EFI em camundongos machos e fêmeas.

Tabela 3. Resumo dos principais resultados obtidos no Capítulo 2.

Testes Comportamentais		**Machos**		**Fêmeas**	
		EFI	4-APSB	EFI	4-APSB
Sensibilidade mecânica		↑	↓	↑	↓
Sensibilidade térmica		↑	↓	↑	↓
Força muscular		↓	↑	↓	↑
Testes comportamentais *(suspensão da cauda)*		**Machos**		**Fêmeas**	
		EFI	4-APSB	EFI	4-APSB
Latência		↓	↑	↓	✕
Imobilidade		↑	↓	↑	↓
Análises *ex vivo*		**Machos**		**Fêmeas**	
		EFI	4-APSB	EFI	4-APSB
Níveis de TBARS	Córtex cerebral	↑	↓	↑	↓
	Medula espinhal	✕	✕	↑	↓
Níveis de NPSH	Córtex cerebral	✕	✕	✕	✕
	Medula espinhal	✕	✕	↓	↑
Atividade da GPx	Córtex cerebral	✕	✕	↑	↓
	Medula espinhal	✕	✕	✕	✕
Atividade da Na^+,K^+-ATPase	Córtex cerebral	✕	✕	↓	↑
	Medula espinhal	✕	✕	↓	↑
Atividade da Mg^{2+}-ATPase	Córtex cerebral	↑	✕	✕	✕
	Medula espinhal	↑	✕	✕	✕
Expressão do NFκB	Córtex cerebral	↑	↓	↑	↓
Expressão do Nrf-2	Córtex cerebral	↑	↓	↑	↓
Expressão OH-1	Córtex cerebral	✕	✕	✕	✕

(↑) Aumento induzido pela exposição ao EFI ou ao tratamento com o 4-APSB; (↓) diminuição induzida pela exposição ao EFI ou ao tratamento com o 4-APSB; (✕) nenhuma alteração induzida pela exposição ao EFI ou ao tratamento com o 4-APSB.

Insights of the imbalance redox signaling in fibromyalgia induced by intermittent cold stress in mice: 4-amino-3-(phenylselanyl) benzenesulfonamide a promising approach to treat fibromyalgia

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Abstract

Fibromyalgia is characterized by a widespread chronic pain associated to mood disorders. The pathophysiology of fibromyalgia is complex and it remains inconclusive. The organoselenium compounds play a role in multiple biological targets. This study investigated the effects of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) in an experimental model of fibromyalgia induced by intermittent cold stress (ICS), in male and female Swiss mice. After the ICS exposure, the animals were treated with 4-APSB (1 mg kg^{-1}) or vehicle, by the intragastric (i.g.) route, until the tenth day. The behavioral tasks were performed on days 5, 8 and 10 of the experimental protocol. The results suggest that 4-APSB suppressed the nociceptive signs and a depressive like-phenotype in male and female mice exposed to ICS, the characteristic symptoms of fibromyalgia. Moreover, 4-APBS normalized the elevated levels of TBARS and the up-regulation of Nrf2 and NFκB expression in the cerebral cortex of ICS-exposed mice. This organoselenium compound also modulated the alterations induced by ICS, regarding the levels of TBARS, NPSH and GPx activity, in the spinal cord of female mice. The 4-APSB attenuated the inhibition of Na^+, K^+ - ATPase activity in the central nervous system (CNS) of female mice exposed to ICS, but this compound did not change the high activity of Mg^{2+}-ATPase in the CNS of male mice exposed to ICS. Therefore, the 4-APBS seems to interact with distinct biological targets altered by ICS at central levels and it might be a promising prototype for treating fibromyalgia in both sexes.

Keywords: fibromyalgia; redox signaling; selenium; sex.

1. Introduction

Fibromyalgia, classified as a widespread chronic pain, is prevalent among 2 - 8% of the general population [1, 2]. Patients diagnosed with this disease generally suffer from mechanical and thermal allodynia, muscle pain and spontaneous nociception [3]. Moreover, fibromyalgia often coexists with other neurological disorders, such as impaired cognition, sleep disturbances and depression [4]. Together, all these symptoms lead to a vicious cycle that impaired the productivity and quality of life of patients [5].

An excessive generation of prooxidants markers stimulate several signaling pathways, including the nuclear factor erythroid 2-related factor 2 (Nrf2). Notably, studies have demonstrated the Nrf2 pathway as an important therapeutic target for chronic painful conditions, including diabetic neuropathy and rheumatoid arthritis [6, 7]. Although the mechanistic aspects of fibromyalgia remain poorly understood, an imbalance between the oxidant and antioxidant signaling molecules has been implicated in the etiopathogenesis of this syndrome and associated comorbidities [8]. However, there is a lack of studies evidencing the role of Nrf2 in animal models of fibromyalgia.

The nuclear factor kappa B (NFκB), another transcript factor, is recognized as a prototypical proinflammatory signaling pathway because it is responsible for regulating the gene expression of chemokines, cytokines, and adhesion molecules [9]. Some evidence obtained using experimental models of fibromyalgia supports the hypothesis that the stimulation of NFκB cascade may promote the release of inflammatory mediators at peripheral levels [10]. Indeed, a complex interplay of oxidative stress markers and cytokines has been evidenced in pain-depression dyad, the hallmark symptoms of fibromyalgia [11]. Of particular importance, studies have also found that the NFκB pathway is sensitive to intracellular redox state and it could easily respond to different reactive species (RS), similar to Nrf2. In this line, both transcript factors exhibit a

dynamic relationship to control the physiological homeostasis of redox signaling and the responses to inflammatory process [12].

The central nervous system (CNS) presents, in its composition, an elevated level of fatty acids and transition metals, a low concentration of antioxidant defenses and a diminished capacity of regeneration. All these factors enhancing the CNS susceptibility to oxidative stress harmful events, including disturbances in the ion pumps activities [13, 14]. Particularly, Na^+, K^+ - and Mg^{+2} - ATPases play a critical role for neuronal function, since these enzymes participate of the nerve impulses conduction, as well as the excitatory neurotransmission [15]. Despite a dysfunction of ATPases has been closely implicated in neuropsychiatry and neurodegenerative diseases [16], few studies have investigated the relevance of these ion pumps in pre-clinical and clinical studies of fibromyalgia.

Several organoselenium compounds have been widely recognized for their ability to modulate the oxidative stress [17]. Besides, the lipophilicity of these compounds allows them to permeate the blood-brain barrier and act in the CNS, interacting with multiples biological targets and displaying pharmacological effects in different animal models [18–21]. Recently, previous data obtained from our research group evidenced that a novel organoselenium compound, the 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB), elicited a promising antinociceptive effect in different animal models of acute pain, mainly through its antioxidant activity [22].

Considering that the management of the painful symptoms and the comorbidities associated to fibromyalgia remains a challenge in the pharmaceutical area because the physiopathology complexity of this syndrome, the purpose of the present study was elucidated the pharmacological effects of 4-APBS in a well-stablished experimental model of fibromyalgia, the intermittent cold stress (CSI). Some markers of oxidative stress, the interplay of Nrf2 and NFκB pathways, as well as the ion pumps activities were

also investigated in attempt to extend knowledge about the mechanisms underlying ICS induced characteristic signs of fibromyalgia.

2. Materials and Methods

2.1 Animals

The experiments were performed using male and female Swiss mice (25-35g, bred in house, two months old). The animals were housed in individually cages (3-4 animals per cage) at an acclimatized (22 ± 2 °C) animal room, with wood shaving bedding and nesting material. Mice were maintained under a 12 h light/12 h dark cycle (the lights were turned on at 07:00 a.m.) and a commercial rodent pellet diet and fresh water were provided *ad libitum*. The animals were acclimatized to the housing environment for at least 7 days before the commencement of the experiments and to the behavioral room for 1 h prior to behavior tests. No previous procedure was performed on these mice.

The experimental protocols were authorized by the Committee on Care and Use of Experimental Resources of the Federal University of Pelotas (Brazil), affiliated to the National Council for the Control of Animal Experimentation, and registered under the number CEEA 28142-2019. All animal handling and experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 823, revised 1978) and International Guiding Principles for Biomedicals Research Involving Animals which every effort was made to reduce both the number of animals used and their discomfort. Behavioral evaluations were blinded to the treatment when performing tests. All experiments were performed between 8:00 a.m. and 5:00 p.m.

2.2 Chemicals

4-APSB (Figure 1) was synthesized and characterized at the Laboratory of Clean Organic Synthesis at the Federal University of Pelotas (Sacramento et al, 2022). Analysis of GC/MS determined the chemical purity of this compound (99.9%). 4-APSB was dissolved in canola oil and administered by intragastric (i.g.) route at a constant volume of 10 $mLkg^{-1}$ body weight. All other chemicals used in this study were of analytical grade and obtained from standard commercial suppliers.

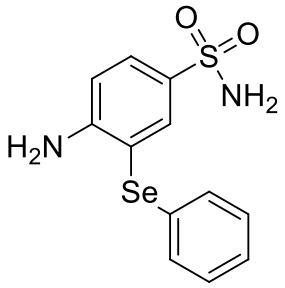

Fig. 1 Chemical structure of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB).

2.3 Experimental design

2.3.1 Intermittent cold stress (ICS)-induced fibromyalgia model

Briefly, the animals (3-4 mice in each cage) were placed in a cold environment (4 ± 2 °C) at 4:30 p.m. on the first day of the experimental protocol (day 1), with feeding and filtered water *ad libitum*. In the next morning (10:00 a.m.), mice were transferred to a temperature room (22 ± 2 °C) during 30 minutes and then, they were put in the cold room again for 30 minutes. This process was repeated until 4:30 p.m. (day 2). Finally, the animals were allocated in the cold room overnight. The same procedure was performed on the next day of the experimental protocol (day 3). In the morning (10:00 a.m.) of the fourth day, following the completion of the ICS exposure, mice were returned and adapted to a normal room temperature (22 ± 2 °C) for at least 2 hours prior to behavioral tests.

Mice in the control group were kept at 22 ± 2 °C during all three days (from 4:30 p.m. on day 1 to 10:00 a.m. on day 4). ICS was developed and validated as an experimental mouse model of fibromyalgia by Nishiyori and Ueda (2008) [23].

Two hours after the adaptation period, the animals were randomly divided into four experimental groups: Control + vehicle, Control + 4-APSB, CSI + vehicle and CSI + 4-APSB. As shown in Figure 2, mice from control + vehicle and CSI + vehicle groups received canola oil (10 mL kg^{-1}), by the intragastric (i.g.) route, whereas the mice from control + 4-APSB and CSI + 4-APSB groups were treated with the compound 4-APSB (1 mg kg^{-1}, i.g. route) until the tenth day of the experimental protocol. Immediately after the behavioral tests, the animals were anesthetized and euthanized by inhalation of isoflurane anesthetic. The samples of spinal cord and cerebral cortex and hippocampus were rapidly dissected, weighed and placed at -20 °C to further *ex vivo* assays.

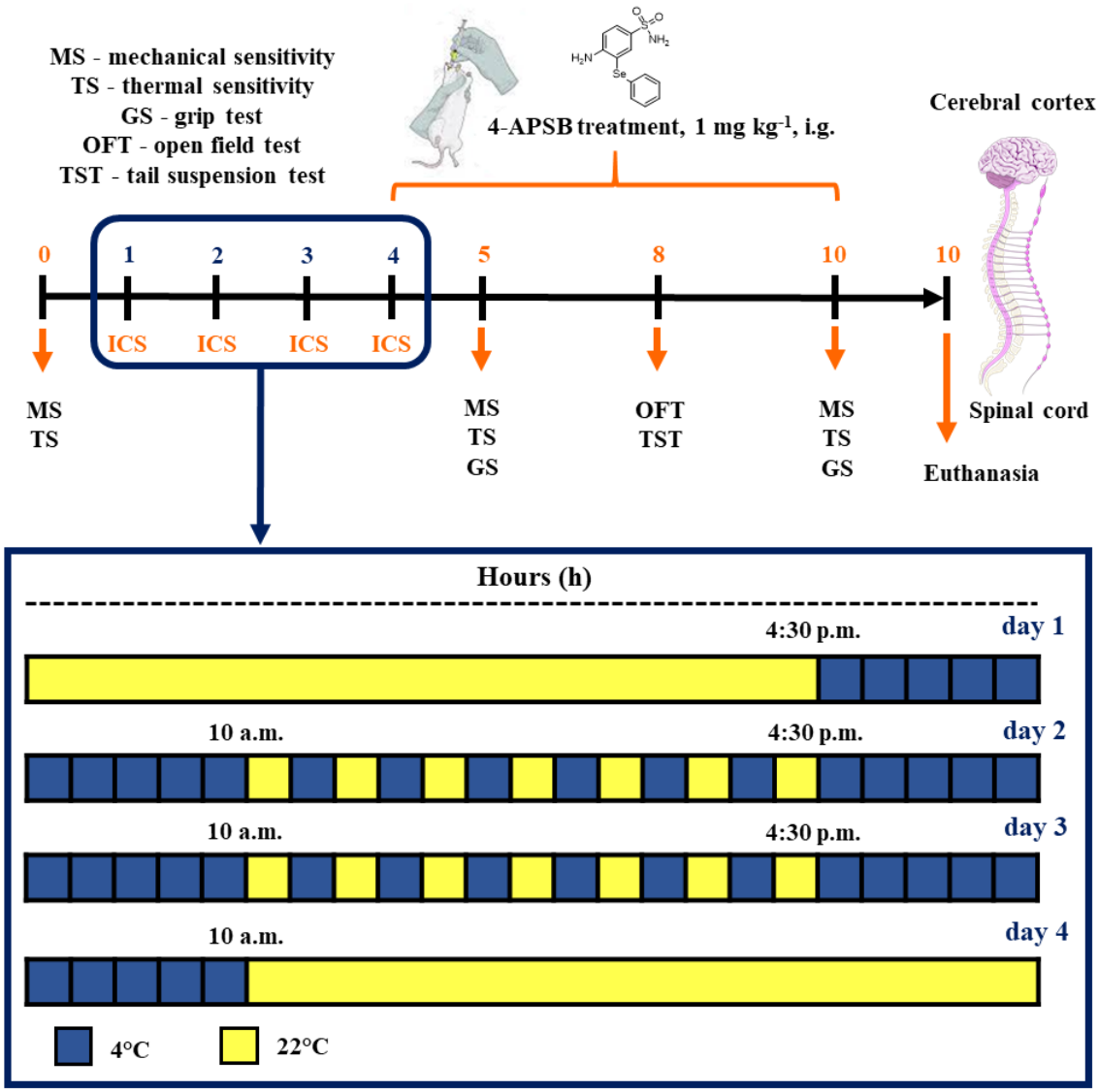

Fig. 2 Schematic representation of the experimental design of the study.

The days of the behavioral tests and the experimental protocol were selected in accordance with a previous study developed by our research group, which evaluated the development of nociceptive sings induced by ICS in male and female mice in an attempt to standardized this experimental model of fibromyalgia [24]. In addition, the dose, the route of administration and the time interval of treatment with 4-APSB were chosen based on the reports that this compound exerted antinociceptive effect in acute models of nociception [22].

2.4 Behavioral tests

2.4.1 Measurement of mechanical sensitivity

As described by Alamri et al. (2018) [24], mechanical sensitivity of mice was estimated using an electronic aesthesiometer (Insight, Ribeirão Preto, SP, Brazil), an apparatus containing a specific paw pressure transducer connected. Briefly, the animals were acclimatized for 30 minutes in individual clear plastic chambers on an elevated wire mesh platform which allowed to access the plantar surface of the paws. The investigator was trained to apply constant progressive pressure on the central area of the hind paw using a blunt-tipped probe connected to a transducer until paw withdrawal followed by clear flinching movements. After this response, the pressure intensity was automatically recorded. The paw withdrawal threshold (g), defined as a measure of mechanical sensitivity, was evaluated on days 0, 5 and 10 of the experimental protocol.

2.4.2 Measurement of thermal sensitivity

In the hot plate test, nociceptive reflexes in response to thermal stimulus were assessed as previously described [25]. Each mouse was placed on a heated metallic surface plate (52 ± 1 ºC) surrounded by a clear acrylic cage. The time between animal placement on the apparatus and the nociceptive response event (jumping off the surface, shaking, or licking the hind paws) was recorded. To avoid any injury on animal paws, a cut-off time of 45 seconds was established. The paw withdrawal latency (s), defined as a measure of thermal sensitivity, was evaluated on days 0, 5 and 10 of the experimental protocol.

2.4.3 Measurement of muscular strength

A simple and non-invasive method, the grip strength test was design to assess mouse muscle strength *in vivo*. The experiment was conducted using a digital force-gauging apparatus (Insight, Ribeirão Preto, SP, Brazil). Mouse was gently pulled parallel away from the bar by the tail until the forelimbs released the bar. The maximum force prior to release of the mouse's paw from the bar was recorded on days 5 and 10 of the experimental protocol. The test was repeated 3 times and an average was reported as the muscle strength (N) [26].

