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ANA KAROLINA DE SANTANA NUNES BERNARDO

AVALIAÇÃO DOS EFEITOS DO INIBIDOR DE FOSFODIESTERASE-5 SOBRE OS MECANISMOS REGULATÓRIOS DA NEUROINFLAMAÇÃO, EM MODELO DE DESMIELINIZAÇÃO INDUZIDO EM CAMUNDONGOS C57BL/6 WILD TYPE E KNOCKOUT PARA iNOS

RECIFE
2016

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Aos meus Pais: José João e Cleide.

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“A tarefa não é tanto ver aquilo que ninguém viu,
mas pensar o que ninguém ainda pensou sobre
aquilo que todo mundo vê.”

Arthur Schopenhauer

RESUMO

O Sildenafil (Viagra®) é um inibidor potente e seletivo da fosfodiesterase-5 (PDE5), enzima responsável pela hidrólise do monofosfato de guanosina cíclico (GMPc). Tem sido demonstrado que Sildenafil tem potencial eficácia em desordens patológicas de carater neuroinflamatório e neurodegenrativo no sistema nervoso central (SNC). A inflamação cerebral é mediada/modulada por citocinas pró-inflamatórias e pelo NO e exerce um papel central em numerosas patologias do cérebro, incluindo a esclerose múltipla (EM). A EM é uma doença inflamatória crônica, com início típico durante os anos produtivos (entre 20 e 50 anos), caracterizada por desmielinização das células nervosas, que leva à severa deficiência psicomotora. Recentemente foi demonstrado que a administração de Sildenafil promoveu a inativação das células microgliais e astrocitária, reduziu os fenômenos inflamatórios e danos causados à mielina em modelo de EM. No entanto, o mecanismo de ação desse fármaco ainda é desconhecido. Desta forma, o presente estudo investigou os efeitos do Sildenafil sobre os mecanismos regulatórios do processso na neuroinflamação em modelo de desmielinização induzido por Cuprizona. Os resultados demonstraram que Sildenafil 25 mg/kg reduziu a expressão das citocinas inflamatórias IL-1β e TNF-α e aumentou a expressão da citocina anti-inflamatória IL-10. Em adição, o tratamento com Sildenafil reduziu a expressão de de GFAP (gliose), NFκB (fator de transcrição nuclear para citocinas), AMPK inativo (proteina reguladora de metabolismo) e iNOS (sintase de óxido nítrico induzível) e aumentou o IKBα (proteína inibitória NFκB). Os efeitos do sildenafil sobre a remielinização foi observado através de imagens por ressonância magnética, que demonstrou recuperação de tecido neuronal degenerado (corpo caloso). Além disso, o tratamento aumentou a expressão de MMP-9,MCP-1/CCR-2, contribuindo possivelmente para a troca de fenótipo microglial, que favorece a limpeza de debris mielínicos. Em paralelo, a integridade da mielina foi demonstrada através do aumento da marcação para oligodendrócitos maduros (GST-pi), proteína básica da mielina (MBP) e organização de lamelas mielínicas. Em camundongos knockout $iNOS^{-/-}$, Sildenafil reduziu os níveis de IL-1β, Iba-1, IFN-γ e não alterou a expressão de GFAP, TNF-α e COX-2. Sildenafil elevou os níveis GST-pi e melhorou a estrutura da mielina.Os efeitos do Sildenafil sobre as células astrocitárias demonstrou que o tratamento preventivo e terapêutico foram eficiente em reduzir a gliose astrocitária induzido por LPS. Além disso, os tratamentos com sildenafil promoveu diminuição das ondas de cálcio e do estresse fibras de actina. Diante disso, o presente estudo propõe que o mecanismo de ação envolve a via AMPK–eNOS-IKβα–NFκB, bem como ação de MMP-9 e MCP-1/ CCR-2 no processo de remielinização. O presente estudo abre novas possibilidades de investigação para o tratamento de doenças neurodegenerativas, tais como a esclerose múltipla

Palavras- chave: Desmielinização, Astrócito, Micróglia, Sildenafil, PDE-5.

ABSTRACT

Sildenafil (Viagra) is a potent and selective inhibitor of phosphodiesterase type 5 (PDE5), the enzyme responsible for the hydrolysis of cyclic guanosine monophosphate (cGMP). It has been shown that sildenafil has potential efficacy in pathological disorders and neuroinflammatory character neurodegenrativo the central nervous system (CNS). Cerebral inflammation is mediated / modulated by pro-inflammatory cytokines and NO by and plays a central role in numerous pathologies of the brain including multiple sclerosis (MS). MS is a chronic inflammatory disease with typical onset during the productive years (20 to 50), characterized by demyelination of the nerve cells, which leads to severe psychomotor impairment. It has recently been shown that sildenafil promoted inactivation of microglial cells and astrocytes, reduced inflammatory phenomena and damage to myelin in MS model. However, the mechanism of action of this drug is still unknown. Thus, the present study investigated the effects of Sildenafil on the regulatory mechanisms in the processso in neuroinflammation model of demyelination induced cuprizone. The results showed that Sildenafil 25 mg / kg reduced the expression of inflammatory cytokines IL-1β and TNF-α expression and increased anti-inflammatory cytokine IL-10. In addition, treatment with sildenafil reduced expression of GFAP (glial), NFκB (nuclear transcription factor cytokines), inactive AMPK (regulating protein metabolism) and iNOS (nitric oxide synthase) and increased IKBα (protein NFκB inhibitory). The effects of sildenafil on remyelination was observed by magnetic resonance imaging, which showed recovery of degenerated neuronal tissue (corpus callosum). Furthermore, the treatment increased the expression of MMP-9, MCP-1 / CCR-2, possibly contributing to the exchange of microglial phenotype, which favors the cleaning of myelin debris. In parallel, the integrity of the myelin has been demonstrated by increasing the marking to mature oligodendrocytes (GST-pi), myelin basic protein (MBP) and myelin lamellae organization. In knockout mice iNOS - / - Sildenafil reduced IL-1β levels, Iba-1, IFN-γ and did not alter the expression of GFAP, TNF-α and COX-2. Sildenafil raised the GST-pi levels and improved the structure of mielina.Os effects of sildenafil on astrocytic cells demonstrated that the preventive and therapeutic treatment were effective in reducing astrocytic gliosis induced by LPS. In addition, treatment with sildenafil promoted reduction of calcium waves and actin stress fibers. Thus, the present study suggests that the mechanism of action involves the AMPK-eNOS-IKβα-NFκB and MMP-9 activity and MCP-1 / CCR-2 in the remyelination process. This study opens new possibilities for research into the treatment of neurodegenerative diseases such as multiple sclerosis

Keywords: demyelination, astrocyte, microglia, Sildenafil, PDE-5.

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LISTA DE ABREVIATURAS E SIGLAS

AMPc	Adenosina Monofosfato Cíclico
AMPK	Proteína quinase ativada por AMP
Ang-1	Angiopoietina-1
BHE	Barreira hemato-encefálica
CCR-2	Quimiocina
CPZ	Cuprizona
DAMP	Padrões moleculares associados ao dano
EM	Esclerose múltipla
eNOS	Sintase de óxido nítrico endotelial
ERRO	Espécies reativas de oxigênio
GCs	Guanilato ciclase solúvel
GFAP	Proteína ácida fibrilar glial
GMPc	Guanosina Monofosfato Cíclico
ICAM	Molécula de adesão intercelular
IFN-γ	Interferon-γ
IKBα	Proteína inibitória fator de transcrição kappa B
IKK	Complexo IKK
IL-1β	Interleucina 1β
ILs	Interleucinas
iNOS	Síntase óxido nítrico induzível
LFB	Luxol fast blue
LPS	Lipopolissacarídeo
MBP	Proteína básica da mielina
MCP-1	Proteína quimioatraente de monócitos

Myd88	Gene de diferenciação mieloíde 88
NFκB	Fator de transcrição kappa B
nNOS	Sintase de óxido nítrico neuronal
NO	Óxido nítrico
NOS	Sintase Óxido nítrico
OL	Oligodendrócitos
OPC	Células precursoras de oligodendrócitos
PAMP	Padrões moleculares associados a patógenos
PDE5	Enzima fosfodiesterase tipo 5
PDEi	Iinibidor de enzimas fosfodiesterases
PKA	Proteína quinase dependente de AMPc
PKG	Proteína quinase dependente de GMPc
SNC	Sistema nervoso central
TLR	Receptor Toll Like
TNF-α	Fator de necrose tumoral-α
VCAM	Molécula de adesão vascular

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1. INTRODUÇÃO

O Sildenafil é um inibidor potente e seletivo da fosfodiesterase-5 (PDE5), enzima responsável pela hidrólise do monofosfato de guanosina cíclico (GMPc) à sua forma inativa 5’ monofosfato de guanosina (5’GMP). Esse fármaco foi aprovado para uso terapêutico na disfunção erétil e, atualmente, vem sendo usado também no tratamento da hipertensão pulmonar, Síndrome de Raynaud e LUTS (Sintomas do trato urinário inferior) (KAMATA; MINOTA, 2014; PEIXOTO; GOMES, 2015; ZHAO, 2002). Embora mantenha um excelente nível de segurança e perfil de tolerabilidade, essas são as únicas patologias tratadas atualmente com Sildenafil. Tem sido demonstrado que Sildenafil tem potencial eficácia terapêutica em desordens relacionadas ao sistema nervoso central (SNC), tais como: acidente vascular cerebral, isquemia cerebral e doenças neurodegenerativas (CHARRIAUT-MARLANGUE et al., 2014; CHEN et al., 2014; CUADRADO-TEJEDOR et al., 2011; HUSSAIN et al., 2001).