2.4.4 Assessment of locomotor and exploratory performance

The open field test evaluates the exploratory behavior and the general locomotor activity of mice in order to rule out non-specific effects, such as psychostimulatory activity. The apparatus was made of plywood (30 H x 45 L x 45 W) in which the floor was divided into 9 quadrants of equal areas (3 rows of 3) by masking tape markers. Each animal was placed at the center of the apparatus and observed during 4 minutes. The locomotor (number of segments crossed with the four paws) and exploratory (number of rearing on the hind limbs) activities were recorded on the eighth day of the experimental protocol [27]. The arena was cleaned with 30% ethanol after each session.

2.4.5 Assessment of emotional domain

The tail suspension test was carried out in a quiet environment wherein the total immobility duration is considered the major parameter measured to assess the “behavioral

despair" in rodents [28]. Each mouse was suspended by the tail with an adhesive tape 50 cm above the floor. For the next 6 minutes, the latency for the first immobility episode (s) and the immobility time (s) were recorded on the eighth day of the experimental protocol. Mice were only considered immobile when passively hung and completely motionless.

2.5 Ex vivo assays

The measurement of oxidative stress and the ionic pumps activity was performed in both cerebral cortex and spinal cord tissues of mice. On the other side, the estimation of mRNA expression levels, through the real-time polymerase chain reaction assay, was evaluated only in the cerebral cortex of mice. It should be noted that the cerebral cortex was more susceptible to biochemical markers changes than the spinal cord, after the ICS exposure in mice. In this line, it is possible that this animal model of fibromyalgia, induced by stress, may primarily affect brain structures overlapping the nociceptive and stress perceptions, such as the cerebral cortex, and later, the spinal region.

2.5.1 Tissues processing for biochemical analyses

Samples of cerebral cortex and spinal cord were homogenized in cold 50 mM Tris-HCl at pH 7.4 (1/5 weight/volume). The homogenates were centrifugated at 3000 rpm for 10 minutes at 4ºC and the supernatant fraction (S_1) was used to biochemical analysis, including thiobarbituric acid reactive substances (TBARS), non-protein thiol (NPSH), glutathione peroxidase (GPx), Na^+, K^+ - ATPase and Mg^{2+} - ATPase. Protein concentration in S_1 was estimated by the method of Bradford (1976) [29], using bovine

serum albumin (1 $mg^{-1}mL^{-1}$) as a standard. The color was measured spectrophotometrically at 595 nm.

2.5.2 TBARS assay

TBARS assay was performed to indirectly determine the malondialdehyde (MDA) levels, an important lipid peroxidation marker. As previously described by Ohkawa et al. (1979) [30], MDA reacts with 2-thiobarbituric acid (TBA) under acidic conditions and high temperatures to yield the chromogen. The S_1 aliquots were incubated with 0.8% TBA, acetic acid buffer (pH 3.4) and 8.1% sodium dodecyl sulfate (SDS) for 2 hours at 95ºC. The color reaction was measured at 532 nm and the results were expressed as nmol of MDA/mg protein.

2.5.3 NPSH content

NPSH content, a non-enzymatic antioxidant defense, was determined by Ellman's method [31]. Briefly, S_1 was mixed (1:1) with 10% trichloroacetic acid (TCA). After centrifugation (3000 rpm for 10 minutes), an aliquot of S_1 containing free SH-groups was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The color reaction was measured at 412 nm and NPSH levels were expressed as nmol of NPSH/g tissue.

2.5.4 GPx activity

GPx activity was estimated spectrophotometrically, as described by Wendel (1981) [32]. An aliquot of the S_1 was added in a system composed by reduced glutathione

(GSH)/NADPH/glutathione reductase (GR). The enzymatic reaction was initiated by the addition of H_2O_2. This assay involves monitoring the dismutation of H_2O_2 in the presence of S_1 at 340 nm. In summary, H_2O_2 is reduced and generates oxidized glutathione (GSSG) from GSH. In turn, GSSG is regenerated back to GSH by the GR present in the analysis medium at the expense of NADPH. The decrease in absorbance is proportional to the oxidation of NADPH to $NADP^+$ and it is indirectly correlate to the GPx activity in the sample. The enzymatic activity was expressed as nmol/min/mg protein.

2.5.5 Na^+, K^+-ATPase and Mg^{2+}-ATPase activities

In this assay, a reaction mixture containing an aliquot of S_1, 3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris-HCl at pH 7.4, in a final volume of 500 µL, was preincubated at 37°C for 10 minutes. Control samples were prepared under the same conditions with the addition of 0.1 mM of ouabain. Considering that ouabain is an inhibitor of the Na^+, K^+ pump, it was possible to observe in this technique the enzyme activity related to the Mg^{2+} pump. To determine the Mg^{2+}-ATPase activity, ouabain (1 mM) was added to the reaction medium. The reactions initiated with the addition of ATP to a final concentration of 3.0 mM. All samples were incubated at 37°C for 30 minutes and the reaction was stopped by adding TCA solution (10%) with 10 mM $HgCl_2$. Enzyme activity was calculated from the difference between amounts of inorganic phosphate (Pi) found after incubation in the absence and presence of ouabain. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow [33]. The results were expressed as nmol Pi/mg protein/min.

2.5.6 RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction

Total mRNA was extracted from 50 – 100 mg of cerebral cortex tissue (n = 6 for each experimental group) using TRIzol reagent (Invitrogen™, Carlsbad, USA) followed by DNase treatment with DNase I Amplification Grade (Invitrogen™, Carlsbad, USA) to ensure minimum DNA contamination of the samples. The total RNA isolated was quantified and its purity (260/280 and 260/230 ratios) was examined by spectrophotometer NanoVue (GE, Fairfield, CT, USA).

The cDNA synthesis was performed using High Capacity cDNA Reverse Transcription kit (AppliedBiosystems™, UK) according to the manufacturer's protocol. For reverse transcription, 1 μg of total RNA was used in a reaction volume of 20 μl. The amplification was made with GoTaq® qPCR Master Mix (Promega, Madison, WI) using the LightCycler® 96 Real-Time PCR System (Roche Molecular Systems Inc., CA, USA) and the sequence of primers used are indicated in Table 1. The qPCR conditions were as follows: 10 minutes at 95°C to activate the hot-start Taq polymerase, followed by 35 cycles of denaturation for 15 seconds at 95°C, primer annealing for 60 seconds at 60°C, and extension for 30 seconds at 72°C (fluorescence signals were detected at the end of every cycle). Baseline and threshold values were automatically set by the LightCycler® 96 Software.

The number of PCR cycles required to reach the fluorescence threshold in each sample was defined as the Ct value. The expression of the target genes NFκB, Nrf2 and hemeoxigenase-1 (HO-1) were determined and the 2-ΔΔCT method was used to normalize the fold change in gene expressions [34], using 18S as housekeeping gene.

Table 1: Primers used for quantitative real-time polymerase chain reaction. Listed are the forward and reverse primer sequences used to amplify each target gene as well as the 18S endogenous control.

Primer Name	Sequence	Reference
Nrf2 Forward	5' CTCGCTGGAAAAAGAAGTG 3'	[35]
Nrf2 Reverse	5' CCGTCCAGGAGTTCAGAGG 3'	
HO-1 Forward	5' CGCCTTCCTGCTCAACATT 3'	[36]
HO-1 Reverse	5' TGTGTTCCTCTGTCAGCATCAC 3'	
NFκB Forward	5' AGAGAAGCACAGATACCACTAAG 3'	[37]
NFκB Reverse	5' CAGCCTCATAGAAGCCATCC 3'	
18S Forward	5' CCTGGATACCGCAGCTAGGA 3'	[38]
18S Reverse	5' GCGGCGCAATACGAATGCCCC 3'	

Nrf2: nuclear factor erythroid 2-related factor 2; NFκB: nuclear factor kappa B; HO-1: heme oxygenase-1.

2.6 Statistical analysis

All experimental results are presented as the mean ± standard error of the mean (SEM). The statistical analyses were performed using GraphPad Prism Software version 6.0 (San Diego, CA, USA). A Gaussian distribution was tested using D'Agostino and Pearson omnibus normality test. The ROUT test (Q = 1.0%) was applied for the detection of outliers. Data were analyzed by two-way analysis of variance (ANOVA), followed by Tukey post hoc test when appropriate. Probability values less than 0.05 ($P < 0.05$) were considered statistically significant. Pearson's correlation coefficient was used for analyze the correlation between variables.

3. Results

3.1 4-APSB treatment attenuates the nociceptive signs-like fibromyalgia induced by ICS in male and female mice

The results compiled in this section demonstrate that 4-APSB treatment alleviated the nociceptive signs induced by ICS in mice of both sexes. Before the ICS exposure, there was no difference in the baseline values of paw withdrawal threshold to mechanical stimulus among all experimental groups. Male and female mice exposed to ICS exhibited a significant reduction on the paw withdrawal threshold when compared with those mice of the control groups from the fifth day that lasted for at least until the tenth day of the experimental protocol. Reductions of 39% and 42% (day 5), as well as 44% and 43% (day 10) on the paw withdrawal threshold were observed in male and female mice exposed to ICS, respectively, suggested that ICS induces a robust mechanical sensitivity in mice of both sexes.

On the other side, acute or repeated treatment with 4-APSB was able to increase the paw withdrawal threshold in male and female mice exposed to ICS, indicating that this compound reversed the mechanical sensitivity induced by ICS model (Figures 3A and 3B). Particularly, this compound led to an enhancement of 38% and 39% (day 5), as well as 42% and 43% (day 10) on the paw withdrawal threshold in male and female mice exposed to ICS, respectively.

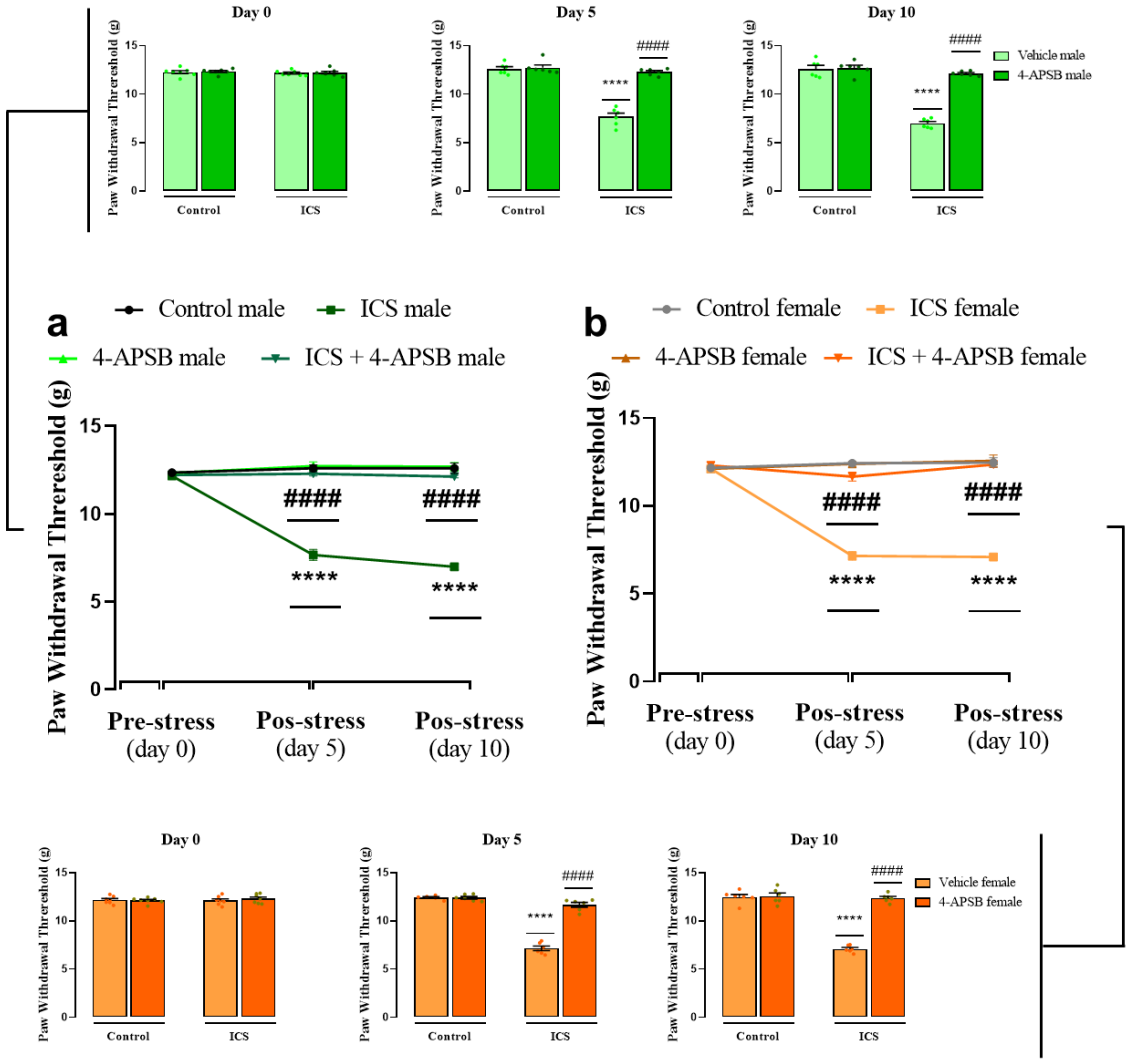

Fig. 3 Effect of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) (1 mg kg^{-1}, i.g.) and the ICS exposure on the paw withdrawal threshold to mechanical stimulus in the von Frey test in male (a) and female (b) mice. Data are expressed as mean ± S.E.M of 7 animals per group. Asterisk denotes significant levels when compared with the control group: (****) P < 0.0001. Hashtag denotes significant levels when compared with the ICS group: ($^{####}$) P < 0.0001 (Two-way ANOVA followed by the Tukey test).

As shown in Figure 4, the baseline values of paw withdrawal latency to thermal stimulus were equivalent among all experimental groups before the ICS exposure. On day 5, male (48%) and female (59%) mice exposed to ICS displayed a significant decrease in

paw withdrawal latency. The thermal sensitivity was sustained in ICS groups until the last day of the experimental protocol, with reductions of 28% and 49% for the paw withdrawal latency in male and female mice, respectively. A single administration of the compound 4-APSB produced an increase in the paw withdrawal latency of female mice exposed to ICS (48%), but not in male mice. Nevertheless, female mice underwent to ICS exposure and treated with a single dose of 4-APSB exhibited a higher thermal sensitivity than female mice of the control group. As expected, repeated administrations of the compound 4-APSB reversed the thermal sensitivity induced by ICS exposure in male (34%) and female (45%) mice. These results indicate that the effect of 4-APSB on thermal sensitivity developed by the ICS model might be time-dependent in male mice, but not in female mice (Figures 4A and 4B).

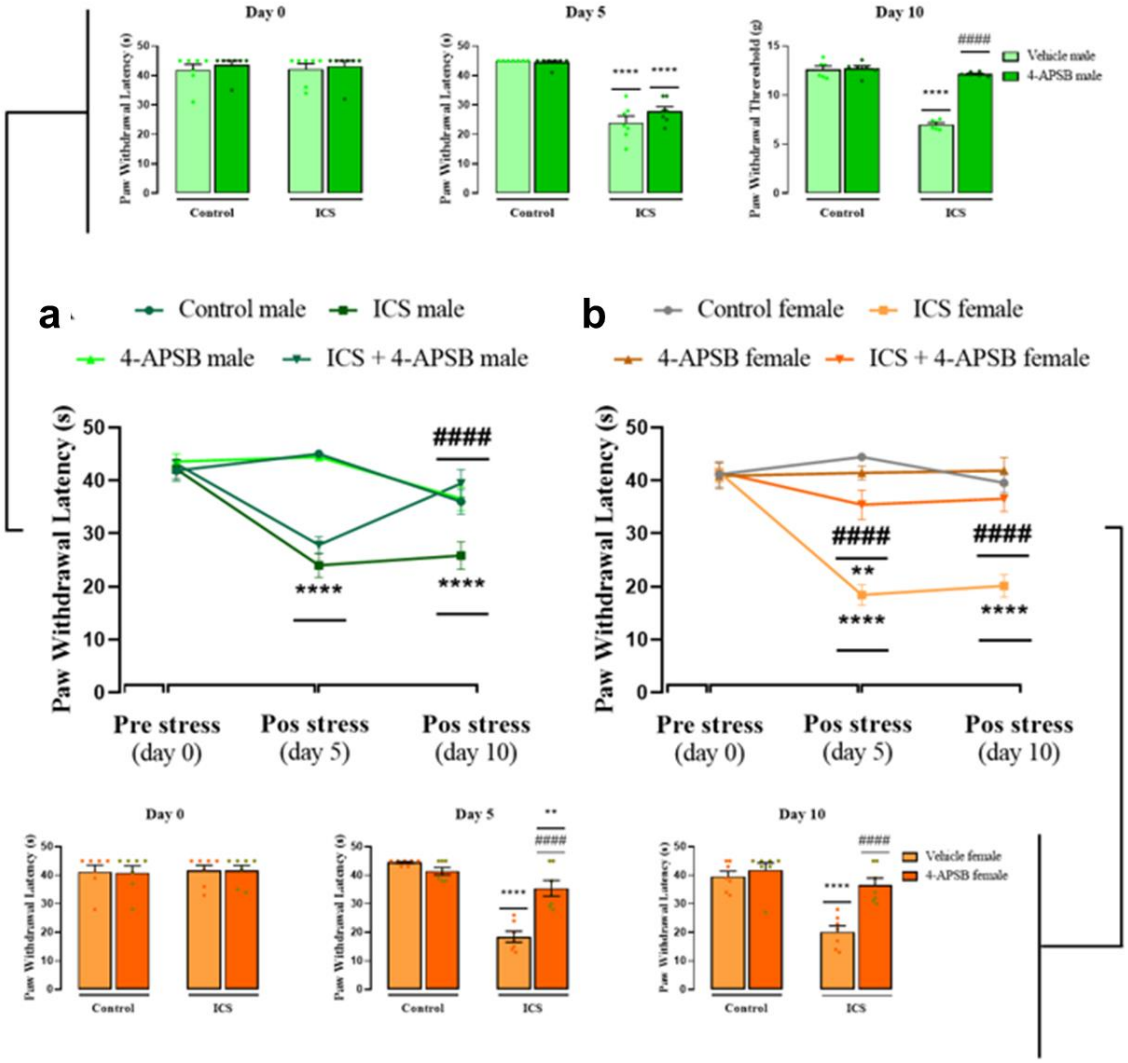

Fig. 4 Effect of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) (1 mg kg^{-1}, i.g.) and the ICS exposure on the paw withdrawal latency to thermal stimulus in the hot plate test in male (a) and female (b) mice. Data are expressed as mean ± S.E.M of 7 animals per group. Asterisk denotes significant levels when compared with the control group: (****) $P < 0.0001$, (**) $P < 0.01$ and (*) $P < 0.05$. Hashtag denotes significant levels when compared with the ICS group: ($^{\#\#\#\#}$) $P < 0.0001$ and ($^{\#\#}$) $P < 0.01$ (Two-way ANOVA followed by the Tukey test).

As shown in Figure 5, the ICS exposure in mice of both sexes produced a significant reduction in the grip strength on days 5 and 10 of the experimental protocol when

compared with mice of the control groups. Reductions of 27% and 29% (day 5), as well as 17% and 30% (day 10) on the grip strength were observed in male and female mice exposed to ICS, respectively, suggesting that ICS triggers the myalgia, a hallmark sign of fibromyalgia. A single (Figures 4A and 4C) or repeated (Figures 4B and 4D) treatment with 4-APSB significantly increased the grip strength in male and female mice exposed to ICS, evidencing that this compound attenuated the deficit in muscle strength. The results revealed that 4-APSB enhanced the grip strength by 26% and 22% on day 5, as well as by 27% and 23% on the last day of the experimental protocol, in male and female mice, respectively. Besides, male mice exposed to ICS and repeatedly treated with 4-APSB exhibited a higher muscular strength than male mice of control group.

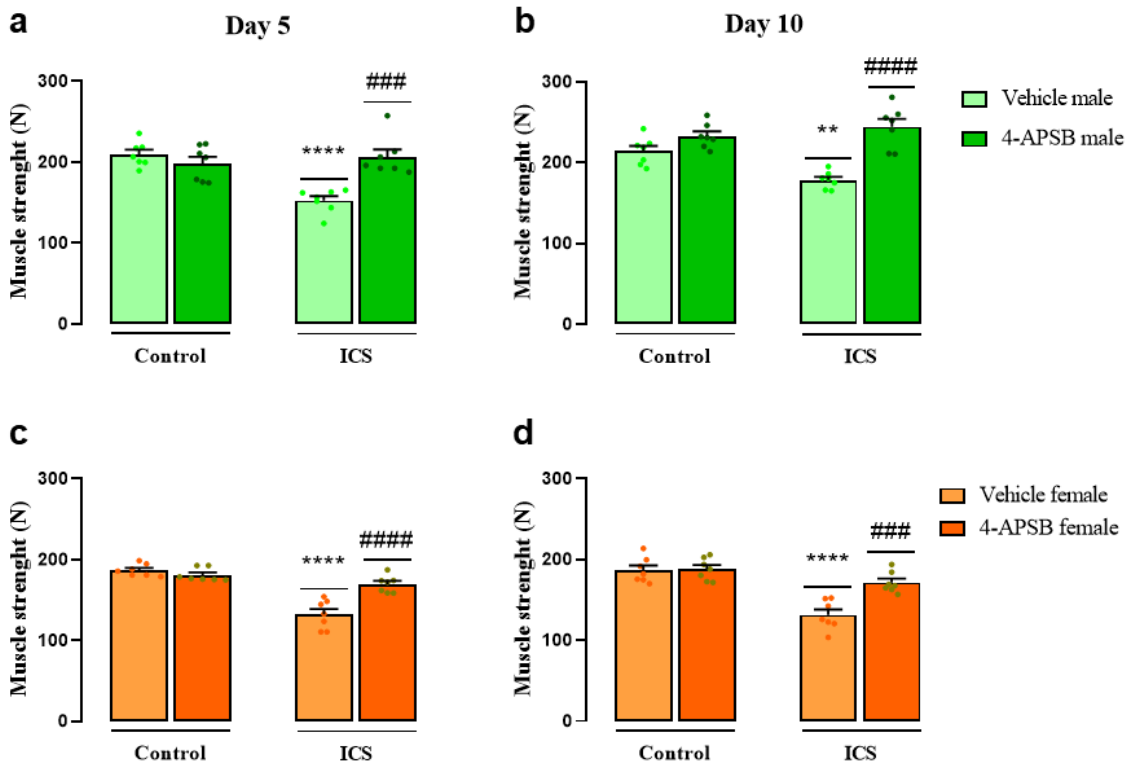

Fig. 5 Effect of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) (1 mg kg^{-1}, i.g.) and the ICS exposure on the muscular strength evaluated with a digital force device in male (a and b) and female (c and d) mice on the fifth and tenth day of the experimental protocol, respectively. Data are expressed as mean ± S.E.M of 7 animals per group.

Asterisk denotes significant levels when compared with the control group: (****) $P < 0.0001$, (**) $P < 0.01$ and (*) $P < 0.05$. Hashtag denotes significant levels when compared with the ICS group: ($^{\#\#\#\#}$) $P < 0.0001$ and ($^{\#\#\#}$) $P < 0.001$ (Two-way ANOVA followed by the Tukey test).

3.2 4-APSB treatment abolishes the depressive-like phenotype in male and female mice exposed to ICS

The Figure 6 shows the effect of 4-APSB treatment and ICS exposure in male and female submitted to tail suspension test. The data demonstrated that ICS exposure significantly decrease the latency time for the first episode of immobility in male and female mice in comparison to control groups. The treatment with 4-APSB was able to increase this parameter in male mice exposed to ICS, but not in female mice (Figures 6A and 6C).

In addition, the exposure to ICS caused a significant increase in the immobility time in male and female mice compared to control groups in the tail suspension test. In contrast, the animals of both sexes exposed to ICS and treated with 4-APBS remained less time immobile than ICS groups (Figures 6B and 6D).

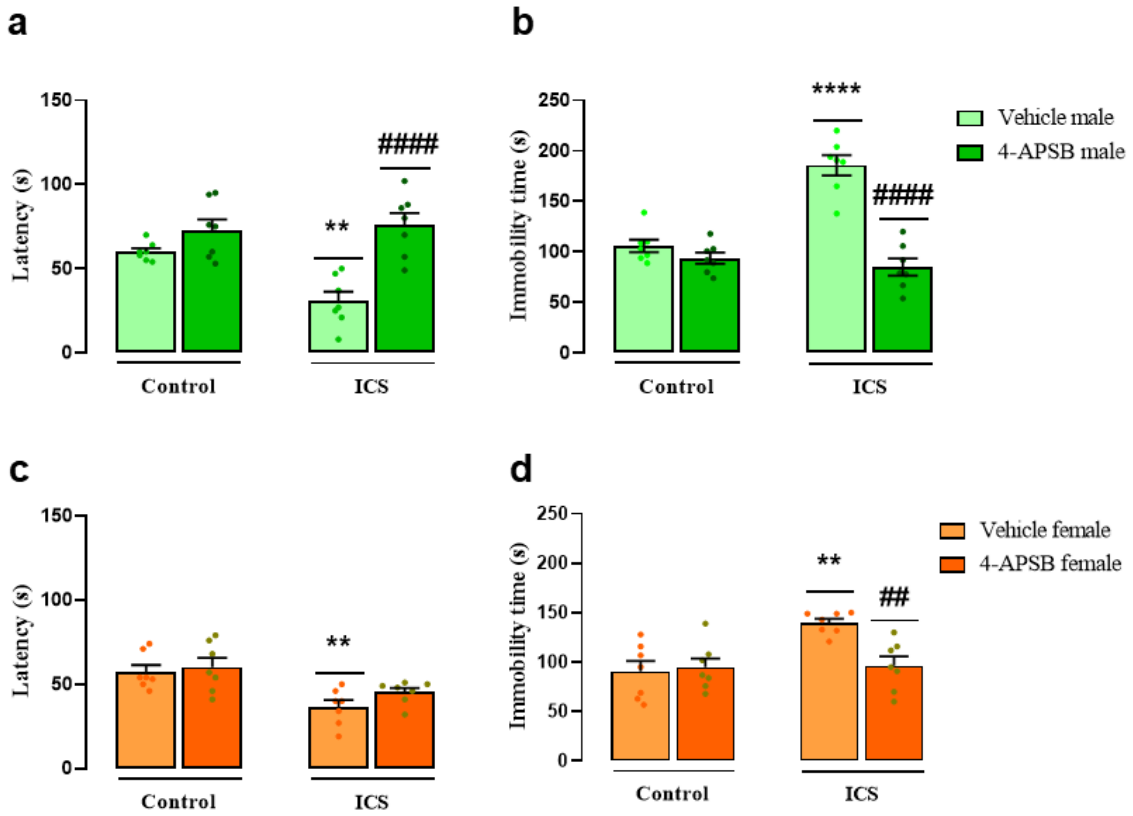

Fig. 6 Effect of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) treatment (1 mg kg^{-1}, i.g.) and the ICS exposure on the latency for the first episode of immobility in the tail suspension test in male (a) and female (c) mice. Effects of 4-APSB treatment (1 mg kg^{-1}, i.g.) and the ICS exposure on the immobility in the tail suspension test in male (b) and female (d) mice. Data are expressed as the mean ± SEM of 7 animals per group. Asterisk denotes significant levels when compared with the control group: (****) $P < 0.0001$ and (**) $P < 0.01$. Hashtag denotes significant levels when compared with the ICS group: ($^{####}$) $P < 0.0001$ and ($^{##}$) $P < 0.01$ (Two-way ANOVA followed by the Tukey test).

3.3 4-APSB treatment and ICS exposure do not alter locomotor and exploratory activities of male and female mice

The locomotor and exploratory activities of male and female mice were evaluated in open field test which there are summarized in Table 2. The data showed that the ICS

exposure or the treatment with the compound 4-APSB did not cause any significant change in the number of crossings or rearing in male and female mice.

Table 2: Effect of 4-APSB treatment on spontaneous locomotor and exploratory activities of male and female mice exposed to ICS-induced fibromyalgia.

Groups	Open Field Test			
	Crossing[a]		Rearing[b]	
	Male	Female	Male	Female
Control + vehicle	106.9 ± 2.14	102.1 ± 2.92	39.7 ± 2.21	34.1 ± 1.03
Control + 4-APSB	106.9 ± 4.89	104.3 ± 3.40	40.3 ± 2.06	35.6 ± 1.74
ICS + vehicle	110.4 ± 3.38	104.1 ± 3.46	40.1 ± 3.11	38.6 ± 1.78
ICS + 4-APSB	107.7 ± 4.23	104.0 ± 2.45	39.4 ± 1.02	36.4 ± 1.27

Data are reported as mean ± SEM for seven animals per group. Statistical analyses were performed by two-way ANOVA, followed Tukey multiple comparison test when appropriate.
[a] Data are expressed as number of crossings.
[b] Data are expressed as number of rearing.

3.4 4-APSB treatment regulates the redox signaling and the inflammatory status within the CNS in male and female mice exposed to ICS

To elucidate the antioxidant properties exerted by the compound 4-APSB, TBARS levels (Figure 7), NPSH content and GPx activity (Figure 8) were evaluated in the spinal cord and cerebral cortex of male and female mice exposed to ICS. In general, TBARS levels remained unchanged among all experimental groups in the spinal cord of male mice, whereas an enhancement in the lipid peroxidation, indirectly measured by the TBARS assay, was observed in female mice exposed to ICS in comparison to control group (Figures 7A Figure 7B). An increase in the levels of TBARS was detected in the cerebral cortex of mice of both sexes exposed to ICS on day 10 of the experimental protocol, when compared with those mice of control groups (Figures 7C and 7D). The results also showed that the treatment with 4-APSB normalized the TBARS levels in the

cerebral cortex of mice of both sexes, as well as, in the spinal cord of female mice exposed to ICS (Figures 7B – 7D).

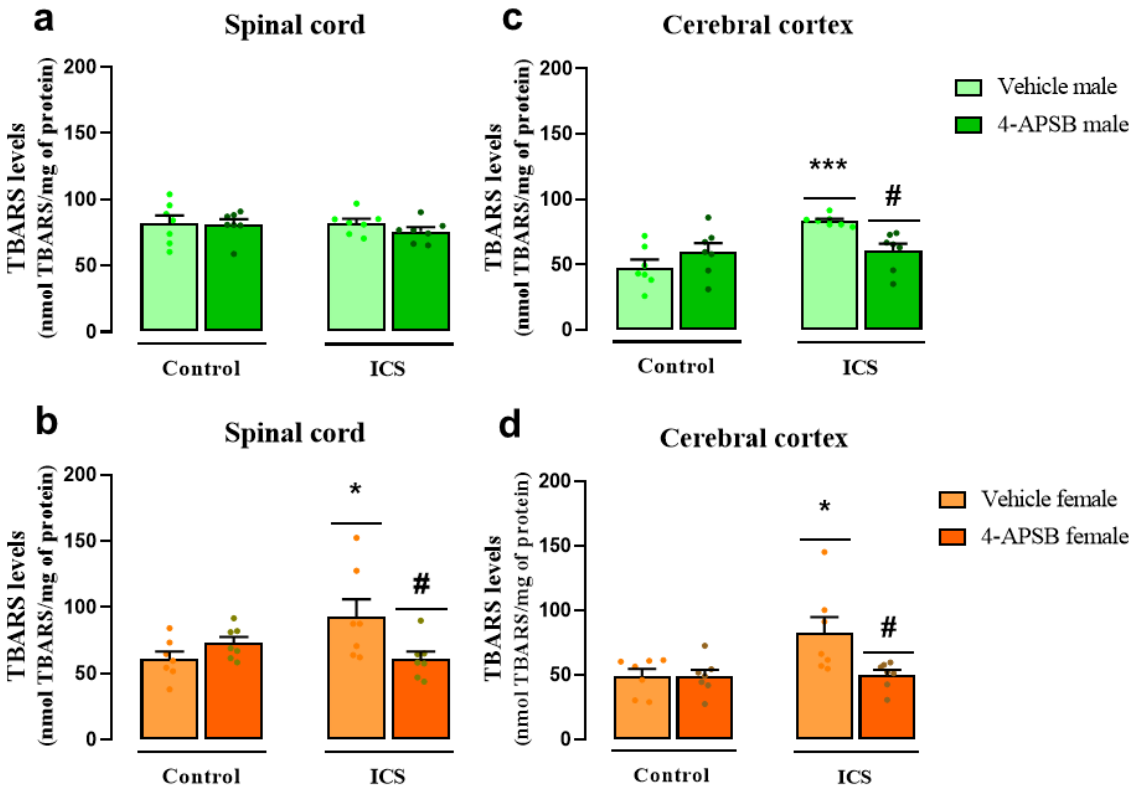

Fig. 7 Effect of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) treatment (1 mg kg^{-1}, i.g.) on the oxidative damage induced by ICS in the CNS of male and female mice. Thiobarbituric acid reactive substances (TBARS) levels in the spinal cord of male (a) and female (b) mice. TBARS levels in the cerebral cortex of male (c) and female (d) mice. Data are expressed as the mean ± SEM of 7 animals per group. Asterisk denotes significant levels when compared with the control group: (***) $P < 0.001$ and (*) $P < 0.05$. Hashtag denotes significant levels when compared with the ICS group: ($^{\#}$) $P < 0.05$ (Two-way ANOVA followed by the Tukey test).