A PDE5 (fosfodiesterase-5) é encontrada em algumas regiões do cérebro e cerebelo, e sua inibição resulta em um acúmulo de GMPc, mensageiro intracelular do óxido nítrico (NO) (PUZZO; SAPIENZA, 2008). A inflamação cerebral é mediada/modulada por citocinas pró-inflamatórias e pelo NO e exerce um papel central em numerosas patologias do cérebro, incluindo a esclerose múltipla (EM) (DUSSE; COOPER; LWALEED, 2007; OVERVIEW, 1996).

A EM é uma doença inflamatória crônica, com início típico durante os anos produtivos (entre 20 e 50 anos), caracterizada por desmielinização das células nervosas, que leva à severa deficiência psicomotora. Acredita-se que essa doença seja uma desordem autoimune mediada por células imunes/inflamatórias ativadas, tais como linfócitos-T e B, micróglia e astrócitos (MC GUIRE et al., 2013). Apesar do caráter autoimune/inflamatório da EM já estar bem elucidado, o controle da doença obtido com imunossupressores e imunomoduladores sempre foi insuficiente e a identificação de novas drogas capazes de interferir na patogênese dessa doença é de grande relevância.

Recentemente foi observado que o acúmulo intracelular de GMPc promove a inativação das células microgliais (SCHEIBLICH et al., 2014), reduz fenômenos inflamatórios e danos causados à mielina (NUNES et al., 2012; PIFARRE et al., 2011).

Diante do exposto, a presente tese propõe a investigação dos efeitos do Sildenafilna evolução clínica e regulação sobre os mecanismos regulatórios do processso na neuroinflamação em modelo de desmilinização por cuprizona induzido em camundongos

C57BL/6 wild type e knockout para iNOS. A hipótese a ser confirmada é se as vias NFkB-eNOS-GMPc e AMPK-eNOS estão envolvidas no mecanismo de ação do Sildenafil. O modelo knockout para iNOS irá elucidar se a neuroinflamação em modelo de EM ocorre por mediação do NO produzido em larga escala pela iNOS e possíveis interações com a fosfodiesterase tipo-5.

2. JUSTIFICATIVA

A EM afeta aproximadamente 2,3 milhões de pessoas em todo o mundo. Apesar do seu caráter autoimune/inflamatório estar bem elucidado, a resposta clínica aos imunossupressores tem sido insuficiente. Por outro lado, a introdução recente de imunomoduladores como o interferon-β apenas induz a diminuição da frequência e severidade das recidivas (VAN RENSBURG; KOTZE; VAN TOORN, 2012).

Tem sido demonstrado que o Sildenafil tem potencial eficácia terapêutica para desordens relacionadas ao SNC, porém existem poucos dados sobre o mecanismo de ação desse fármaco na evolução clínica e nos fenômenos patológicos das doenças que acometem o SNC (CUDRICI et al., 2006; PALMERI et al., 2013; PIFARRE et al., 2011; PUZZO et al., 2014).

Estudos recentes do nosso laboratório demonstraram que o Sildenafil inibe o processo de desmielinização e modula a inflamação, diminuindo a micro- e astrogliose e a expressão de citocinas pró-inflamatórias (TNF-α, IFN-γ e IL-1β) em modelo de desmielinização, indicando, portanto, que este fármaco pode ser uma ferramenta terapêutica importante para o tratamento da EM (NUNES et al., 2012). Esses resultados relevantes nos conduziram a investigar melhor os mecanismos de ação do Sildenafil envolvidos em processos neuroinflamatórios, cuja descrição poderá abrir novas possibilidades de aplicação desse fármaco.

O uso do *knockout* para iNOS poderá esclarecer a via de ação do Sildenafil no modelo de EM, uma vez que a ausência da produção de NO pela iNOS irá interferir na ação do Sildenafilatravés da via NO/GMPc e, conseqüentemente, nos efeitos desse fármaco sobre a remielinização e neuroinflamação.

Portanto, este estudo se propõe a analisar a ação do Sildenafil sobre a regulação dos mecanismos neuroinflamatórios, em modelo experimental de desmielinização por cuprizona induzido em camundongos C57BL/6 *wild type* e *knockout* para iNOS, esclarecendo o seu efeito sobre essa desordem do SNC.

3. OBJETIVOS

3.1. Objetivo Geral:

Caracterizar o efeito e o mecanismo de regulação do Sildenafil sobre a neuroinflamação em modelo de desmielinização *in vivo* induzido por cuprizona, em camundongos C57BL/6 *wild type* e *knockout* para iNOS e em modelo de *in vitro* induzido por LPS em linhagem de astrócitos.

3.2. Objetivos Específicos:

- ***Artigo 1 - "Sildenafil(Viagra) protective effects on neuroinflammation: The role of iNOS/NO system in an inflammatory demyelination model"***

a) Caracterizar o efeito e o mecanismo de ação do Sildenafil25 mg/kg, *in vivo*, em camundongos *konckout* iNOS sobre a evolução clínica e o processo neuroinflamatório;

b) Avaliar ativação microglial e astrocitária, através de análises moleculares e imunohistoquímicas;

c) Avaliar processo de remielinização através de coloração especifica para mielina, microscopia de eletrônica de transmissão e técnicas moleculares;

d) Caracterizar, através de análises moleculares e imunohistoquímicas, a expressão do COX-2, TNF-α e eNOS.

- ***Artigo 2 – "Involvement of AMPK, IKβα-NFκB and eNOS in the sildenafil anti-inflammatory mechanism in a demyelination model"***

a) Caracterizar o mecanismo de ação anti-inflamatória do Sildenafil25 mg/kg, *in vivo*, administrados em tempo inicial e tardio, em camundongos *wild type*;

b) Caracterizar, através de análises moleculares, imunohistoquímicas e imunoenzimáticos, a expressão do NF-κB (fator de transcrição nuclear Kappa B) e de sua proteína regulatória IκBα, além de citocinas e óxido nítrico;

c) Avaliar os efeitos do Sildenafil na recuperação tecidual, através de análises moleculares e imunohistoquimicas, avaliando as proteínas: Fator de crescimento neuronal (NGF) e Interleucina-10 (IL-10);

e) Esclarecer se o Sildenafil atua na via regulada pelo AMPK (proteina kinase ativada por AMP), modulando a inflamação e alterando o metabolismo celular, através de análises moleculares e imunohistoquimicas.

- ***Artigo 3 – "Phosphodiesterase-5 inhibition promotes remyelination by MCP-1/CCR-2 and MMP-9 regulation in a cuprizone-induced demyelination model"***

a) Caracterizar o mecanismo de ação no processo de remielinização do Sildenafil25 mg/kg, *in vivo*, administrados em tempo inicial e tardio, em camundongos *wild type*;

b) Analisar o efeito do Sildenafilsobre a metalloproteinase-9 (MMP-9) e a quimiocina MCP-1/CCR-2, através de análises moleculares;

c) Avaliar áreas do SNC afetadas pelo processo de neuroinflamação através de imagens detectadas por ressonância magnética nuclear;

d) Avaliar o processo de remielinização, através de coloração específica para mielina e microscopia eletrônica de transmissão.

- ***Artigo 4 - "Phosphodiesterase-5 inhibitors: Action on the signaling pathways of neuroinflammation, neurodegeneration, and cognition"***

Revisar na literatura científica artigos que relacionem a aplicação de inibidores de fosfodiesterase-5 em processos neuroinflamatório, neurodegenarativo e cognitivos.

- ***Artigo 5 – "Sildenafil(Viagra®) prevents and restores inflammation induced by LPS in astrocytes"***

a) Caracterizar ação preventiva e protetora do Sildenafil em astrócitos estimulados por por LPS;

b) Avaliar a conservação do fenótipo das células astrocitária, através de análises por imunofluorescência;

c) Analisar a resposta da rede astrocitária através do ensaio de ondas de cálcio latente;

d) Analisar a preservação do citoesqueleto através da marcação para fibras de estresse (actina) através de análises por imunofluorescência.

CAPÍTULO 1

4. REVISÃO DA LITERATURA

4.1 Comunicação celular na fisiopatologia do sistema nervoso central (SNC): o sistema mensageiro

Os nucleotídeos cíclicos, monofosfato cíclico de adenosina (AMPc) e monofosfato cíclico de guanosina (GMPc) exercem várias ações fisiológicas tais como a regulação de canais de íons, relaxamento do músculo liso, imunomodulação, agregação de plaquetas, fototransdução, a sobrevivência neuronal e a consolidação da memória (QI CUI, 2004; RENEERKENS et al., 2012).

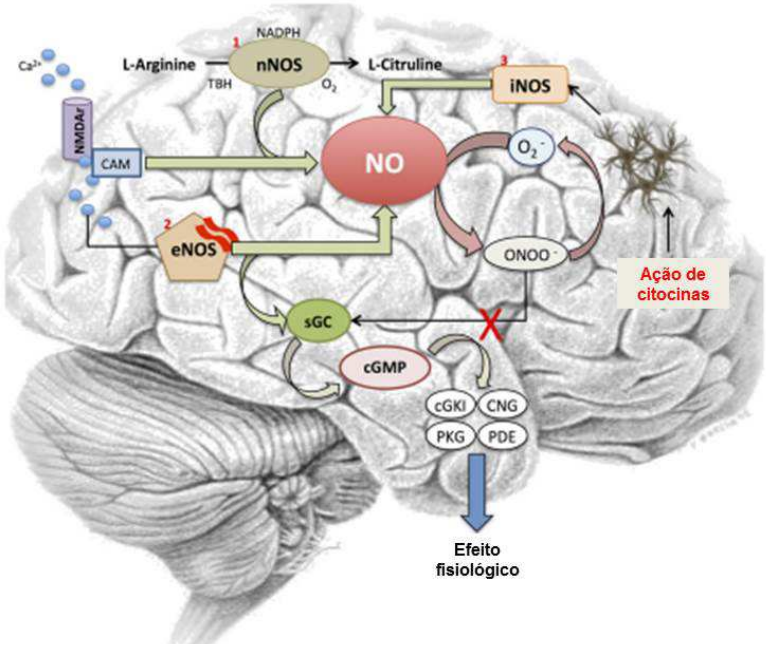
O AMPc é sintetizado a partir do ATP por uma proteína transmembrana multipasso chamada adenilato ciclase e é degradado rápida e continuamente por uma ou mais fosfodiesterases (PDEs) de AMPc, que o hidrolisa à sua forma inativa, adenosina 5'monofosfato (AIZAWA et al., 2003). Este nucleotídeo cíclico exerce seus efeitos através da interação com uma proteína receptora intracelular, a proteína quinase dependente de AMPc (PKA), que catalisa a transferência do grupo fosfato terminal do ATP para serinas ou treoninas de determinadas proteínas-alvo, regulando suas atividades (LEE, 2015). O GMPc, por sua vez, é produzido pela enzima guanilato ciclase (GC) transmembrana e GC solúvel (GCs) presente no citoplasma, e é também degradado por uma ou mais PDEs (BOLLEN; PRICKAERTS, 2012). É reconhecido como um importante segundo mensageiro de sinais extracelulares provenientes do óxido nítrico (NO) e peptídeos natriuréticos (DUSSE; COOPER; LWALEED, 2007). Os efeitos fisiológicos deste nucleotídeo cíclico são determinados pelas atividades de três tipos de receptores intracelulares: proteínas quinases dependentes de GMPc (PKG), canais iônicos regulados por GMPc e PDEs reguladas por GMPc (CORBIN; FRANCIS, 1999). O GMPc também pode ativar vias do AMPc através de sua ligação aos receptores intracelulares de AMPc, tal como a proteína PKA. Desta forma, o GMPc pode alterar a função celular através da ativação ou inativação de proteínas por fosforilação (PUZZO; SAPIENZA, 2008). Algumas ações fisiológicas mediadas pelo GMPc têm sido descritas como fatores regulatórios clássicos no relaxamento do músculo liso, inibição da agregação plaquetária, degranulação de neutrófilos, transdução de sinal visual, entre outras (LEONI et al., 2013).

No sistema nervoso central (SNC), o GMPc desempenha um papel importante como mediador da ação do óxido nítrico (NO) e peptídeos natriuréticos. O NO é um radical livre

gasoso que atua como um sinal importante de processos intra e extracelulares, e é sintetizado intracelularmente por três isoformas da enzima sintase do óxido nítrico (NOS) (DUSSE; COOPER; LWALEED, 2007): 1) formas constitutivas dependentes de Ca2+, que consistem na forma endotelial ou do tipo III (eNOS ou NOS-III), e na forma neuronal ou tipo I (nNOS ou NOS-I), presente em células endoteliais, neurônios e células gliais, que produzem NO sob condições fisiológicas; 2) forma induzível independente de Ca2+ (iNOS) ou do tipo II, presente em macrófagos, hepatócitos, músculo liso, endotélio e células gliais, produzida após estimulação imunológica (IFN-γ, TNF-α, LPS) (PUZZO; SAPIENZA, 2008).

A via NO-GMPc parece estar envolvida na modulação do desenvolvimento do cérebro, plasticidade sináptica, secreção neuroendócrina, processamento sensorial e fluxo sanguíneo cerebral (OTA et al., 2010; RIZZO et al., 2010). Essa via tem sido também reconhecida como mediadora de fenômenos neuropatológicos em condições como epilepsia, derrame e desordens neurodegenerativas (BALTRONS et al., 2003; CHEN et al., 2014). Nessas condições, a via NO-GMPc pode contribuir para a morte celular excitotóxica e dano celular por neuroinflamação. O potencial papel neurotóxico do NO tem sido focado em muitos estudos mostrando que a ativação da iNOS na micróglia está envolvida na patogênese de doenças neurodegenerativas, como, por exemplo, em resposta ao peptídeo β-amilóide (doença de Alzheimer) e na severidade da desmielinização e alteração axonal (fenômenos que ocorrem na esclerose múltipla – EM) (GONZÁLEZ et al., 2014; STREIT, 2000; VOSS et al., 2012; WAKITA et al., 2003). A ativação microglial, com consequente produção de NO em larga escala e neuroinflamação, portanto, é um dos mecanismos de injúria que pode acometer o SNC e que envolve, possivelmente, a via NO-GMPc (PERRY; NICOLL; HOLMES, 2010). O papel do NO na inflamação é complexo. Em nível micromolar, NO produzido por iNOS exerce efeitos citotóxicos e pró-inflamatórios que são opostas às que foram induzidas por concentrações nanomolares de NO produzido pela isoforma eNOS e nNOS, que apresenta efeitos anti-inflamatórios através da via do GMPc-PKG (RIZZO et al., 2010) (Figura 1).

Figura 1 - Via de sinalização do óxido nítrico. Diferentes estágios na cascata de sinalização de NO e a sua interação com vários elementos envolvidos na via de sinalização. (1) Reação catalisada pela nNOS que converte a L-arginina. (2) Ca2+ intracelular ativa eNOS para libertar NO que se liga aos receptores guanilato ciclase solúvel (GCs). Além disso, a CGs é também criticamente afetada pelo estado redox. (3) o NO é sintetizado seguindo a expressão transcricional de um Ca2+ independente de iNOS em células gliais, os astrócitos e microglia após exposição citocinas.



|Fonte: Adaptado de YUSTE et al., 2015.

Por outro lado, tem sido demonstrado que o acúmulo de AMPc e GMPc pode inibir a inflamação e estresse oxidativo (BALTRONS et al., 2003; LJUBISAVLJEVIC; STOJANOVIC, 2015), prevenindo os tecidos contra danos causados por esse processo. Esse papel modulador da inflamação exercido pelo AMPc e GMPc é uma ferramenta potencial para interferir na evolução de doenças nas quais o processo inflamatório exerce um papel central, como, por exemplo, a EM.