The NPSH content remained unchanged among all experimental groups in the spinal cord of male mice, as well as, in the cerebral cortex of mice of both sexes (Figures 8A, 8C and 8D). In contrast, a reduction in the NPSH levels, a non-enzymatic antioxidant

defense, was observed in the spinal cord of female mice exposed to ICS compared with control group. The repeated treatment with 4-APSB normalized this antioxidant biomarker in the spinal cord of ICS exposed female mice to the control levels (Figure 8B). Moreover, the results also demonstrated that the ICS exposure and/or 4-APSB treatment did not change the enzymatic activity of GPx in the spinal cord of mice of both sexes and in the cerebral cortex of male mice (Figures 8E – 8G). On the other side, the cerebral cortex of female mice exposed to ICS exhibited an enhancement in the GPx activity, whereas this increase was markedly suppressed by the treatment with 4-APSB (Figure 8H).

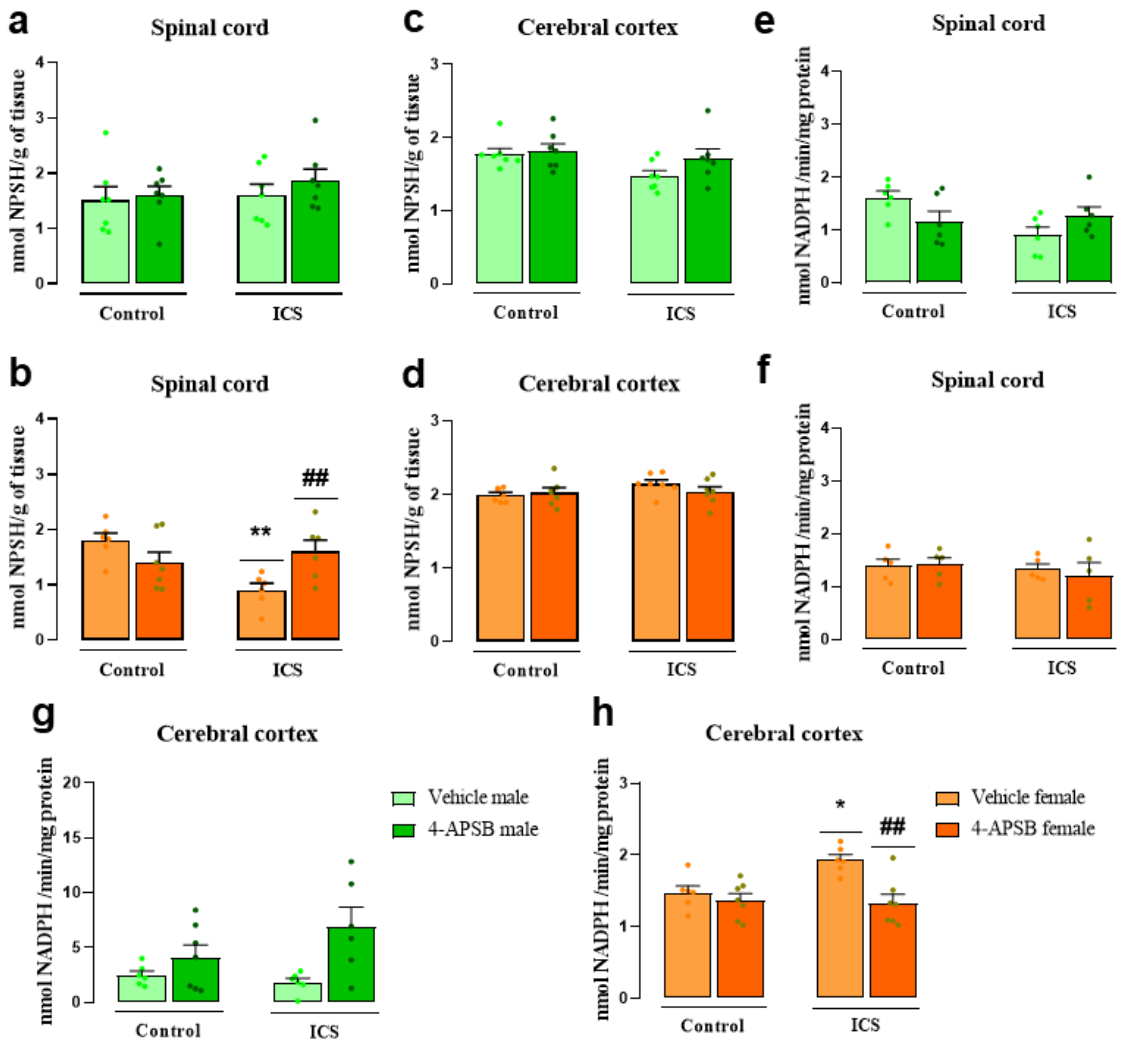

Fig. 8 Effect of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) treatment (1 mg kg^{-1}, i.g.) on the antioxidant defense system profile in the CNS of mice of both sexes exposed to ICS. Non-protein thiols (NPSH) content in the spinal cord of male (a) and female (b) mice. NPSH levels in the cerebral cortex of male (c) and female (d) mice. Glutathione peroxidase (GPx) activity in the spinal cord of male (e) and female (f) mice. GPx activity in the cerebral cortex of male (g) and female (h) mice. Data are expressed as the mean ± SEM of 7 animals per group. Asterisk denotes significant levels when compared with the control group: (*) $P < 0.05$. Hashtag denotes significant levels when compared with the ICS group: ($^{\#\#}$) $P < 0.01$ and ($^{\#}$) $P < 0.05$ (Two-way ANOVA followed by the Tukey test).

In order to further reinforce the antioxidative potential of the compound 4-APSB, the redox signaling pathway-related genes, including Nrf2, HO-1 and NFκB, were estimated in the cerebral cortex of male and female mice exposed to ICS (Figure 9). The results showed that the ICS exposure significantly upregulated NFκB and Nrf2 mRNA expression levels in the cerebral cortex of male and female mice, when compared with those of control groups. The 4-APSB treatment suppressed the increased levels of NFκB and Nrf2 mRNA expressions in the cerebral cortex of mice of both sexes exposed to ICS (Figures 9A - 9D).

On the other side, neither ICS exposure nor 4-APSB treatment promoted any significant change in the levels of HO-1 mRNA expression in the cerebral cortex of male and female mice (Figures 9E and 9F).

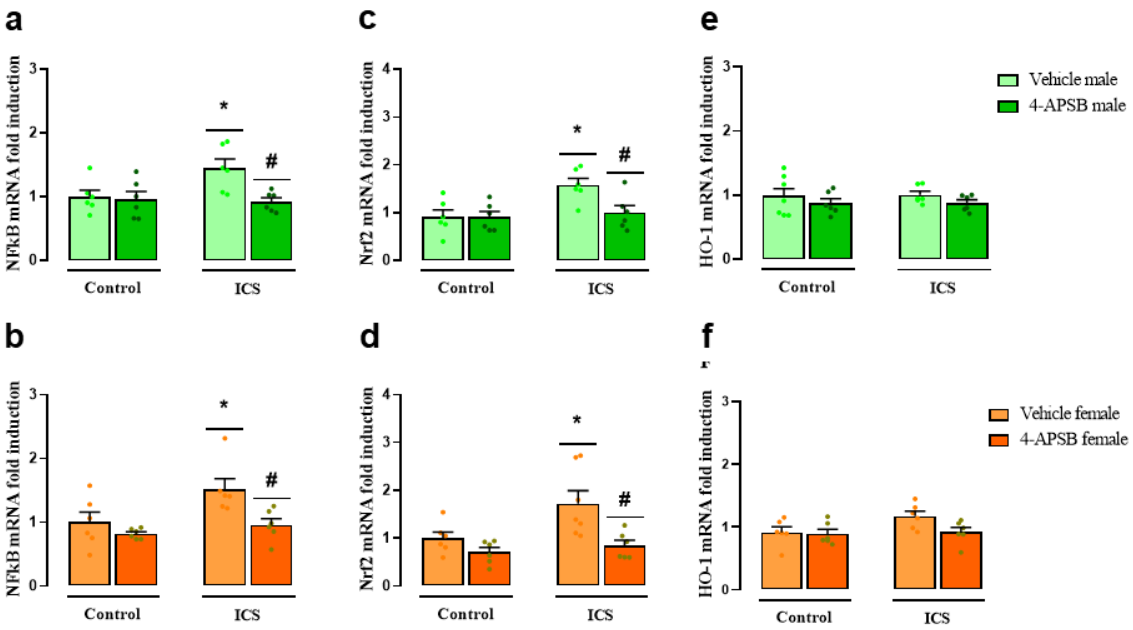

Fig. 9 Effect of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) treatment (1 mg kg^{-1}, i.g.) on the redox signaling pathway-related genes in the cerebral cortex of mice of both sexes exposed to ICS. mRNA expression levels of nuclear factor kappa B (NFκB) in the cerebral cortex of male (a) and female (b) mice. mRNA expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) in the cerebral cortex of male (c) and female (d) mice. mRNA expression levels of heme oxygenase-1 (HO-1) in the cerebral cortex of male (e) and female (f) mice. Data are expressed as the mean ± SEM of 6 animals per group. Asterisk denotes significant levels when compared with the control group: (*) $P < 0.05$. Hashtag denotes significant levels when compared with the ICS group: ($^{\#\#}$) $P < 0.01$ and ($^{\#}$) $P < 0.05$ (Two-way ANOVA followed by the Tukey test).

3.5 Correlation between Nrf2 and NFκB mRNA expression levels after ICS exposure

The data depicted in Figure 10 showed the correlation coefficient between the NFκB and Nrf2 mRNA expression levels in the cerebral cortex of mice exposed to ICS

(r = 0.830; P < 0.0001), using Pearson's correlation test. The result indicates that the parameters evaluated exhibited a significant positive correlation.

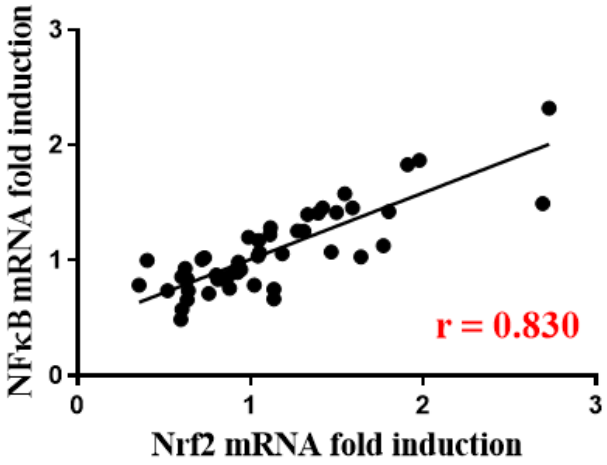

Fig. 10 Correlation between NFκB and Nrf2 mRNA expression levels after the ICS exposure in mice. The data was analyzed by Pearson's correlation coefficient (r) between the NFκB and Nrf2, expressed as mRNA fold induction, in the cerebral cortex of male and female mice exposed to ICS. The P value was used to validate the correlation between these variables.

3.6 4-APSB treatment modulates the imbalance in neuronal ionic gradient induced by ICS in male and female mice

The Figure 11 illustrate the effects of ICS exposure and/or the 4-APSB treatment on the neuronal ionic gradient throughout the Na^+, K^+ - ATPase and Mg^{2+} - ATPase activities in the CNS in male and female mice. The results demonstrated that the exposure to ICS and/or 4-APSB treatment did not promote any alteration in the Na^+, K^+ - ATPase activity within the CNS of male mice (Figures 11A and 11C). In contrast, an impairment in the enzymatic activity of Na^+, K^+ - ATPase was evidenced in the spinal cord and cerebral cortex of female mice exposed to ICS, when compared with the control group. As

expected, the treatment with 4-APSB in female mice exposed to ICS significantly reversed the inhibition of Na^+, K^+ - ATPase activity in the CNS in comparison with the ICS group (Figures 11B and 11D).

Regarding another ionic pump, the activity of Mg^{2+} - ATPase in the CNS of female mice was similar among all experimental groups (Figures 11E and 11F) whereas the ICS exposure triggered an increase in the activity of this enzyme in the spinal cord and cerebral cortex of male mice when compared with the control group (Figures 11G - 11H). On the other side, the 4-APSB treatment did not restore the Mg^{2+} - ATPase activity in the CNS of male mice exposed to ICS.

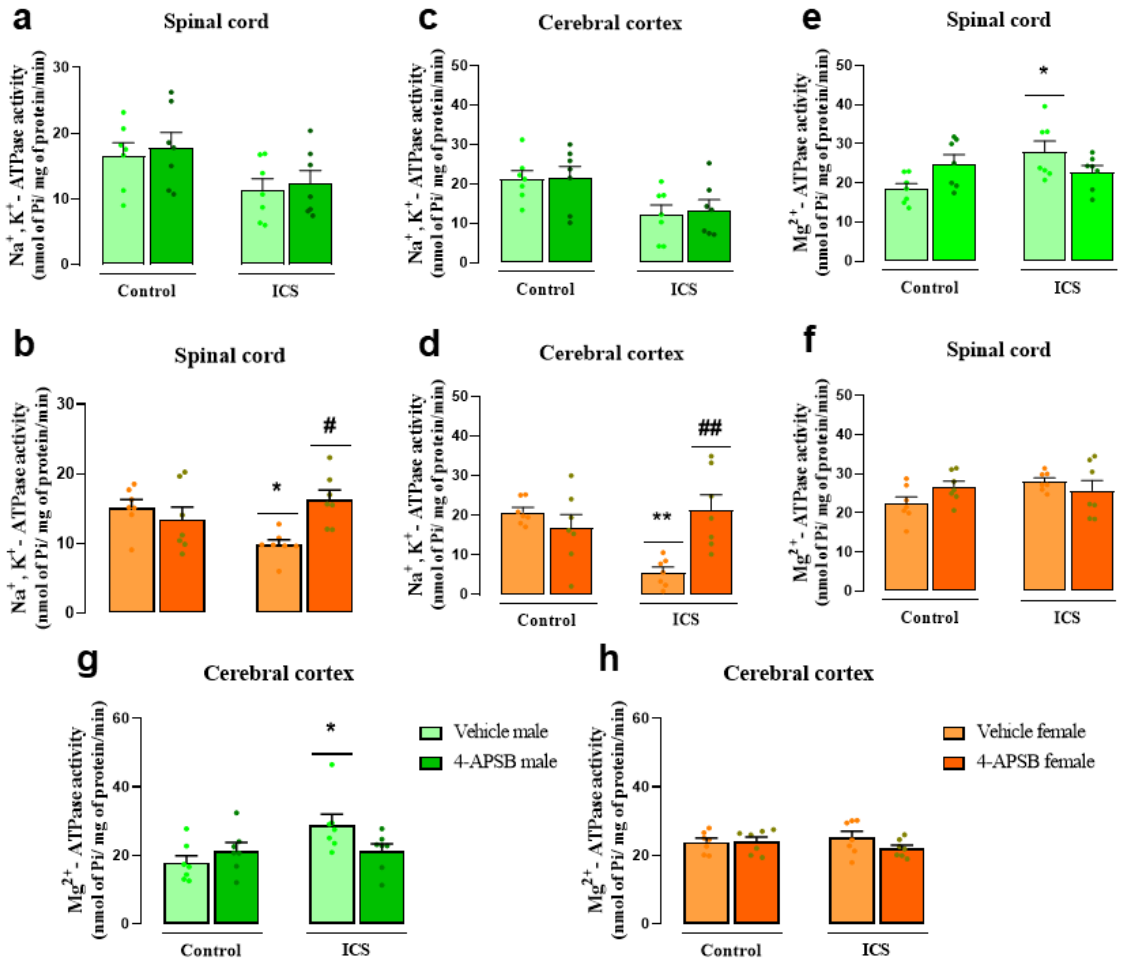

Fig. 11 Effect of 4-amino-3-(phenylselanyl) benzenesulfonamide (4-APSB) treatment (1 mg kg^{-1}, i.g.) on neuronal ionic gradient imbalance induced by ICS in mice of both sexes.

Na^+, K^+ - ATPase activity in the spinal cord of male (a) and female (b) mice. Na^+, K^+ - ATPase activity in the cerebral cortex of male (c) and female (d) mice. Mg^{2+} - ATPase activity in the spinal cord of male (e) and female (f) mice. Mg^{2+} - ATPase activity in the cerebral cortex of male (g) and female (h) mice. Data are expressed as the mean ± SEM of 7 animals per group. Asterisk denotes significant levels when compared with the control group: (**) $P < 0.01$ and (*) $P < 0.05$. Hashtag denotes significant levels when compared with the ICS group: ($^{\#\#}$) $P < 0.01$ and ($^{\#\#}$) $P < 0.05$ (Two-way ANOVA followed by the Tukey test).

4. Discussion

Fibromyalgia is recognized as a painful refractory syndrome, marked by diffuse fatigue and sensitivity, related to the development of psychological disturbances that negatively impact the patients' quality of life [39]. Because there is no established specific pathophysiology for this disease, an appropriate diagnosis or even treatment persist as a challenge. In the present study, we demonstrated that 4-APSB, a novel organoselenium compound, alleviated the nociceptive signs and a depressive-like phenotype in male and female mice exposed to ICS, an experimental model of fibromyalgia. Moreover, biochemical analyses performed in this study provided evidence that the treatment with 4-APBS reestablished the Na^+, K^+ - ATPase function as well as it modulated some markers related to the cellular redox and inflammatory status in the cerebral cortex and spinal cord of mice exposed to ICS.