4.2 Inibidor de fosfodiesterase tipo 5 – PDE5 (Sildenafil)

As PDEs de mamíferos formam uma grande família e estão subdivididas de acordo com a ordem de descoberta, sequência de aminoácidos e características catalíticas e regulatórias. Estas enzimas hidrolisam os nucleotídeos cíclicos, AMPc e GMPc, às suas formas inativas 5´monofosfatos (ZHANG et al., 2006).

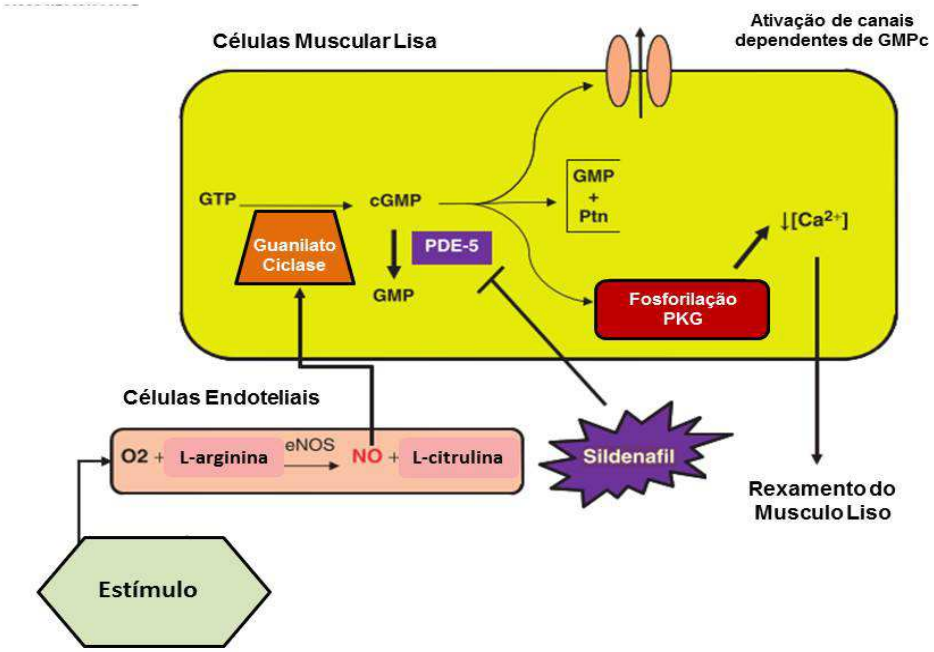
O Sildenafil, designado quimicamente como 1-[4-etoxido-3-(6,7-dihidro-1-metil-7-oxo-3-propil-1H-pirazolo[4,3-d]pirimidina-5-il)fenilsulfonil]-4-metilpiperazina citrato, é um inibidor potente e seletivo da PDE5, presente em vários tecidos tais como o vascular, o

muscular liso e o neural. Este fármaco foi resultado de um programa que iniciou em 1985, na Sede de Investigação Européia Pfizer em Sandwich, UK, com finalidade de desenvolver um inibidor de PDE5 para estimular a via NO-GMPc para o tratamento de angina pectoris em pacientes com doença arterial coronária. Este fármaco foi patenteado em 1996, e aprovado para o uso terapêutico na disfunção erétil (DE) em 1998 (Viagra®, Pfizer) (CORBIN; FRANCIS, 1999; MENNITI; FARACI; SCHMIDT, 2006). Em 2005, a ANVISA (Agência Nacional de Vigilância Sanitária) aprovou o uso do Sildenafil (nome comercial: Revatio, Pfizer) no tratamento da hipertensão pulmonar (ZHAO, 2002).

A PDE5 hidrolisa especificamente o GMPc, contribuindo para o controle da concentração intracelular deste nucleotídeo. Esta enzima é um homodímero, e cada monômero é uma proteína quimérica composta por um domínio regulatório (R) e um catalítico (C) que transforma o GMPc em 5´-GMP. O domínio R contém vários subdomínios funcionais, incluindo sítios de fosforilação, sítios alostéricos de ligação de GMPc e contatos de dimerização. A ligação do GMPc ao sítio alostérico da PDE5 ativa a fosforilação da enzima pela PKG, resultando em uma maior afinidade deste nucleotídeo cíclico ao domínio R e aumento de sua taxa de hidrólise pelo sítio catalítico. Este efeito está envolvido na regulação por *feedback* negativo dos níveis celulares de GMPc. O Sildenafil é um inibidor competitivo da catálise pela PDE5, e representa um análogo não hidrolisável pelo sítio catalítico da enzima. Sua administração promove um efeito de *feedback* positivo, representado pela inibição da degradação, com acúmulo do GMPc, através da interação do Sildenafil com o domínio C (CORBIN; FRANCIS, 1999)

Esse fármaco tem sido amplamente utilizado no tratamento da DE, descrita como a inabilidade do homem em atingir e manter a ereção do pênis. A ereção normalmente ocorre em resposta à estimulação sexual, que promove a liberação de NO a partir de neurônios e do endotélio dos corpos cavernosos do pênis. O NO ativa a GCs, levando à formação de GMPc, que causa relaxamento do músculo liso por reduzir a concentração de cálcio intracelular. Os espaços lacunares do tecido são preenchidos com sangue e a pressão intracavernosa aumenta, tornando o pênis ereto. Em resposta ao estímulo sexual, o Sildenafil promove a ereção através da inibição seletiva da PDE5. Logo, a inibição desta enzima eleva a concentração de GMPc e, com isso, potencializa o relaxamento do músculo liso dos corpos cavernosos. A dose oral recomendada de Sildenafil é de 50mg em uma dose única, administrada aproximadamente uma hora antes da atividade sexual, e com frequência de uma vez ao dia (LANGTRY & MARKHAM, 1999; PFIZER, 1998) (Figura 2).

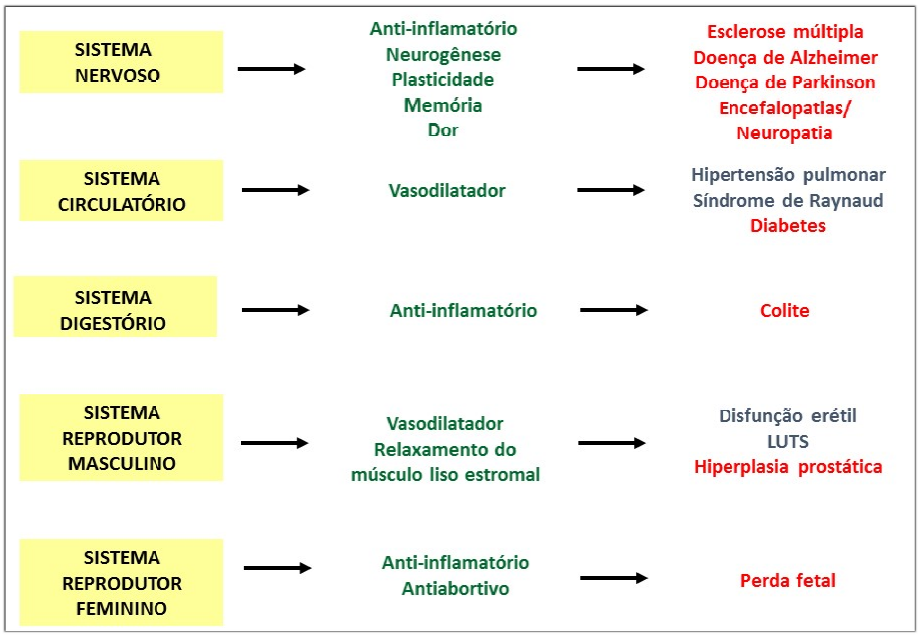
Figura 2 – Mecanismo de ação do sildenafil. eNOS - sintase de óxido endotelial; NO - óxido nítrico; GTP – guanosina trifosfato cíclico; GMPc – guanosina monofosfato ciclico; Ptn – proteína; PDE-5 – fosfodiesterase-5; PKG – proteína quinase G.



Fonte: Adaptado de LEONI et al., 2013.

Além de manter um excelente nível de segurança e perfil de tolerabilidade no tratamento da DE, Sildenafil também parece fornecer um benefício prolongado para várias outras doenças. Entretanto, não obstante o grande potencial desses inibidores (Figura 3), as únicas patologias tratadas atualmente com os inibidores de PDEs são a DE, hipertensão pulmonar, síndrome de Raynaud e, mais recentemente Sintomas do trato urinário inferior (LUTS) (KAMATA; MINOTA, 2014; PEIXOTO; GOMES, 2015). Estudos têm relatado uma importante ação anti-inflamatória do Sildenafil em diversos modelos experimentais, tais como colite (PATIL et al., 2005), diabetes (DAS et al., 2015), perda fetal (LUNA et al., 2015), bem como desordens neurodegenerativas. Entretanto, existem poucos dados sobre o efeito anti-inflamatório e remielinizante do Sildenafil sobre o SNC. Portanto, estudos precisam explorar e esclarecer a influência desse fármaco na evolução clínica e nos mecanismos patológicos das doenças que acometem o SNC.