Herein, the ICS exposure effectively evoked long-lasting mechanical and thermal sensitivities, as well as a muscle strength loss in male and female mice. Clinically, the major symptoms of fibromyalgia, including pain, rigidity and tender points in the body, occur as a result of a disfunction in several brain regions, especially in those involved in

pain signaling, probably due to the development of central sensitization [40]. Moreover, pain and emotions have a bidirectional relationship, as negative emotions could increase pain sensation or vice-versa [41].

In this line, fibromyalgia is frequently related to comorbid mood disorders [8]. In this study, male and female mice exposed to ICS exhibited a depressive like-phenotype, as evidenced by a decrease in the latency for the first episode of immobility and an increase in the immobility time in the tail suspension test. A deficit in the grip test, commonly used to measure forelimb motor function, has been previously related to depression because it could show the unwillingness of the mouse to persevere [42]. Therefore, the ICS model shares the characteristic sings and the comorbidity associated to fibromyalgia.

The findings of the present study showed that a single administration of 4-APSB attenuated the nociceptive signs induced by ICS in mice of both sexes, except the thermal sensitivity in male mice. This data suggests that sex affected the antinociceptive response exerted by this compound against a thermal stimulus, which requires a longer period of treatment in male than in female mice to obtain the same effect. Although the 4-APSB treatment decreased the depressive-like behavior in mice of both sexes exposed to ICS, its only increased the latency for the first episode of immobility in male mice. It should be noted that neither ICS exposure, nor 4-APSB treatment altered the locomotor and exploratory activities in mice of both sexes.

Besides that, male mice treated with this organoselenium compound and exposed to ICS exhibited a higher muscular strength than control group. In this sense, the increase in the muscular strength observed might be influencing the responses of male mice in the tail suspension test, which could reflect the resilience of mouse to persevere. Importantly, our results highlighted that the 4-APSB treatment improved the major behavioral signs

altered by ICS in mice, regardless the sex, and may be a promisor and useful strategy for the fibromyalgia management.

The pathophysiology of fibromyalgia is considered highly complex and varied. Indeed, a relationship between excessive oxidants levels and the reduction in the antioxidant defenses at central and peripheral levels is well-stablished with the development of fibromyalgia symptoms, including pain and muscle fatigue [43, 44]. In agreement, our results showed the ICS exposure triggered a lipid peroxidation, as evidenced by an increase of TBARS levels in the cerebral cortex of mice of both sexes as well as in the spinal cord of female mice. The lipid peroxidation products can easily form adducts with the cell components, especially peptides and proteins, hence promoting disturbances in cell signaling and metabolism [45].

As a compensatory mechanism, it was evidenced an increase in the GPx activity accompanied by a reduction of the NPSH content in the cerebral cortex and the spinal cord of female mice exposed to ICS, respectively. A decay in the NPSH content could be explained due to a greater consumption of glutathione (GSH), the major non-protein thiol quantified in this assay. In addition, studies have found that the Nrf2 promotes the expression of antioxidant response elements, including superoxide dismutase (SOD), GSH, GPx and HO-1 biosynthesis, in order to detoxify the oxidative treats and inflammatory cascades within the cells [46, 47]. In this sense, the ICS exposure promoted a higher mRNA expression levels of Nrf2 without changed the HO-1 in the cerebral cortex of male and female mice.

Taken together, the present study provided evidences that an ensemble of enzymatic and non-enzymatic antioxidant defenses was stimulated, in an attempt to counteract the increase in the lipid peroxidation and the oxidative damage triggered by ICS within the CNS, especially in females but also in male mice. The most active estrogen

circulating, 17β-estradiol, binds to estrogen receptors ERα and ERβ that mediate some physiological responses related to oxidative stress. In the cytoplasm, the ERs may stimulate the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA)/ cAMP response element-binding protein (CREB) cascade. In turn, CREB can promote the expression of antioxidant proteins [48–50]. Interestingly, 17β-estradiol may also directly upregulate the expression of Nrf-2, through the ERα [51]. In this study, the different responses to oxidative stress observed in male and female mice may be explained by the estrogen function in regulation of the redox state.

Notably, our data also demonstrated that 4-APSB treatment modulated TBARS and NPSH levels, GPx activity and Nrf2 mRNA expression levels in the cerebral cortex and spinal cord of male and female mice exposed to ICS, indicating that this compound exhibited an antioxidant effect at central levels that was able to maintain the cellular redox homeostasis, sex-independently. Consistent with our findings, a previous study reported that 4-APSB elicited a promising antinociceptive effect in an acute inflammatory nociceptive model, mainly through its ability to modulate the oxidative stress on the local injury [22].

The activation of NFκB has been involved in the progression of painful conditions and mood disorders because it regulates the transcription of several proinflammatory mediators [52, 53]. In line with this, our data revealed a higher NFκB mRNA expression levels in the cerebral cortex of male and female mice exposed to ICS. Indeed, Kaur et al. (2019) reported that, via N-methyl-D-aspartate (NMDA) receptors, the NFκB activation may induce the expression of pro-inflammatory proteins and hence, mediated the neuronal degeneration process in the CNS of reserpine-treated mice, another animal model of fibromyalgia [10]. Of particular importance, the treatment with 4-APSB reversed the increase of NFκB mRNA expression levels in the cerebral cortex of mice

exposed to ICS, suggesting that the sulfonamide moiety, present in some nonsteroidal anti-inflammatory drugs (NAIDs), into the backbone of this organoselenium compound may contribute to its anti-inflammatory effect, through the suppression of NFκB signaling.

A crosstalk between Nrf2 and NFκB signaling pathways has been documented under stress and a diversity of pathologies [54]. Despite the most studies proposed that the Nrf2 and NFκB pathways inhibit each other at their transcription level, some authors suggest that both nuclear factors may be activated reciprocally. The mutual stimulation of Nrf2 and NFκB pathways could be explained for two reasons: the oxidative stress promoted the backward-activation of NFκB, favoring the Nrf2 migration into the nucleus to protected the cells from inflammation and oxidative injury. On the other side, the Nrf2 may enhanced the proteasome activity leading to the IκB degradation and hence, the release of NFκB from cytoplasm to nucleus [55–57]. Indeed, as previously reported, an oxidative environment may induce the release of these nuclear factors from their repressors, stimulating both Nrf2 and NFκB downstream pathways [45].

According to these evidences and the data obtained in this study, it is plausible to suggest that the ICS exposure produced an elevated amount of the lipid peroxidation products that might trigger a higher NFκB and Nrf2 expression levels in the cerebral cortex. Therefore, such biochemical alterations may be associate with the development of a nociceptive and a depressant-like behaviors in male and female mice. In fact, the interconnection between Nrf2 and NFκB pathways has been poorly explored in fibromyalgia. In this case, we highlighted that one of the most relevant findings of our study is that the interplay of these nuclear factors, evidenced by a positive correlation between Nrf2 and NFκB mRNA expression levels, could be a mechanism underlying ICS induced adaptative behaviors in mice, similar to fibromyalgia.

The enzyme Na^+, K^+ - ATPase has a crucial relevance in several aspects of the neuronal function because it might affect directly or indirectly the neurotransmitter release processes, the signaling transmission and neurogenesis [16]. The data of the present study revealed that only female mice exposed to ICS exhibited an inhibition of the Na^+, K^+ - ATPase activity in both cerebral cortex and spinal cord. In line with our evidence, it is well documented that a lipid environmental can disrupted the neuronal membrane integrity and fluidity, affecting the activity of this enzyme in the CNS [58]. Also, the inhibition of Na^+, K^+ - ATPase activity may induce hyperexcitability due to the large Na^+ influx into axons, resulting in a vicious cycle of reactive species (RS) generation, increased Ca^{2+} membrane permeability, mitochondrial dysfunction and neurodegeneration [59, 60].

Further, we also demonstrated that only male mice exposed to ICS elevated the Mg^{2+}-ATPase activity in the cerebral cortex and in the spinal cord. Importantly, Mg^{2+} ions have been reported as a relevant cofactor for Na^+, K^+ - ATPase activity [61]. In view of this, we hypothesized that the increased Mg^{2+}-ATPase activity in the CNS, especially in male mice exposed to ICS, may occur as a compensatory mechanism in order to maintain the electrochemical gradient homeostasis in the neurons. It is known that Mg^{2+} regulates the redox balance and all metabolic pathways [62]. In this sense, disturbances in the Mg^{2+} concentration might be related to a higher amount of RS production and the upregulation of pro-inflammatory pathways, thereby contributing for the development of many diseases, including peripheral neuropathy [62–64]. However, we emphasize that further studies are necessary to better elucidate the relationship between the sexual dimorphism and the activity of Na^+, K^+ - and Mg^{2+}- ATPases, after the ICS exposure.

In general, our findings clearly indicate that alterations in the Mg^{2+}, Na^+ and K^+ concentrations in the CNS also contribute to the onset of nociceptive and depressive-like

signs associated with fibromyalgia. On the other side, the treatment with 4-APSB promoted an enhancement in the Na^+, K^+ - ATPase activity in the spinal cord and cerebral cortex of female mice, without affecting the activity of Mg^{2+}- ATPase in the CNS of male mice, after the ICS exposure. Interestingly, our results reinforce that ICS exposure caused distinct adaptations in the CNS of male and female mice which could explain the different effects exerted by the compound 4-APSB according to sex in this model. Cumulatively, the results of the current study provided evidences that the mechanisms underlying the compound 4-APSB mitigated the nociceptive signs and a depressive-like phenotype in mice of both sexes exposed to ICS might be related to its ability to restored the Na^+, K^+ - ATPase activity as well as the Nrf2 and NFκB transcription levels, mediated by a reduction of lipid peroxidation products within the CNS. Therefore, we highlight the beneficial effects of the 4-APSB treatment in an animal model of fibromyalgia.

Some studies also described the beneficial use of different coumarins and monoterpenes in experimental models of fibromyalgia [10, 65–67]. After a detailed revision and comparing the pharmacological effects of the compound 4-APSB with other natural products in animal models of fibromyalgia, we can infer that this organoselenium compound exerted an antinociceptive and an antidepressant-like actions at the dose 10 times lower than the furanocoumarins [10, 67] reinforcing the pharmacological potential of this synthetic compound.

5. Conclusion

Therefore, the present study for the first time demonstrated that 4-APSB treatment attenuated mechanical and thermal sensitivities, increased the muscular strength and improved the depressive-like phenotype in mice of both sexes exposed to ICS. Pain and depression are considered the main complaints of patients with fibromyalgia and for this

reason, the antinociceptive and the antidepressant-like effects of 4-APSB are one of the most relevant findings of this study. In fact, compounds with dual-action have been emerged as an attractive alternative to treat physical and somatic symptoms of fibromyalgia, avoiding the polypharmacy and discontinuing the pharmacological treatment. Despite the treatment with 4-APSB exhibited clear behavioral differences regarding the sex, the compound exerted promising effects in this model of fibromyalgia induced by ICS in male and female mice. In this sense, our findings further reinforce that the compound 4-APSB, due to its antioxidant property, was able to restored the Na^+, K^+ - ATPase activity and modulated the Nrf2-NFκB axis, thereby resulting in the cellular redox homeostasis within the CNS. Such interactions between the structure of 4-APSB and those molecular targets might contribute to this compound improved the nociceptive signs and a depressive-like phenotype in mice of both sexes exposed to ICS.

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Author contributions

C.C.M., A.S.R., K.P.M., C.L. and E.A.W. conceived and designed the study. C.C.M., A.S.R. and K.P.M. conducted all behavioral tests and biochemical analyses. M.S., J.A.R., D. A. synthesized the compound. E.B.B and V.F.C. were responsible for performed the qRT-PCR assay. C.C.M. and E.A.W. wrote and reviewed the manuscript. E.A.W. supervised the study. All authors approved the final version of the manuscript.

Data availability

The dataset generated or analyzed during this study are included in this article. Materials are available upon request.

Compliance with Ethical Standards

Animal care and all experimental procedures were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80-23, revised in 1996) and in accordance with the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil (CEEA 28142-2019). All efforts were made to minimize the number of animals used and their suffering.

Conflict of interest

The authors declare that they have no conflicts of interest.

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4.3 CAPÍTULO 3

Os resultados deste capítulo da tese estão apresentados sob a forma de resultados preliminares Os itens Materiais e métodos, Resultados e Referências Bibliográficas encontram-se dispostos no próprio manuscrito.

Os dados na Tabela 4 demonstram que a exposição ao EFI causa distintas alterações na expressão de microRNAs e de seus genes alvos em camundongos machos e fêmeas, à nível central e periférico.

Tabela 4. Resumo dos principais resultados obtidos no Capítulo 3.

Análises *ex vivo*		**Machos**	**Fêmeas**
		EFI	EFI
miR-155-5p	Córtex cerebral	↑	↑
	Plasma	✕	✕
BDNF	Córtex cerebral	↓	↓
	Plasma	↑	↓
Nrf-2	Córtex cerebral	✕	✕
	Plasma	↑	✕
miR-338-3p	Córtex cerebral	✕	✕
	Plasma	↓	↑
TRPV1	Córtex cerebral	✕	✕
	Plasma	↑	↓

(↑) Aumento induzido pela exposição ao EFI; (↓) diminuição induzida pela exposição ao EFI; (✕) nenhuma alteração induzida pela exposição ao EFI.

MicroRNA expression profiles and pathological responses in fibromyalgia induced by intermittent cold stress in mice: the role of sex differences

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Materials and methods

Animals

The experiments were performed using male and female Swiss mice (25-35g, bred in house, two months old). The animals were housed in individual cages (3-4 animals per cage) at an acclimatized (22 ± 2 °C) room. Mice were maintained under a 12 h light/12 h dark cycle (the lights were turned on at 07:00 a.m.) with free access to food and fresh water. No previous procedure was performed on these mice. The experimental protocol was approved by the Committee on Care and Use of Experimental Resources of the Federal University of Pelotas (Brazil), affiliated to the National Council for the Control of Animal Experimentation, and registered under the number CEEA 28142-2019. All animal handling and experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 823, revised 1978) and International Guiding Principles for Biomedicals Research Involving Animals. All efforts were made to reduce the number of animals used in this study and to minimize their suffering.

Experimental procedures

Intermittent cold stress (ICS)-induced fibromyalgia model

The ICS model was developed and validated as an experimental mouse model of fibromyalgia by Nishiyori and Ueda (2008). Briefly, the animals (3-4 mice in each cage) were placed in a cold environment (4 ± 2 °C) at 4:30 p.m. on the first day (day 1), with feeding and filtered water *ad libitum*. In the next morning (10:00 a.m.), mice were transferred to a temperature room (22 ± 2 °C) during 30 minutes and then, they were put in the cold room again for 30 minutes. This process was repeated until 4:30 p.m. (day 2). Finally, the animals were allocated in the cold room overnight. The same procedure was performed on the next day of the experimental protocol (day 3). In the morning (10:00 a.m.) of the fourth day, following the completion of the ICS exposure, mice were returned and adapted to a normal room temperature (22 ± 2 °C) for at least 2 hours. Mice from the control group were kept at a constant temperature of 22 ± 2 °C during all four days of the ICS model (from 4:30 p.m. on day 1 to 10:00 a.m. on day 4).

Two hours after the adaptation period, the animals were anesthetized by adding isoflurane-soaked cotton balls into a clear glass cage until loss of consciousness and euthanized through cardiac puncture for blood collection from the heart ventricle. The samples of cerebral cortex were dissected, weighed and frozen at -80°C (Figure 1).

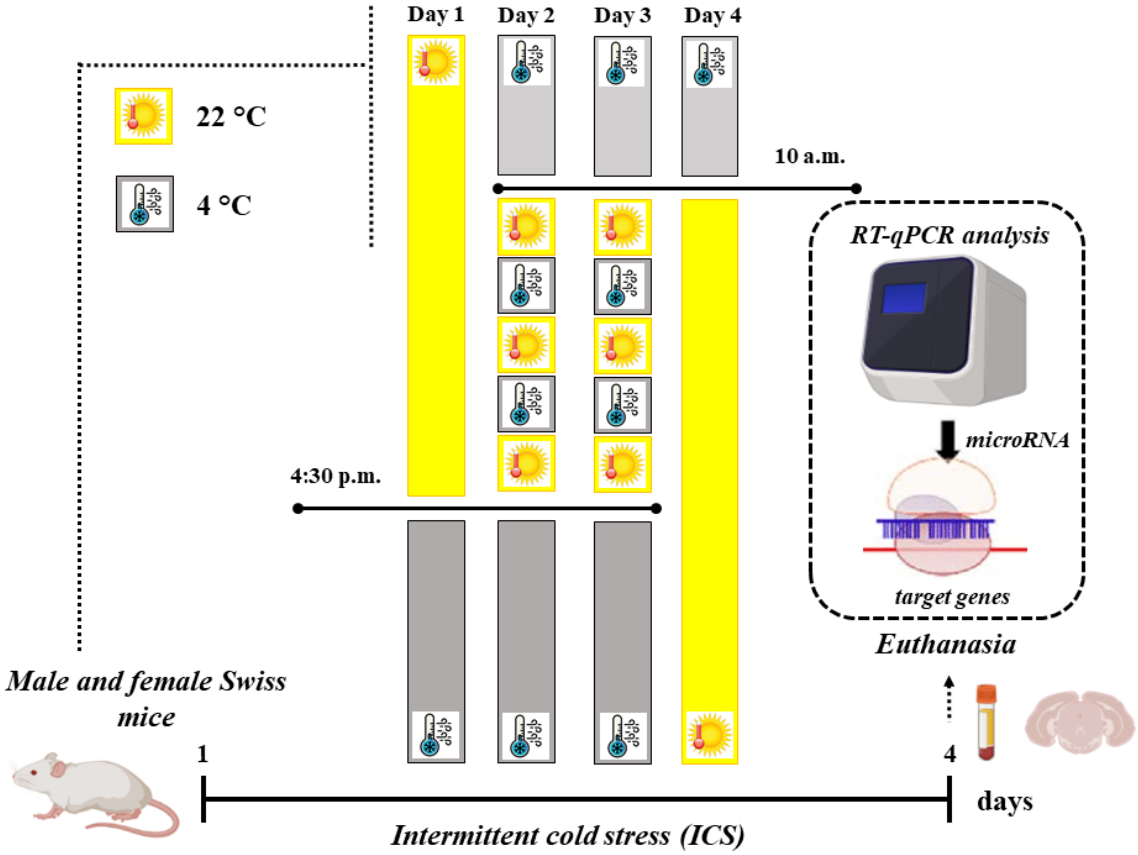

Figure 1: Schematic representation of the experimental design of this study.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from plasma and cerebral cortex samples using TRIZOL Reagent (Thermo Fisher Scientific, USA), following manufacturer instructions. RNA was then treated with DNase using DNAse-free kit (Ambion, USA) to remove genomic DNA contamination. Subsequently, the total RNA concentration isolated and its purity were measured using a NanoVue Plus spectrophotometer (GE Healthcare Life Science, USA), followed by storage at − 80 °C.

For the expression analysis of the predicted microRNAs, the complementary DNA (cDNA) was prepared using the stem loop RT primers and PCR was performed with the

microRNA-specific forward primer and the universal reverse primer, as mentioned by Singh et al. (2010). For the expression analysis of the target genes, the cDNA was synthetized using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to manufacturer instructions at a total final concentration of 750 ng of cDNA. Finally, it was stored at − 20 °C until further use.

For reverse transcription, 1 μg of total RNA was used in a reaction volume of 20 μl. The amplification was made with GoTaq® qPCR Master Mix (Promega, Madison, WI) using the LightCycler® 96 Real-Time PCR System (Roche Molecular Systems Inc., CA, USA). The primers used in this study were previously validated in mice. The specific stem loop primers for miR-155-5p and miR-338-3p were designed at the Laboratory of Structural Genomics. We also used specific primers for brain-derived neurotrophic factor (BDNF), nuclear factor erythroid 2-related factor 2 (Nrf-2) and transient receptor potential vanilloid 1 (TRPV1) previously described from literature. A complete list of the primer sequences used in the present study is shown in Table 1. The qPCR conditions were as follows: 10 minutes at 95°C to activate the hot-start Taq polymerase, followed by 35 cycles of denaturation for 15 seconds at 95°C, primer annealing for 60 seconds at 60°C, and extension for 30 seconds at 72°C (fluorescence signals were detected at the end of every cycle). Baseline and threshold values were automatically set by the LightCycler® 96 Software.

The number of PCR cycles required to reach the fluorescence threshold in each sample was defined as the Ct value. The $2^{-\Delta\Delta CT}$ method was used to calculate the fold change in gene expression and the data were normalized to the reference genes U6 (microRNA) or 18s (mRNA), (Livak and Schmittgen, 2001).

Table 1: Primers used for quantitative real-time polymerase chain reaction. Listed are the forward and reverse primer sequences used to amplify each target gene as well as the 18S endogenous control.

Primer Name	Sequence	Reference
miR-155-5p Loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG CACCAGAGCCAACACCCCT	-
miR-155-5p Forward	GGGGGTTAATGCTAATTGTGAT	
miR-155-5p Reverse	GTGCAGGGTCCGAGGT	
miR-338-3p Loop	CTCAACTGGTGTCGTGGAGTCGGCAATTCAG TTGAGCAACAAAA	-
miR-338-3p Forward	ACACTCCAGCTGGGTCCAGCATCAGTGATT	
miR-338-3p Reverse	GTGTCGTGGAGTCGGCAATTCAGTTGAG	
U6 Loop	AACGCTTCACGAATTTGCGT	-
U6 Forward	CTCGCTTCGGCAGCACA	
U6 Reverse	AACGCTTCACGAATTTGCGT	
BDNF Forward	GCTCACACTCCACTGCCCAT	Gremmelspacher et al. (2017)
BDNF Reverse	TCCCTGACCCATGCCAGAAGA	
Nrf-2 Forward	GATCCGCCAGCTACTCCCAGGTTG	Rabaneda-Lombarte et al. (2021)
Nrf-2 Reverse	CAGGGCAAGCGACTCATGGTCATC	
TRPV1 Forward	CGAGGATGGGAAGAATAACTCACTG	Wang et al. (2017)
TRPV1 Reverse	GGATGATGAAGACAGCCTTGAAGTC	
18S Forward	CCTGGATACCGCAGCTAGGA	Li et al. (2019)
18S Reverse	GCGGCGCAATACGAATGCCCC	

Statistical analysis

The experimental data are presented as the mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism (version 7.00, San Diego, USA). Previously, the values were tested by the D'Agostino and Pearson omnibus normality test and then the Student's *t*-test was performed to evaluate the differences between groups. Probability values less than 0.05 ($P < 0.05$) were considered statistically significant.

Results

The gene expression of miR-155-5p, BDNF and Nrf-2 in male and female exposed to ICS

The molecular analysis showed that the mRNA expression levels of miR-155-5p was significantly increase in the cerebral cortex of male and female mice exposed to ICS when compared with the control groups samples. The cortical mRNA expression levels of BDNF, a target gene of miR-155-5p, was found remarkably reduced after the ICS exposure in male and female mice in relation with the control groups. In contrast, cortical the mRNA expression levels of Nrf-2, another target gene of miR-155-5p, were similar between control and ICS groups in male and female mice (Figure 2A and 2B).

No significant change was observed in the mRNA expression levels of miR-155-5p in the plasma of animals submitted to ICS. In the plasma samples, the ICS exposure caused an upregulation of BDNF and Nrf-2 mRNA expression levels in male mice compared to control group. On the other hand, a reduction of BDNF mRNA expression levels was observed in female mice when compared with the control group, whereas no significant alteration in the mRNA expression levels of Nrf-2 was detected in female mice followed the ICS exposure (Figure 2C and 2D).

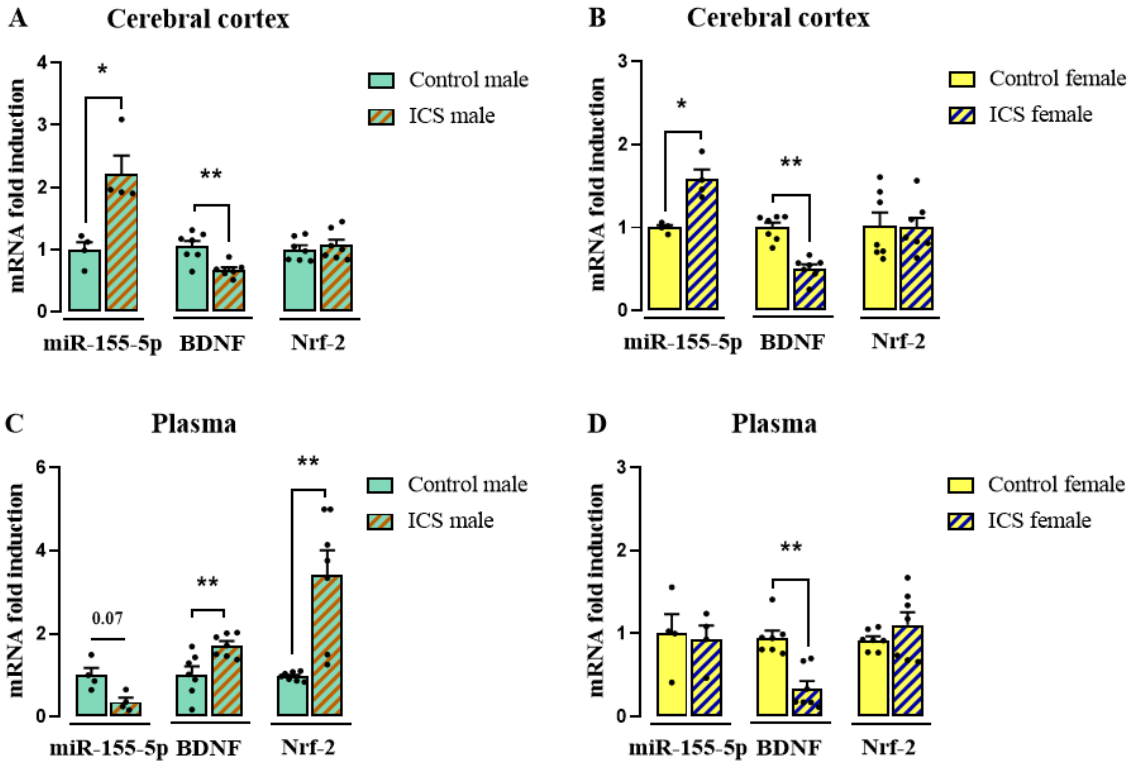

Figure 2: The gene expression of miR-155-5p, BDNF and Nrf-2 at central and peripheral levels of mice exposed to ICS. The mRNA expression levels of miR-155-5p, BDNF and Nrf-2 in the cerebral cortex of male (A) and female (B) mice. The mRNA expression levels of miR-155-5p, BDNF and Nrf-2 in the plasma of male (C) and female (D) mice. Data are present as the mean ± SEM (n = 4 animals per group for microRNA and n = 7 animals per group for gene expression). Asterisk denotes significant levels when compared with the control group: (**) $P < 0.01$ and (*) $P < 0.05$. The data obtained were analyzed using Student's *t*-test.

The gene expression of miR-338-3p and TRPV1 in male and female exposed to ICS

The RT-qPCR analysis showed that the ICS exposure did not cause any significant alteration in the mRNA expression levels of miR-338-3p and TRPV1 in the cerebral cortex of male and female mice, in relation to the control groups (Figure 3A and 3B). However, the miR-338-3p mRNA expression levels were reduced in the plasma samples of male mice exposed to ICS when compared with the control group. In turn, the mRNA expression levels of TRPV1 in the plasma of male mice were higher in the ICS group than that in the control group (Figure 3C). Differently from male mice, it was observed an enhancement of miR-338-3p mRNA expression levels, followed by a significant decrease of TRPV1 mRNA expression levels in the plasma of female mice exposed to ICS compared with the control group (Figure 3D).

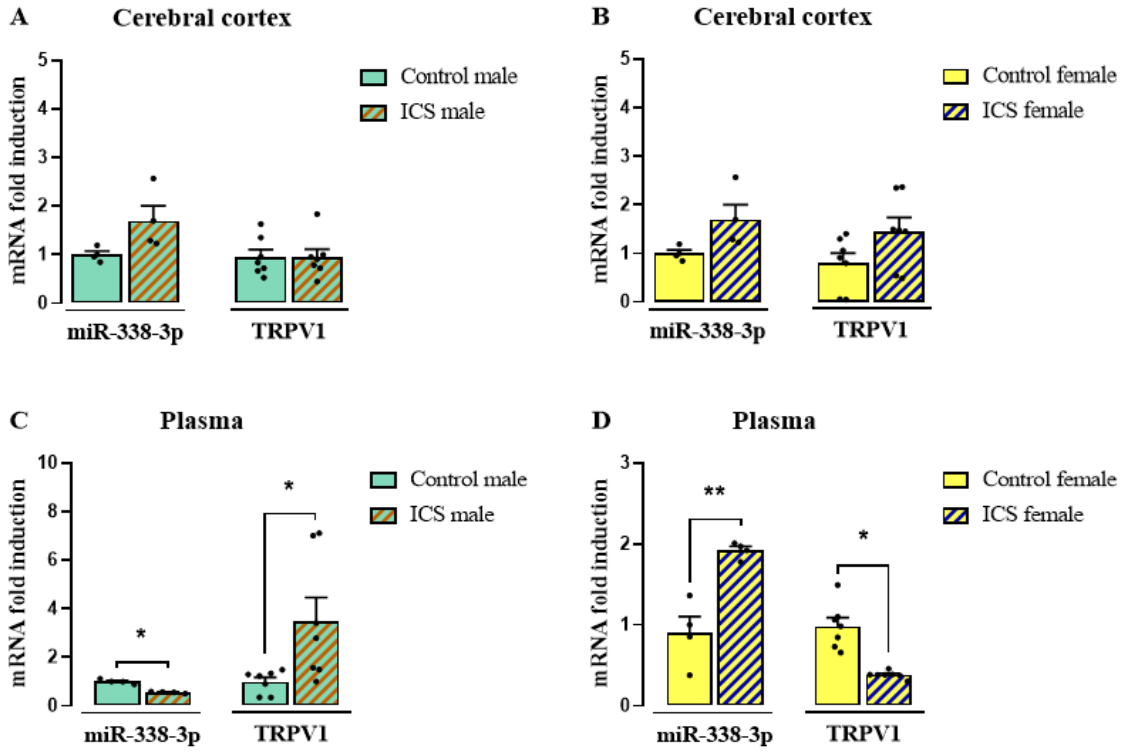

Figure 3. The gene expression of miR-338-3p and TRPV1 at central and peripheral levels of mice exposed to ICS. The mRNA expression levels of miR-338-3p and TRPV1 in the cerebral cortex of male (A) and female (B) mice. The mRNA expression levels of miR-338-3p and TRPV1 in the plasma of male (C) and female (D) mice. Data are present as the mean ± SEM (n = 4 animals per group for microRNA and n = 7 animals per group for gene expression). Asterisk denotes significant levels when compared with the control group: (**) $P < 0.01$ and (*) $P < 0.05$. The data obtained were analyzed using Student's *t*-test.

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Author contributions

C.C.M., A.S.R., K.P.M., E.N.D., N.M.G., V.F.C., C.L. and E.A.W. conceived and designed the study. C.C.M., A.S.R. and K.P.M. conducted the *in vivo* experiments. E.N.D., N.M.G. and V.F.C. were responsible for performed the qRT-PCR assay. C.C.M. and E.A.W. wrote and reviewed the manuscript. E.A.W. supervised the study. All authors approved the final version of the manuscript.

Data availability

The dataset generated or analyzed during this study are included in this article. Materials are available upon request.

Compliance with Ethical Standards

Animal care and all experimental procedures were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80-23, revised in 1996) and in accordance with the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil (CEEA 28142-2019). All efforts were made to minimize the number of animals used and their suffering.

Conflict of interest

The authors declare that they have no conflicts of interest.

5. DISCUSSÃO

Os resultados apresentados nessa tese ampliam o conhecimento sobre o papel do sexo nos mecanismos fisiopatológicos envolvidos na fibromialgia, no modelo do EFI utilizando camundongos machos e fêmeas. A partir das evidências obtidas no primeiro e segundo estudo, as propriedades farmacológicas do composto 4-APSB foram elucidadas nesse modelo, como uma promissora estratégia terapêutica para o tratamento da fibromialgia considerando as especificidades do sexo. Notavelmente, os resultados apresentados no primeiro estudo sugerem que o sexo modula diferentemente os mecanismos fisiopatológicos subjacentes à fibromialgia no modelo do EFI. De fato, evidências sugerem que as mulheres apresentam maior susceptibilidade às condições dolorosas crônicas do que os homens, tais como a fibromialgia e a síndrome da fadiga crônica (MOGIL, 2012). Estudos clínicos que induzem dor experimentalmente também comprovaram essas diferenças sexuais (AYESH et al., 2007; GARCIA et al., 2007; NISHINO et al., 2008). Além disso, as mulheres são mais sensíveis e menos tolerantes aos estímulos dolorosos do que os homens (SARLANI et al., 2007).

No primeiro estudo foi demonstrado que camundongos expostos ao EFI exibiram um aumento nas sensibilidades mecânica e térmica, assim como um déficit na força muscular, de forma semelhante em ambos os sexos. O estresse ao frio gerado pela baixa temperatura, associado a alta periodicidade de alterações de temperatura são responsáveis pelo desenvolvimento da alodínia estável e duradoura em camundongos (NISHIYORI e UEDA, 2008). Tais resultados reforçam que a exposição ao EFI desencadeia nocicepção generalizada em roedores, de modo análogo àquela observada nos quadros clínicos, contribuindo, assim, com a validade facial desse modelo.