Figura 3 - Potencial terapêutico do inibidor de PDE-5. Esquema apresenta as principais aplicações do inibidor de PDE-5. Efeitos observados em modelo experimental e testes clínicos após tratamento com o inibidor de PDE-5 (verde). Em vermelho, possíveis aplicações baseadas em evidências experimentais. Em azul, atual uso clínico.



Fonte: Nunes AKS., Peixoto C., 2016.

4.3 Neuroinflamação e o envolvimento das células gliais

A neuroinflamação, inflamação do SNC, é reconhecida como uma característica proeminente de várias condições patológicas (GLASS et al., 2010). Com isso, várias linhas de evidência sugerem fortemente que neuroinflamação é um processo crucial envolvida na progressão da degeneração neuronal, uma característica comum observada em várias perturbações neurodegenerativas (GLASS et al., 2010).

Até recentemente o SNC era considerado imunologicamente privilegiado, visto que muitos anticorpos e células do sistema imune periférico são geralmente bloqueados através da barreira hematoencefálica (BHE), uma estrutura especializada composta por células endoteliais, pericitos, astrócitos e micróglia. A BHE mantém a composição química do microambiente neuronal, que é necessário para o funcionamento adequado dos circuitos neuronais, transmissão sináptica, remodelação sináptica, angiogênese e neurogênese (ZLOKOVIC, 2008). O sistema imune influencia o funcionamento da BHE, que por sua vez, afeta o funcionamento do SNC tanto em condições fisiológicas como patológicas. Em alguns casos, a BHE separa o SNC do sistema imune, em outros atua como mediador de interações

neuroimunes, e ainda em outras pode atuar como alvo de ataques do sistema imunológico (ERICKSON; BANKS, 2013). Em condições fisiológicas, células imunes atravessam a BHE em uma taxa muito baixa, através de interações específicas (BLECHARZ et al., 2015; XU et al., 2003). Por outro lado, em doenças neurodegenerativas, a BHE é danificada. Células perivasculares (pericitos, astrócitos e micróglia), além das células endoteliais produzem diversos fatores inflamatórios que afetam a permeabilidade da BHE e a expressão de moléculas de adesão. Citocinas podem estimular a expressão de moléculas de adesão nas células endoteliais permitindo a passagem de leucócitos ativados para o SNC (DIETRICH, 2002). O tráfego de células imunes através da BHE deve iniciar ou contribuir para um ciclo vicioso resultando em uma progressiva disfunção sináptica e perda neuronal em desordens com a Doença de Alzheimer (AD), Doença de Parkinson, Esclerose Amiotrófica Lateral (ELA), Esclerose Múltipla (EM) e NeuroAIDS (PETITO et al., 2006; ZLOKOVIC, 2008).

No processo inflamatório observa-se aumento do fluxo sanguíneo e da permeabilidade vascular, dilatação venular e recrutamento de células para o sítio inflamatório. Espécies reativas de oxigênio têm papel importante no processo inflamatório, incluindo danos à célula endotelial e aumento na permeabilidade microvascular, produção de fatores quimiotáticos, recrutamento de neutrófilos, oxidação e peroxidação lipídica (MENEGAZZI et al., 2008). Esses mediadores inflamatórios desempenham um papel regulatório no crescimento, diferenciação e ativação de células imunes (SCHINS et al., 2000b).

As células da glia (micróglia, astrócitos e oligodendrócitos) definem a homeostase cerebral e são responsáveis pela defesa contra injúrias ao tecido neural e em condições patológicas assumem estado reativo contribuindo para o processo inflamatório (JENSEN; MASSIE; DE KEYSER, 2013; PARPURA; VERKHRATSKY, 2012).

4.3.1 Astrócitos

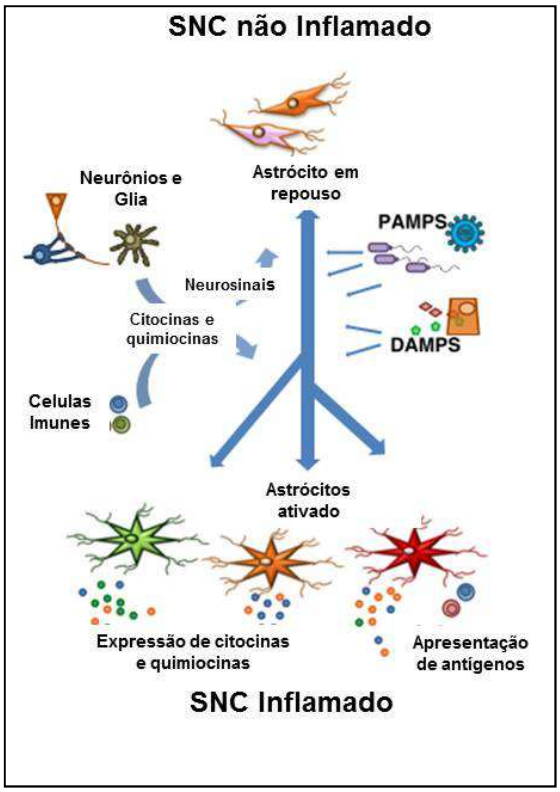
Os astrócitos constituem uma população muito heterogênea de células, as quais regulam o pH, os níveis extracelulares de íons e de neurotransmissores e o metabolismo energético. Estão envolvidos na formação e funcionamento da BHE (FIGLEY; STROMAN, 2011) e também participam ativamente da neurotransmissão (SANTELLO; VOLTERRA, 2012). Em situações de danos ao SNC, a resposta típica é algum grau de gliose reativa (Colodner et al., 2006), uma resposta astrocitária que envolve a regulação gênica positiva de proteínas do citoesqueleto (p.ex. proteína ácida fibrilar glial - GFAP), hipertrofia, hiperplasia e rearranjo dos astrócitos, podendo formar cicatrizes gliais (JENSEN; MASSIE; DE

KEYSER, 2013). Os astrócitos ativados também aumentam a expressão da proteína de ligação a cálcio S100β (ROTHERMUNDT et al., 2003; SEDAGHAT; NOTOPOULOS, 2008), um marcador de ativação de astrócitos particularmente associado com proliferação de astrócitos (SELINFREUND et al., 1991).

Além disso, os astrócitos exercem papel importante na imunidade central. A resposta imune inata é ajustada de forma precisa pela identificação do tipo de ameaça que está presente. Estruturas moleculares associadas à ameaça são reconhecidas por Receptores de Reconhecimento de Padrão (Pattern Recognition Receptors, PRRs). PRRs reconhecem padrões moleculares associados a patógenos (PAMPs) expressos por bactérias, fungos e vírus, ou padrões moleculares associados ao dano (DAMPs) expressos por células e tecidos em situação de estresse ou injúria (JENSEN; MASSIE; DE KEYSER, 2013). Uma das principais classes de PRRs em mamíferos é a dos receptores tipo Toll (Toll Like Receptors, TLRs). A resposta é rápida tanto à presença de patógenos, quanto a outros tipos de danos (estéreis) ao tecido, assumindo alguns papéis das células do sistema imune, liberando citocinas e quimiocinas, influenciando outras células e modulando a BHE (JENSEN; MASSIE; DE KEYSER, 2013).

Os astrócitos expressam receptores da família do tipo Toll (STEVENS et al., 2013). Encéfalo e medula espinhal de pacientes com EM mostraram TLR3 e TLR4 aumentados em astrócitos em regiões de inflamação (Bsibsi et al., 2012). A maioria dos TLRs, após detectarem seus respectivos ligantes, inicia um sinal que é mediado pelo gene de diferenciação mieloide 88 (Myd88) e resulta na ativação do fator nuclear de transcrição NFκB. A translocação do NFκB para o núcleo culmina na secreção de moléculas pró-inflamatórias (IL-1β, IL-6, TNF-α, IL-12) (Figura 5). Astrócitos ativados podem também produzir quimiocinas que recrutam células microgliais, linfócitos e células dendríticas para o local da injúria (GONZÁLEZ et al., 2014).

Figura 4 – Esquema de atividade astrocitária durante a inflamação. No cérebro não inflamado, astrócitos em repouso executam uma variedade de funções não-imunes. Os astrócitos detectam ameaças imunes através da expressão de receptores de reconhecimento de padrão, respondendo através de liberação de citocinas no SNC. Quando uma ameaça é detectada, os astrócitos exibem funções imunológicas adequadas à ameaça imunológica (gliose).