Consistente com os achados de NISHIYORI e UEDA (2008), de que camundongos fêmeas são mais susceptíveis ao modelo do EFI, os nossos resultados também evidenciaram que camundongos fêmeas submetidos ao EFI apresentaram sensibilidade mecânica por um período mais prolongado do que em machos. Cabe ressaltar que o aumento na sensibilidade mecânica foi observado após a exposição ao EFI em camundongos de ambos os sexos, com uma recuperação progressiva ao longo dos dias. Entretanto, os camundongos

machos e fêmeas atingiram a resposta basal nos dias 16 e 26 após a indução, respectivamente. Apesar do relativo conhecimento sobre a diferença entre os sexos em estudos relacionados à fibromialgia, a influência do sexo sobre os mecanismos fisiopatológicos subjacentes a essa síndrome é consideravelmente escassa.

A fisiopatologia da fibromialgia é considerada complexa pois envolve variados processos neurobiológicos, os quais ainda não estão completamente elucidados. No entanto, há um consenso geral de que a sensibilização central promove adaptações nas vias responsáveis pelo processamento da dor, podendo, assim, ser uma das principais causas da fibromialgia (BORTOLATO et al, 2016). Diante dessa condição, os impulsos nociceptivos transmitidos para os neurônios da medula espinhal, amplificam a atividade dos neurônios glutamatérgicos. Particularmente, a estimulação exacerbada dos receptores NMDA pode alterar o funcionamento das cascatas de sinalização intracelular, resultando, portanto, na intensificação da percepção do estímulo doloroso (CHOY, 2015). Somando-se a isso, a depleção nos níveis das monoaminas biogênicas, serotonina e norepinefrina, pode desregular as vias nociceptivas descendentes facilitando, assim, a transmissão da dor (STAUD e RODRIGUEZ, 2006; NAGAKURA et al, 2009; PAREDES et al, 2019).

No primeiro estudo, as análises farmacológicas forneceram evidências de que os receptores serotoninérgicos 5-$HT_{1A/1B}$, 5-$HT_{2A/2C}$, 5-HT_3, assim como a cascata de sinalização do NMDA/ON/GMPc contribuem para o desenvolvimento da sensibilização central, resultando nos sinais nociceptivos induzidos pela exposição ao EFI em camundongos de ambos os sexos. Em contrapartida, a administração da L-arginina, o precursor do ON, em camundongos fêmeas expostos ao EFI reduziu a sensibilidade mecânica, enquanto que em camundongos machos não houve alteração nesse parâmetro nociceptivo quando comparados ao grupo exposto ao EFI. A partir desses dados, pode-se inferir que o envolvimento do ON no desenvolvimento da fibromialgia parece ser dependente do sexo. Portanto, o conjunto dos dados demonstrou que a via de sinalização do NMDA/NO/GMPc e os receptores serotoninérgicos contribuem para o desenvolvimento dos sinais nociceptivos induzidos pela exposição do EFI em camundongos machos e fêmeas.

Nos últimos anos, alterações na expressão de proteínas têm sido propostas como uma das principais causas da sensibilização central e periférica em pacientes que sofrem com condições dolorosas crônicas (ANDERSEN et al., 2014). Os microRNAs, por sua vez, podem desempenhar um papel crítico na patogênese de diversas doenças, uma vez que foram identificados como importantes moduladores da expressão gênica (SCHMIEDEL, et al., 2015; TANG et al., 2021). Contextualizando com a fibromialgia, a identificação de alterações na expressão de microRNAs e nos seus genes alvos pode fornecer uma melhor compreensão sobre os mecanismos patológicos envolvidos nessa doença.

Os resultados encontrados no terceiro estudo demonstraram um aumento na expressão gênica do miR-155-5p no córtex cerebral de camundongos de ambos os sexos expostos ao EFI. Dentre os microRNAs, o miR-155-5p participa de várias vias fisiológicas, incluindo inflamação e neuroplasticidade (FARAONI et al., 2009). Nesse sentido, também foi observado uma diminuição na expressão gênica do BDNF no córtex cerebral de camundongos machos e fêmeas expostos ao EFI. Diante das evidências, é plausível sugerir que este modelo experimental de fibromialgia induz um aumento na expressão do miR-155-5p, o qual pode estar relacionado com a reduzida expressão do mRNA que codifica o BDNF.

Em conformidade com os nossos resultados, LEE et al. (2018) reportaram que camundongos submetidos ao EFI apresentaram sinais nociceptivos e fenótipo do tipo depressivo, seguido do aumento nos níveis de corticosterona e da diminuição nos níveis proteicos do BDNF e da proteína de ligação ao elemento de resposta ao AMPc (CREB) à nível central (LEE et al., 2018). De acordo com esses dados, pode-se inferir que em modelos de fibromialgia mediados pelo estresse, o desenvolvimento dos sinais nociceptivos pode estar relacionado com a diminuição na via de sinalização do BDNF devido ao aumento nos níveis de corticosterona induzido pelo estresse.

Por outro lado, no terceiro estudo, demonstramos que a expressão do miR-155-5p não modula as alterações encontradas na expressão gênica do BDNF em camundongos submetidos ao EFI, à nível periférico. Enquanto a exposição ao EFI causa um aumento na expressão de BDNF no plasma de camundongos machos, foi observado uma redução na expressão desse gene em camundongos fêmeas. Diante do exposto, a diminuição na expressão gênica do BDNF, à nível central e periférico, em camundongos fêmeas pode ser

considerada um fator interessante para explicar a alta prevalência da fibromialgia em mulheres em todas as culturas.

Os resultados também demonstraram que a exposição ao EFI não alterou a expressão gênica do miR-338-3p e do TRPV1 no córtex cerebral de camundongos de ambos os sexos. Entretanto, no plasma de camundongos machos expostos ao EFI, foi detectado uma diminuição na expressão do miR-338-3p, acompanhado de um aumento na expressão do gene que codifica o TRPV1. Já as fêmeas submetidas ao EFI apresentaram um aumento na expressão do miR-338-3p, assim como uma diminuição na expressão do TRPV1 no plasma. De particular importância, uma redução na expressão do miR-338-3p já foi reportada no sangue de pacientes com fibromialgia (CERDÁ-OLMEDO et al., 2015). Os dados obtidos neste estudo confirmam que o modelo do EFI mimetiza, pelo menos em partes, os aspectos fisiopatológicos da fibromialgia. Vale salientar que o miR-338-3p também pode afetar outros genes alvos com respostas fisiológicas diferentes, incluindo genes relacionados ao sistema imune (MAHURKAR-JOSH et al., 2021).

De fato, estudos anteriores mostraram que a ativação do TRPV1 contribui para o desenvolvimento da hiperalgesia crônica em um modelo de fibromialgia induzido pela solução salina ácida em camundongos (YÜKSEL et al.,2017). O TRPV1 é um canal iônico permeável ao Ca^{2+} ativado por múltiplos estímulos sensoriais, como calor, prótons e vaniloides (JARA-OSEGUERA e ROSENBAUM, 2008). O TRPV1 é reconhecido como um importante mediador da dor porque também pode ser ativado por diferentes mediadores inflamatórios, incluindo prostaglandinas, bradicinina e o fator de crescimento nervoso (NGF) (CHUANG et al., 2001). Além dos mediadores inflamatórios, o NGF pode translocar o TRPV1 para a superfície da membrana celular e ativá-lo, por meio da sinalização do TRkA. No entanto, alguns estudos sugerem que o estrogênio reduz os níveis de mRNA de NGF e de TRkA em roedores e, assim pode suprimir a sinalização do TRPV1 (JI et al., 2002; ZHANG et al., 2005; SHANG et al., 2021). Dessa forma, a diferença na expressão gênica do TRPV1 observada entre os sexos pode ser explicada pela função do estrogênio em inibir a via do NGF/TRkA. Em conjunto, as evidências indicam que, independentemente do sexo, a expressão do miR-338-3p e do TRPV1 participam dos processos

patológicos envolvidos nesse modelo experimental de fibromialgia à nível periférico, mas não central.

Tendo em vista a complexidade e pouca compreensão sobre a fisiopatologia da fibromialgia, o tratamento aprovado pela FDA para essa síndrome inclui apenas medicamentos que exercem função neuromoduladora, tais como os inibidores da recaptação de serotonina/ norepinefrina (duloxetina e milnacepram) e os moduladores dos canais de cálcio (pregabalina). No entanto, a limitada eficácia desses fármacos em amenizar os sintomas da fibromialgia, bem como o aparecimento dos efeitos adversos, permite que muitos pacientes descontinuem o tratamento farmacológico (TZADOK e ABLIN, 2020).

Nesse sentido, a busca por estratégias terapêuticas mais eficazes para o tratamento da fibromialgia impulsionou o desenvolvimento do terceiro estudo, por meio do qual avaliou-se as propriedades farmacológicas do 4-APSB, um novo composto orgânico de selênio, em camundongos machos e fêmeas expostos ao EFI. Os resultados do segundo estudo demonstraram que o tratamento com o 4-APSB atenuou todos os sinais nociceptivos característicos da fibromialgia em camundongos de ambos os sexos. No entanto, a sensibilidade térmica de camundongos machos expostos ao EFI não foi alterada após uma única administração desse composto. Esse dado sugere que o efeito antinociceptivo exercido pelo 4-APSB frente a um estímulo térmico, parece ser dependente do número de administrações em machos, mas não em fêmeas.

Além disso, camundongos de ambos os sexos expostos ao EFI exibiram um fenótipo do tipo depressivo no teste da suspensão da cauda. Nesse contexto, a perda ou um déficit na função motora dos membros anteriores foi associada a depressão, uma vez que também poderia refletir na falta de vontade dos animais em perseverar (MOSER, 2011). Apesar de ter aumentado a latência para o primeiro episódio de imobilidade apenas em camundongos machos, o tratamento com 4-APSB aumentou o tempo de imobilidade em camundongos de ambos os sexos expostos ao EFI. Cabe ressaltar que os camundongos machos expostos ao EFI e tratados com o 4-APSB apresentaram maior força muscular do que os animais do grupo controle. Nesse sentido, esses resultados indicam que o aumento na força muscular, proporcionado pelo composto 4-APSB, poderia influenciar positivamente nas respostas ao teste da suspensão da cauda em camundongos machos.

As condições dolorosas crônicas, incluindo a fibromialgia, têm sido associadas ao desequilíbrio na sinalização redox, caracterizado pela produção anormal de marcadores oxidativos acompanhado pela depleção das defesas antioxidantes endógenas (SINGH et al, 2019). As evidências do primeiro estudo indicam que a exposição ao EFI desencadeou estresse oxidativo no córtex cerebral de camundongos machos, mas não no de fêmeas. Ainda, nenhuma alteração nos marcadores de estresse oxidativo (níveis de ER, de NPSH e a atividade da GPx) na medula espinhal foram evidenciadas em camundongos de ambos os sexos expostos ao EFI. Logo após a exposição ao EFI, sugere-se que o córtex cerebral de camundongos machos esteja mais vulnerável ao desenvolvimento do estresse oxidativo do que o das fêmeas.

Sob condições de estresse oxidativo, elevados níveis de ER induzem a produção do fator nuclear eritroide 2 relacionado ao fator 2 (Nrf-2) (KRYSZCZUK e KOWALCZUK, 2022). Diante do exposto, os resultados obtidos no terceiro estudo evidenciaram um aumento na expressão gênica do Nrf-2 no plasma de camundongos machos expostos ao EFI, mas não no de fêmeas. Particularmente em camundongos machos, é possível que o aumento na expressão do Nrf-2 nesse modelo animal de fibromialgia possa estar atrelado ao acúmulo de produtos do estresse oxidativo. Essas evidências, juntamente com os resultados obtidos no primeiro estudo, sugerem que a exposição ao EFI em camundongos machos provoca alterações em diferentes componentes do sistema redox que podem contribuir para o desenvolvimento dos sinais nociceptivos que mimetizam os da fibromialgia, tanto à nível central como periférico.

O miR-155-5p parece estar envolvido de forma direta ou indireta no estresse oxidativo, reduzindo ou neutralizando as ER (CHEN et al., 2019). No entanto, nenhuma alteração na expressão gênica do miR-155-5p no plasma de camundongos machos e fêmeas foi encontrada após a exposição ao EFI. Portanto, o conjunto dos dados sugere que o EFI, um modelo experimental de fibromialgia, promove alterações na expressão do Nrf-2 de forma dependente do sexo, as quais não tem relação com a expressão do miR-155-5p.

Ainda, um aumento na expressão gênica do miR-155-5p no córtex cerebral de camundongos machos e fêmeas expostos ao EFI foi observado. Entretanto, a expressão gênica do Nrf-2 não foi alterada nessa estrutura cerebral em camundongos de ambos os sexos, após a exposição ao EFI. Tais indícios

reforçam que, apesar do miR-155-5p contribuir para o desenvolvimento de diversas patologias por meio da regulação da via de sinalização do Nrf-2 (WAN et al., 2016; GU et al., 2017), o miR-155-5p não participa da modulação da expressão do Nrf-2, à nível central e periférico, neste modelo de fibromialgia induzido pela exposição ao EFI. Cabe salientar que o miR-155-5p pode ter como alvo vários outros genes que afetam principalmente o desenvolvimento e a progressão da inflamação (WANG et al., 2018).

De fato, a interação entre as vias de sinalização do Nrf-2 e do fator nuclear kappa B (NFκB), um regulador da resposta inflamatória, foi documentada em inúmeras patologias (KRAJKA-KUZNIAK e BAER-DUBOWSKA 2021). Assim, no segundo estudo foi demonstrado que, 6 dias após a exposição ao EFI, houve um aumento na expressão gênica do NFκB e do Nrf-2 no córtex cerebral de camundongos de ambos os sexos, indicando que há uma possível interação entre essas vias. Apesar da maioria dos estudos propor que as vias do Nrf-2 e do NFκB inibem uma à outra, alguns autores sugerem que ambos os fatores nucleares podem ser ativados reciprocamente. A estimulação mútua das vias do Nrf-2 e do NFκB pode ser explicada por dois motivos: o estresse oxidativo pode promover a ativação retrógrada do NFκB, favorecendo a migração do Nrf-2 para o núcleo para proteger as células da inflamação e da lesão oxidativa. Por outro lado, o Nrf-2 pode potencializar a degradação do IκB, o repressor do NFκB, por meio do aumento da atividade do proteassoma, facilitando assim a translocação do NFκB do citoplasma para o núcleo (BELLEZZA et al, 2010; WAKABAYASHI et al., 2010; AMBROZOVA et al., 2017). De extrema relevância, um ambiente oxidativo pode induzir a liberação desses fatores nucleares de seus repressores, estimulando as vias do Nrf2 e do NFκB (GEGOTEK e SKRZYDLEWSKA, 2017).

Dentro desse contexto, no segundo estudo foi demonstrado que, 6 dias após a exposição ao EFI, houve um aumento na peroxidação lipídica no córtex cerebral de camundongos machos, indicando dano oxidativo nessa estrutura. Também foi evidenciado o desenvolvimento do estresse oxidativo na medula espinhal e no córtex cerebral de camundongos fêmeas, 6 dias após a exposição ao EFI. De forma geral, a fibromialgia pode ser desencadeada por inúmeros estressores físicos e psicossociais, bem como pela maior vulnerabilidade ao estresse. Diante do exposto, as evidências desta tese sugerem que esse modelo de fibromialgia induzido pelo estresse pode afetar, primeiramente, as estruturas

cerebrais que sobrepõem as percepções da dor e do estresse, como o córtex cerebral, e, posteriormente, a região espinhal.

Além disso, esses dados também indicam que a exposição ao EFI altera a homeostasia da sinalização redox no córtex cerebral à curto e à longo prazo, resultando nos sinais nociceptivos e no fenótipo do tipo-depressivo em camundongos machos e fêmeas. Ainda, o desenvolvimento do estresse oxidativo na medula espinhal de camundongos fêmeas, 6 dias após a exposição ao EFI, poderia explicar o aumento na sensibilidade mecânica por um período mais prolongado em fêmeas do que em machos, assim como a prevalência da fibromialgia em mulheres. Portanto, o conjunto dos resultados reforçam que a exposição ao EFI desencadeia distintas adaptações no sistema nervoso central de acordo com as especificidades do sexo.

No segundo estudo, o tratamento com o composto 4-APSB modulou todos os marcadores de estresse oxidativo alterados no córtex cerebral e na medula espinhal de camundongos machos e fêmeas expostos ao EFI. Os resultados desse estudo, pela primeira vez, elucidaram os efeitos antinociceptivo e do tipo-antidepressivo do composto 4-APSB em uma modelo animal de fibromialgia, uma condição dolorosa crônica. Ainda, os dados presentes nesta tese reforçam as propriedades antioxidantes desse composto orgânico de selênio, uma vez que ele modulou o estresse e o dano oxidativo no SNC de camundongos machos e fêmeas expostos ao EFI.

A produção exacerbada de moléculas oxidantes inibe a atividade, reduz a expressão na membrana plasmática e oxida a proteína Na^+, K^+-ATPase (DADA et al, 2003; PETRUSHANKO et al, 2014). As evidências na literatura sugerem que tanto a diminuição da expressão, como a inibição da atividade da enzima Na^+, K^+-ATPase, por ser amplamente distribuída ao longo dos axônios, pode comprometer diretamente a condução do impulso axonal em direção às fibras nervosas, resultando em alterações na percepção da dor (KURNELLAS et al., 2005). Consistente com esses achados, os resultados do primeiro estudo demonstraram que a exposição ao EFI inibiu a atividade da Na^+, K^+-ATPase no córtex cerebral de camundongos machos.

Em contrapartida, foi observado um aumento na atividade dessa enzima no córtex cerebral e na medula espinhal de camundongos fêmeas expostos ao EFI. Alguns estudos propõem que o aumento na atividade da Na^+, K^+-ATPase

pode ocorrer devido a uma inibição na fosforilação da Na^+, K^+-ATPase mediada pela PKC, bem como pela ativação da calcineurina, resultando em um aumento na atividade dessa enzima (MARCAIDA et al., 1996; BERTUCCIO et al., 2007). SCAVONE et al (2005) evidenciaram que o glutamato estimula a ON sintase e a síntese de GMPc, os quais também poderiam ser responsáveis pelo aumento na atividade da Na^+, K^+-ATPase. Portanto, a desregulação na atividade da Na^+, K^+-ATPase, independentemente da direção, pode contribuir para o desenvolvimento dos sinais nociceptivos induzidos pelo EFI em camundongos machos e fêmeas.

No sengundo estudo foi evidenciado que, 6 dias após a exposição ao EFI, a atividade da enzima Na^+, K^+-ATPase estava inibida no córtex cerebral e na medula espinhal de camundongos fêmeas, mas não nos de machos. De acordo com os resultados obtidos nessa tese, é possível sugerir que a inibição dessa enzima em camundongos de ambos os sexos expostos ao EFI, pode ser atribuída ao desenvolvimento do estresse oxidativo no SNC. Contudo, esse modelo de fibromialgia induzido pelo estresse promove alterações no sistema redox em camundongos machos e fêmeas, de forma dependente do tempo. Diante do exposto, sugere-se que o SNC de camundongos fêmeas esteja mais susceptível ao desenvolvimento do estresse oxidativo do que o dos machos, após um período de sensibilização ao estresse. Essas diferenças bioquímicas observadas entre os sexos após a exposição ao EFI, também ajudam a entender porque a hipersensibilidade mecânica permanece por um período mais prolongado em fêmeas do que em machos.

Em relação a enzima Mg^{2+}-ATPase, foi evidenciado um aumento na atividade dessa enzima no SNC de camundongos machos expostos ao EFI, mas não no de fêmeas. É importante ressaltar que os íons Mg^{2+} já foram descritos como cofator para a atividade da Na^+, K^+-ATPase (APPEL et al., 2017). Diante disso, nós propusemos que o aumento da atividade da Mg^{2+}-ATPase no SNC, principalmente em camundongos machos expostos ao EFI, pode ocorrer como um mecanismo compensatório para manter a homeostase do gradiente eletroquímico nos neurônios.

Por outro lado, o tratamento com o composto 4-APSB restaurou a atividade da Na^+, K^+-ATPase aos níveis basais no córtex cerebral e na medula espinhal de camundongos fêmeas, sem afetar a atividade da Mg^{2+}-ATPase no SNC de camundongos machos, após a exposição ao EFI. Os nossos achados

reforçam a propriedade antioxidante deste composto, uma vez que ele foi capaz de modular a atividade de enzimas e a expressão de genes relacionados ao estresse oxidativo, assim como a atividade da Na^+, K^+-ATPase. Por meio dessas vias de sinalização, o composto 4-APSB atenuou os sinais nociceptivos e o fenótipo de tipo-depressivo em camundongos de ambos os sexos expostos ao EFI. Os nossos resultados também indicam que a exposição ao EFI causou distintas adaptações no SNC de camundongos machos e fêmeas, o que poderia explicar os diferentes efeitos exibidos pelo composto 4-APSB de acordo com o sexo neste modelo. Os efeitos da exposição ao EFI e do tratamento com o composto 4-APSB nos testes comportamentais e nas análises bioquímicas realizadas no córtex cerebral de camundongos machos e fêmeas estão ilustrados na Figura 5.

Diante dos indícios de que não só as alterações comportamentais podem ser influenciadas pelo sexo, mas também as vias de sinalização envolvidas nesse modelo de fibromialgia, esses dados são considerados fundamentais para uma reflexão sobre a limitada eficácia das opções terapêuticas disponíveis para o tratamento desta doença. Além disso, ressalta-se que apesar de ser evidenciado algumas diferenças entre os sexos, o composto 4-APSB apresentou efeitos farmacológicos promissores em camundongos de ambos os sexos exposto ao EFI. Portanto, torna-se necessário a realização de mais estudos para uma melhor compreensão sobre a influência do sexo nos mecanismos fisiopatológicos envolvidos na fibromialgia.

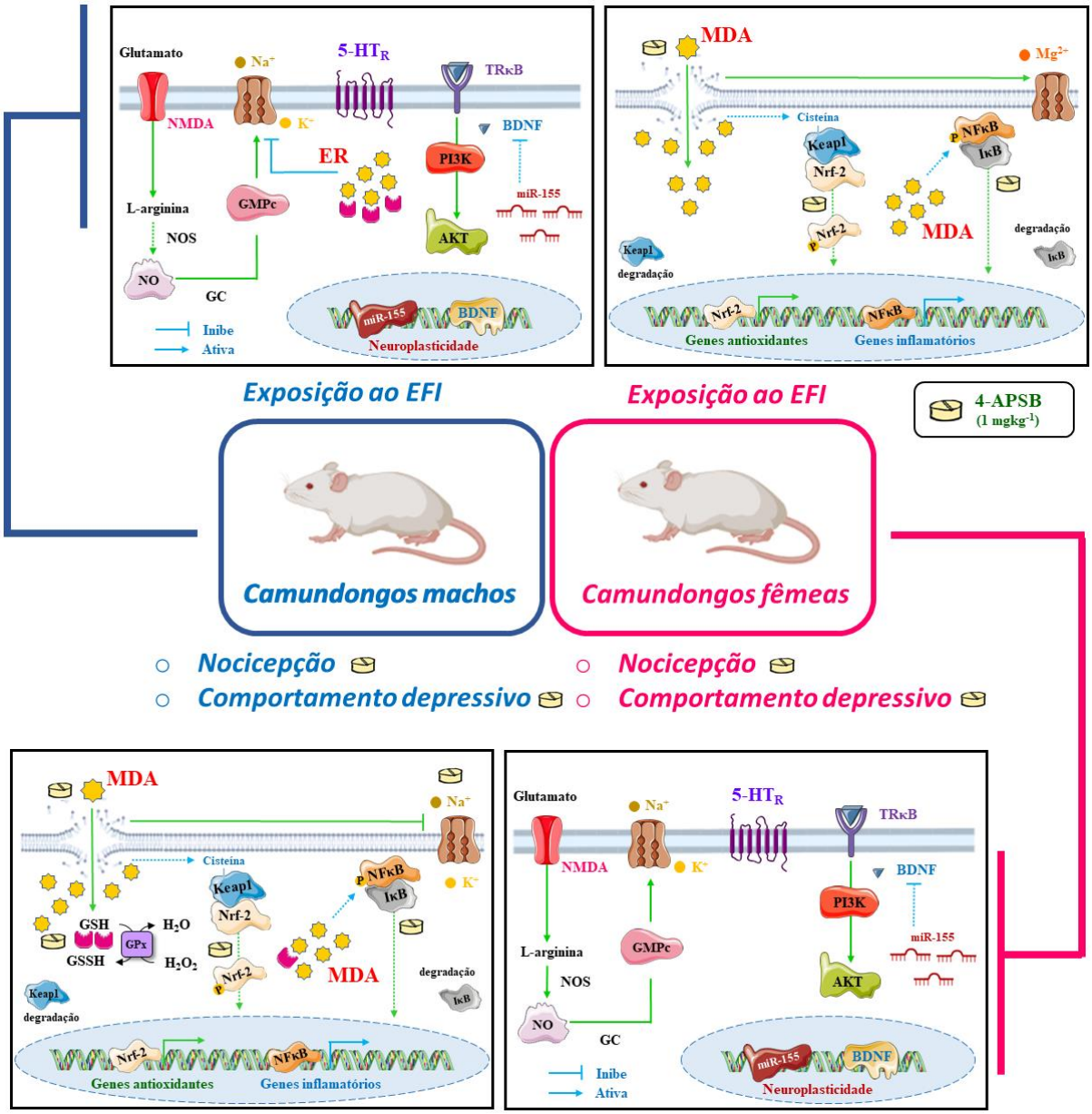

Figura 5. Representação esquemática dos principais resultados obtidos na tese. Efeitos da exposição ao EFI e do tratamento com o composto 4-APSB nos testes comportamentais e nas análises bioquímicas realizadas no córtex cerebral de camundongos machos e fêmeas.

6. CONCLUSÃO

Neste estudo foi demonstrado que a exposição de camundongos ao EFI induz alterações comportamentais, bioquímicas e moleculares, semelhantes às observadas em pacientes diagnosticados com fibromialgia. No entanto, há uma nítida diferença entre os sexos. A partir dos resultados obtidos foi possível elucidar que a exposição ao EFI desencadeia os sinais nociceptivos e o fenótipo do tipo-depressivo em camundongos de ambos os sexos, ou seja, os sintomas característicos dessa doença. Considerando que a prevalência da fibromialgia é maior em mulheres, o presente estudo proporcionou uma melhor compreensão sobre a influência do sexo nos mecanismos fisiopatológicos envolvidos nessa doença. Particularmente, os dados demonstraram que há uma diferença temporal para o desenvolvimento do estresse oxidativo em camundongos machos e fêmeas expostos ao EFI. Por sua vez, o desequilíbrio entre os marcadores oxidantes e o sistema antioxidante favoreceu a inibição da atividade da enzima Na^+, K^+-ATPase. Além disso, os receptores serotoninérgicos e a via de sinalização do NMDA/ON/GMPc parecem contribuir para o desenvolvimento da fibromialgia. Dentre os microRNAs analisados, o miR-155-5p e o miR-338-3p podem estar envolvidos nos processos fisiopatológicos dessa doença, ao modular a expressão gênica do BDNF e do TRPV1 à nível central e periférico, respectivamente. Entretanto, as diferenças sexuais podem interferir na expressão de microRNAs e de seus genes alvos neste modelo experimental de fibromialgia. Destaca-se que a compreensão sobre o dimorfismo sexual na sinalização neurológica está em estágio inicial, sendo que a maioria das informações são inconclusivas. Por outro lado, o tratamento baseado na administração do composto 4-APSB atenuou os sinais nociceptivos e o fenótipo do tipo-depressivo em camundongos machos e fêmeas expostos ao EFI. Além disso, os efeitos farmacológicos do 4-APSB parecem ser decorrentes de ações antioxidantes e anti-inflamatórias, sobretudo pela capacidade desse composto de restabelecer a atividade da enzima Na^+, K^+ -ATPase e a expressão gênica do Nfr-2 e do NFκB. De forma geral, os resultados indicam que o composto 4-APSB pode ser um bom protótipo para o tratamento da fibromialgia, independentemente do sexo.

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ANEXOS

ANEXO A

Carta de aprovação do protocolo experimental pela Comissão de Ética e Experimentação Animal da Universidade Federal de Pelotas

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº **61/2019/CEEA/REITORIA**
PROCESSO Nº 23110.028142/2019-16

Certificado

Certificamos que a proposta intitulada **"Investigação dos processos fisiopatológicos e das comorbidades envolvidas em um modelo de fibromialgia em camundongos machos e fêmeas."**, registrada com o nº 23110.028142/2019-16, sob a responsabilidade de **Ethel Antunes Wilhelm** - 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 **16/07/2019**.

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	01/08/2019 a 01/08/2024
Espécie/linhagem/raça	*Mus musculus*/Swiss e C57BL/6
Nº de animais	312 Swiss e 312 C57BL/6
Idade	60 dias
Sexo	312 machos (156 Swiss e 156 C57BL/6) e 312 fêmeas (156 Swiss e 156 C57BL/6)
Origem	Biotério Central - UFPel

Código para cadastro nº **CEEA 28142-2019**

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Documento assinado eletronicamente por **ANELIZE DE OLIVEIRA CAMPELLO FELIX**, **Médico Veterinário**, em 19/07/2019, às 14:54, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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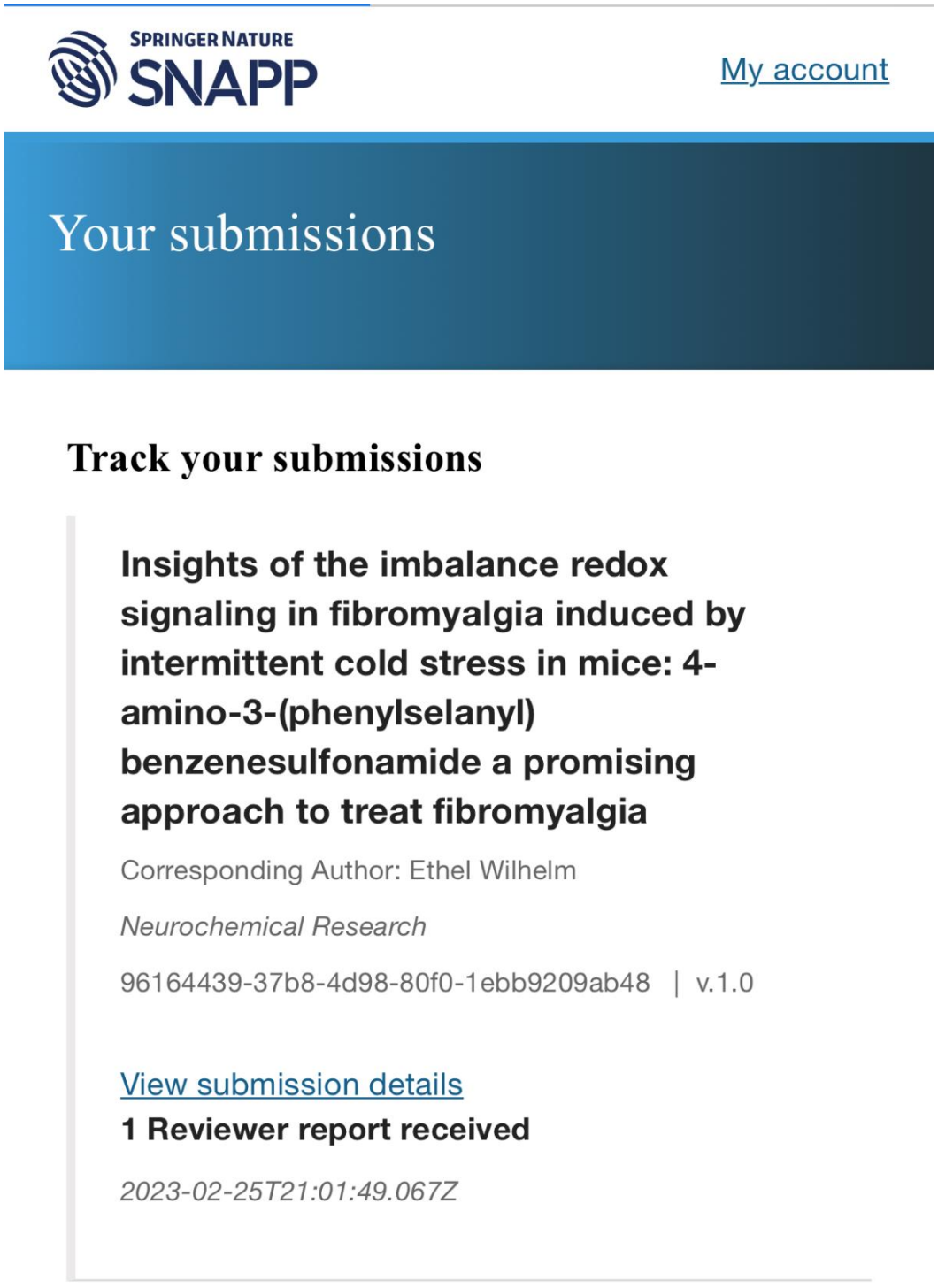
Referência: Processo nº 23110.028142/2019-16 SEI nº 0627723

ANEXO B

Comprovante de submissão do manuscrito científico intitulado: Insights of the imbalance redox signaling in fibromyalgia induced by inttermitent cold stress in mice: 4-amino-3-(phenylselanyl) benzenesulfonamide a promising approach to treat fibromyalgia



Progress so far

- Submission received
- Initial technical check
- Peer review

Your submission

Title

Insights of the imbalance redox signaling in fibromyalgia induced by intermittent cold stress in mice: 4-amino-3-(phenylselanyl) benzenesulfonamide a promising approach to treat fibromyalgia

Type

Research

Journal

Neurochemical Research

Submission ID

96164439-37b8-4d98-80f0-1ebb9209ab48