Fonte: Adaptado de JENSEN; MASSIE; DE KEYSER, 2013.

Na resposta astrocitária, além do aumento da expressão de TLR4 levando à expressão de uma variedade de quimiocinas e citocinas (FORSHAMMAR et al., 2011; STEVENS et al., 2013), outros processos importantes ocorrem, tais como alteração da sinalização do cálcio intracelular. Sob condições que levam à neuroinflamação no SNC, como na exposição ao LPS (lipopolissacarídeo), a sinalização pelo Ca2+ na rede de astrócitos é superativada, desencadeando ativação astrocitária. Ocorre inibição das junções comunicantes (junções gap), com alterações nas ondas intercelulares de Ca2+ e na atividade da Na+/K+-ATPase, resultando em desorganização dos filamentos de actina (fibras de estresse) (FORSHAMMAR et al., 2011, 2013).

Evidências indicam que a via GMPc-PKG está envolvida na regulação da atividade astrocitária (BORÁN; BALTRONS; GARCÍA, 2008). Foi demonstrado que o óxido nítrico (NO), através da via GMPc-PKG, diminuiu o cálcio intracelular em astrócitos, reduzindo as ondas de cálcio intercelulares (WILMS et al., 2010). Além disso, GMPc inibiu a expressão de MHC-II induzida por IFN-γ, bem como a expressão de MMP-9 e TNF-α induzida por LPS em

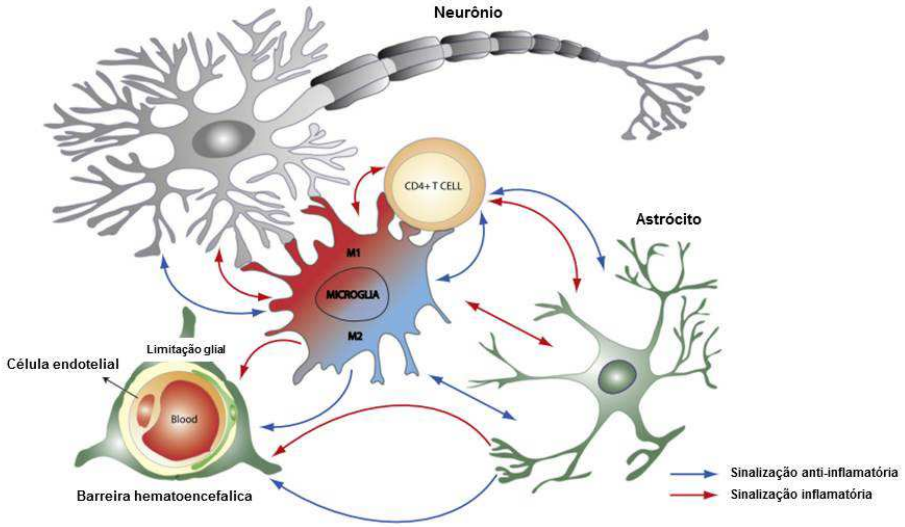
astrócitos em cultura (BORÁN; BALTRONS; GARCÍA, 2008; SHIN et al., 2007). Esses dados apoiam a hipótese de que a via GMPc-PKG exerce um papel em células astrocitárias que colabora para a resolução da neuroinflamação.

4.3.2 Micróglia

A micróglia, por sua vez, constitui as células que fazem parte do sistema imune inato e são, portanto, consideradas como os sensores patológicos do encéfalo. As alterações fenotípicas da micróglia após ativação são funcionalmente idênticas às observadas em macrófagos (DAVID; KRONER, 2011; STREIT, 2000). As ações fisiológicas da micróglia são importantes para a manutenção da homeostase. Eles remodelam sinapses por fagocitose (PAOLICELLI et al., 2011), secretam fatores neurotróficos, tais como o fator neurotrófico derivado do cérebro (BNDF) (Kettenmann et al., 2011) e removem agregados de proteínas (JOLIVALT et al., 2010). No entanto, quando expostas a infecções, lesões ou disfunção do sistema nervoso, as células microgliais se convertem em células microgliais ativadas. As alterações fenotípicas após a ativação da micróglia são funcionalmente idênticas às observadas em macrófagos. Na ausência de patologia, a micróglia “em repouso” são células pequenas com processos longos e finos ("fenótipo ramificado"). Quando ativada, a micróglia perde as longas extensões típicas da micróglia inativa, e passa apresentar extensões ameboides ("fenótipo ameboide") (OLSON, 2010) (Figura 5). Esta transformação fisiológica está associada a alterações na expressão de antígenos de superfície e de liberação de citocinas, cujos efeitos supressores podem contribuir para o prejuízo da plasticidade sináptica e agravamento de doenças neurodegenerativas (HENEKA; GOLENBOCK; LATZ, 2015).

Células microgliais ativadas tornam-se uma fonte de TNF-α, IL1-β, IL1-α, superóxido, óxido nítrico (NO), quimiocinas e glutamato, o que pode promover a morte neuronal. O TNF-α, secretado tanto por micróglia como astrócitos, pode promover diretamente a morte neuronal por ligação aos seus receptores correspondentes (TNFRs). Evidências indicam que o TNF-α induz apoptose de oligodendrócitos maduros em doenças desmielinizantes inflamatórias tais como a esclerose múltipla (EM) (CAMPBELL et al., 2012; WILMS et al., 2010), e desempenha um papel fundamental na neurodegeneração como na doença de Parkinson AD (GONZÁLEZ et al., 2014). Por outro lado, em condições normais o TNF- α modula a sinapse positivamente. (PEREA; ARAQUE, 2005).

Figura 5 – Papel da micróglia na neuroinflamação. Interação entre micróglia e outros tipos de células no parênquima cerebral, que regulam neurodegeneração. Micróglia inflamatória exibe o fenótipo M1 (vermelho) e estimulam a ativação de astrócitos, lesão neuronal, a ativação de células T e a perturbação da BHE. Em contraste, micróglia exibindo o fenótipo anti-inflamatório M2 (azul) atenua os efeitos inflamatórios e neurotóxicos induzidos por M1, apoiando a sobrevivência neuronal, o controle da permeabilidade e a reparação do tecido danificado.



Fonte: Adaptado de GONZÁLEZ et al., 2014.

4.3.3 Oligodendrócitos

Os oligodendrócitos são células mielinizantes do SNC que surgem a partir de células progenitoras de oligodendrócitos (OPCs). Uma vez que a OPC se diferencia em oligodendrócito maduro ou mielinizante (OL), seus prolongamentos são fixados individualmente em axônios para que sejam geradas as camadas concêntricas de membrana que compõem a mielina (GOLDMAN; KUYPERS, 2015; PEDRAZA et al., 2014). A presença de oligodendrócitos é mais comum na substância branca do tecido neuronal, como por exemplo: corpo caloso e cerebelo, e menos frequente na substância cinzenta (GOLDMAN; KUYPERS, 2015). Em ambos os compartimentos, de mielina é necessário para a condução saltatória dos potenciais de ação ao longo de axônios (NAVE; WERNER, 2014).

Além de sintetizar a mielina, os oligodendrócitos também promovem sua manutenção. Há um intenso “cross-talk” entre oligodendrócitos e as outras células gliais. Células microgliais ativadas são consideradas iniciadores fundamentais do processo de desmielinização mediada por espécies reativas de oxigênio (ERO), que é um importante indutor de dano aos oligodendrócitos (PEFEROEN et al., 2014). Em contrapartida, durante o

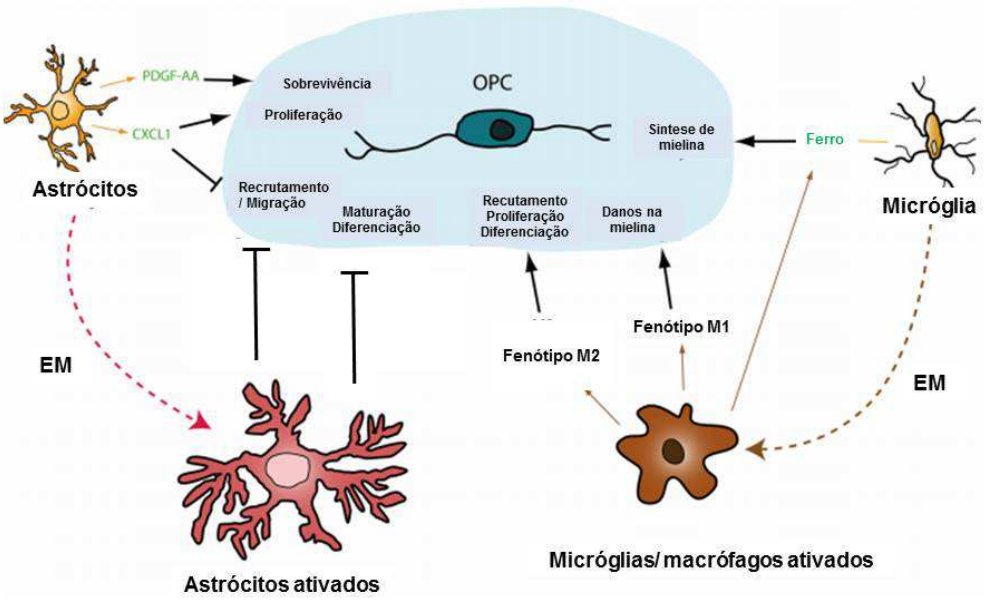
desenvolvimento os fatores tróficos liberados pela micróglias são necessários para a mielinização (PAOLICELLI et al., 2011). Os astrócitos têm um papel importante no sentido de facilitar a mielinização no SNC, no entanto, sua disfunção em estados patológicos contribui para patologias de mielinas (LUNDGAARD et al., 2014) (Figura 6).

A disfunção de oligodendrócitos e anormalidades de mielina são encontrados numa ampla variedade de doenças neurológicas e podem estar envolvidos na patofisiologia de várias doenças, incluindo leucodistrofias genéticas, esquizofrenia e a doença bipolar (HAYASHI et al., 2012), lesão cerebral (KINNUNEN et al., 2011). As doenças desmielinizantes envolvem a disfunção ou perda de oligodendrócitos e consequentemente, a perda de mielina central. Eles incluem as desordens neurodegenerativas adquiridas em adultos, tal como a esclerose múltipla, acidente vascular cerebral e perda de matéria branca da substância branca relacionada com a idade, assim como a falha mielinização precoce na paralisia cerebral e distúrbios metabólicos de perda de mielina (FRANK-CANNON et al., 2009; RAWJI; YONG, 2013; SUN et al., 2013).

Na tentativa de reparar os danos à mielina, pode ser percebido o aumento da diferenciação de OPCs em oligodendrócitos maduros, para promover a remielinização (BRADL; LASSMANN, 2010). Em estágios mais avançados da injúria, entretanto, as OPCs também entram em apoptose. Estudos do nosso grupo de pesquisa mostraram que o inibidor de fosfodiesterase 5, Sildenafil, aumenta os níveis da proteína expressa por oligodendrócitos, MBP (proteína básica da mielina), além de restaurar a morfologia da bainha de mielina, indicando remielinização. Além disso, o Sildenafil induz a diferenciação de OPCs em oligodendrócitos maduros, demonstrado através do aumento de GST-pi (marcador de oligodendrócitos maduros) (Rapôso et al., 2014).

Os oligodendrócitos não são células imunológicas inertes, mas secretam uma grande variedade de mediadores inflamatórios, tais como as citocinas pró-inflamatórias IL-1-β e IL-6, e as quimiocinas CCL2 e IL-8 envolvidas no recrutamento de células imunes durante inflamação aguda (Ramesh et al., 2012). Em modelos experimentais EM, os oligodendrócitos em apoptose também expressam aumento dos níveis da COX-2 no início da desmielinização, que parece estar a tornar estas células mais susceptíveis à morte por excitotoxicidade mediada por glutamato (Carlson et al., 2010).

Figura 6 - Representação esquemática da interação das células gliais durante o desenvolvimento e patologia. Mielinização é o resultado de uma variedade de processos que afetam as OPC (sombra azul). Durante o desenvolvimento, astrócitos e micróglia liberam várias moléculas (em verde) que controlam diversos aspectos da oligodendrogênese. Ambos os astrócitos e microglia são capazes de se transformar em estado ativado em condições patológicas, tais como a EM.



Fonte: Adaptado: CLEMENTE et al., 2013.

4.4 Sildenafil na neuroinflamação

A administração crônica de Sildenafil em pacientes com diabete tipo 2 reduz os níveis de marcadores inflamatórios vasculares tais como endotelina, proteína-C reativa, interleucina-6, molécula de adesão intercelular (ICAM) e molécula de adesão vascular (VCAM), além de reduzir os níveis de nitrito/nitratos (AVERSA et al., 2008).

Além disso, o Sildenafil reduz déficits neurológicos, aumenta a recuperação funcional e a neurogênese após derrame em ratos (ZHANG et al., 2002). Outros estudos indicam que este fármaco aumenta os níveis de GMPc no encéfalo e exerce efeitos neuroprotetores em doenças inflamatória crônica do SNC (FIRESTEIN; BREDT, 1998).

De acordo com os elegantes estudos realizados por Wang et al (2011), ratos diabéticos tipo II apresentam super-expressão de PDE5 no nervo ciático, enquanto que a espessura da bainha de mielina, a proteína básica da mielina (MBP), e as fibras nervosas subcutâneas encontram-se significativamente reduzidas. O tratamento com Sildenafil reverteu significativamente estes efeitos e melhorando a função neurológica. Análises *in vivo* e *in vitro* demonstraram que a análise de PDE5/GMPc regula a expressão de BDNF nas células de Schwann, contribuindo para a formação de mielina do nervo ciático. O mesmo grupo de

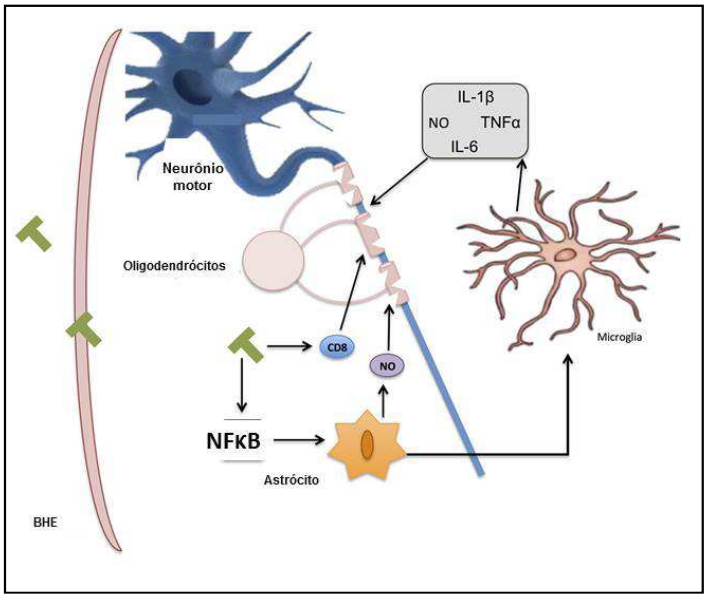
pesquisa estudou também o efeito terapêutico do Sildenafilem ratos diabéticos de meia-idade com neuropatia periférica, concluindo que o Sildenafil promove efeitos benéficos sobre a função do Sildenafil neurovascular em ratos diabéticos através da angiopoietina-1 (Ang1) e seu receptor Tie-2 (WANG et al., 2015).

4.4.1 Esclerose Múltipla

A EM é uma doença inflamatória de etiologia desconhecida em que o isolante mieliníco do SNC é danificado (DUNCAN; HEALES, 2005), esta doença é caracterizada por infiltração de células mononucleares inflamatórias no SNC através da BHE danificada, provocando a liberação de mediadores inflamatórios e citotóxicos, incluindo NO (SMITH; LASSMANN, 2002). A neuroinflamação é gerada a partir de infiltração de linfócitos T, recrutamento de macrófagos e ativação de astrócitos e micróglias (FRANK-CANNON et al., 2009; GAY et al., 1997). Embora haja uma forte correlação entre neuroinflamação e lesão axonal o mecanismo exato do dano ainda não foi elucidado (YUSTE et al., 2015) (Figura 7).

Uma vez que a expressão de NO participa na manutenção da homeostase da permeabilidade da BHE, esta molécula pode ter um papel essencial no EM. Além disso, diferentes vias de degradação podem estar envolvidas na indução da lesão neuronal e perda de oligodendrócitos (SMITH; LASSMANN, 2002). Embora não haja evidências consideráveis, estudos sugerem que as três isoformas de NOS estão envolvidos na fisiopatologia da EM(LJUBISAVLJEVIC, 2014). No entanto, o papel da iNOS é particularmente importante, especialmente no início do patologia (DUNCAN; HEALES, 2005).

Figura 7 - Inflamação na esclerose múltipla. As células T imunitárias (em verde) atravessam a BHE, afetam a estrutura de oligodendrócitos e ativam a resposta gliais através do NFkB e AP-1. Espécies reativas de oxigênio (ERO) e óxido nítrico (NO) secretados por micróglia e astrócitos ativados contribuem para danos de mielina, a degradação do axônio, e da morte neuronal final.



Fonte: Adapatdo de YUSTE et al., 2015.

A ação de Sildenafil na melhoria dos sintomas clínicos da EM inicialmente foi atribuída à indução da neurogênese, mas informações recentes apontam também para o papel do fármaco como um modulador da inflamação e a proteção da bainha de mielina (NUNES et al., 2012; PIFARRE et al., 2011; RAPOSO et al., 2013; RAPÔSO et al., 2014).

Nunes et al (2012) demonstraram que o Sildenafil inibiu o processo de desmielinização, reduziu micro e astrogliose, bem como a expressão de citocinas pró-inflamatórias (TNF-α, IFN-γ e IL-2 IL-1β) em um modelo de desmielinização induzida por cuprizona. Em outro estudo do nosso grupo, o Sildenafil aumentou os níveis de proteína expressa por oligodendrócitos, MBP e restaurou a morfologia da bainha de mielina, indicando a remielinização. Além disso, o Sildenafil promoveu a diferenciação de OPC em oligodendrócitos maduros, demonstrada por um aumento da GST-pi (RAPÔSO et al., 2014).

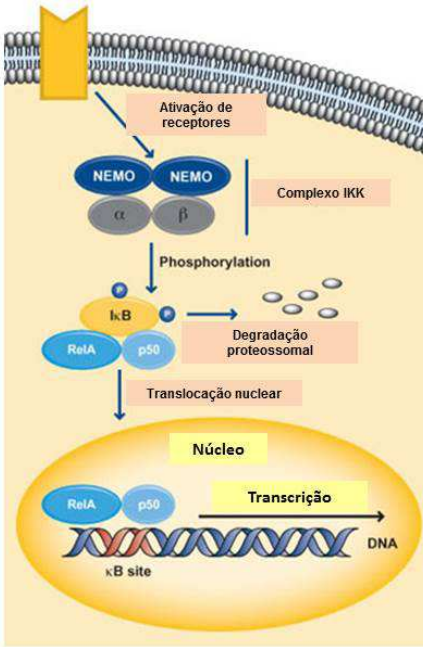
O acúmulo de GMPc é, portanto, uma ferramenta potencial para modular a neuroinflamação e reduzir danos à bainha de mielina, na patogênese da EM e embora tenha sido demonstrado que o Sildenafil, de fato, tem ação protetora no SNC, ainda não foi esclarecido o mecanismo pelo qual este fármaco age no processo inflamatório. Estudos que esclareçam o mecanismo de ação do Sildenafil na neuroinflamação e lesão da mielina precisam ser desenvolvidos e poderão abrir novas possibilidades de aplicação dessa droga.

4.5 Mecanismos envolvidos no controle da inflamação

Vários estudos têm demonstrado que o fator nuclear-κB (NFκB) desempenha um papel importante na regulação dos genes responsáveis pela geração de mediadores da resposta inflamatória, tais como TNF-α e IL-1β, desempenhando um papel regulatório no crescimento, diferenciação e ativação de células imunes (SCHINS et al., 2000a) et al., 2000).

O NF-κB reside no citosol sob a forma inativa, como um dímero das subunidades RelA e p50. Devido à sua ligação com a proteína inibitória IκB, conhecida também como IkBα, o NFκB é incapaz de se translocar para o núcleo. Na sua via clássica de ativação, o IkBα é fosforilado e subsequentemente degradado no proteossomo. Desta forma, o NFκB é liberado e migra para o núcleo, onde ativará a transcrição de vários genes pró-inflamatórios (KALTSCHMIDT; KALTSCHMIDT, 2009) (Figura 8).

Figura 8 - A ativação da via de NFκB na resposta inflamatória celular. Diversos estímulos extracelulares causam a fosforilação do IkB, mediada pela complexo quinase (IKK). A fosforilação do IkB libera o NF-κB de forma que ele possa atuar sobre um gene alvo no núcleo, enquanto o IkB é degradado.



Fonte: Adaptado de BONIZZI et al., 2004.

O NO tem um importante papel na regulação do NFκB. O NO produzido pela enzima eNOS, em concentrações nanomolares, tem efeitos anti-inflamatórios através da via de sinalização do GMPc. Nesse caso, a via NO-GMPc pode inibir diretamente a atividade do NFκB por ativar o IKBα (sua proteína inibitória), ou indiretamente por ativar a Proteína

Kinase-A (PKA). Além disso, a eNOS pode inibir a transcrição gênica de NFκB, limitando o processo inflamatório local (AIZAWA et al., 2003; CONNELLY et al., 2003; GRUMBACH et al., 2005; MATTHEWS et al., 1996; SPIECKER; PENG; LIAO, 1997).

Estudo recente demonstrou que o Sildenafil atenua resposta inflamatória induzida por LPS, pela via proteína quinase ativada por AMP (AMPK)-NFκB (ZHAO et al., 2011). Pesquisas relatam que essa proteína também tem um papel anti-inflamatório por ativar a eNOS. O NO pode atuar como um ativador endógeno do AMPK, sugerindo uma relação de reciprocidade entre AMPK e a eNOS (ZHANG et al., 2008). Além disso, há evidências recentes de que a sinalização do AMPK pode suprimir a ativação do NFkB, contribuindo também para o controle da inflamação (ZHAO et al., 2011). Entretanto, a AMPK é conhecida principalmente como um regulador do metabolismo energético intracelular (ASHABI et al., 2014; NATH et al., 2009). Esta proteína responde às exigências metabólicas, seja estimulando a produção de energia e o catabolismo de glicose e lipídios, seja inibindo as vias de consumo de energia (MYERBURG et al., 2010; PERERA; TURNER, 2015).

4.6 Modelo animal

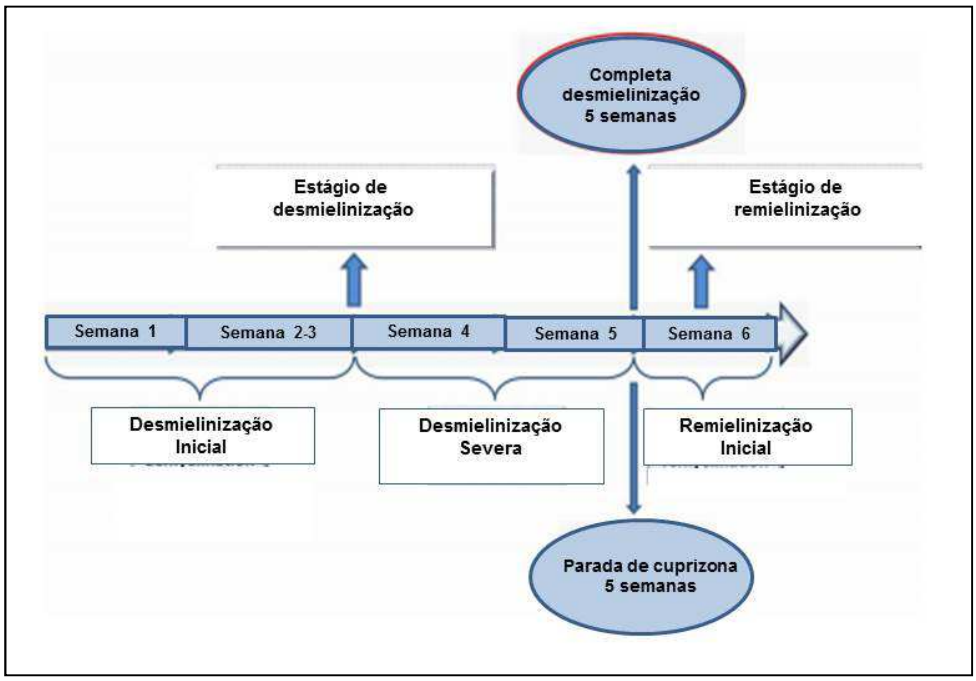
O cobre é um elemento essencial para uma série de metaloenzimas, incluindo cobre-zinco superóxido dismutase e ceruloplasmina, presentes no SNC. A cuprizona (oxalic-bis-cyclohexylidenehydrazide) é um quelante de cobre produzido para uso em pesquisa animal. A administração de cuprizona leva a uma redução na atividade cerebral de citocromo-oxidase bem como de outras enzimas ligadas à mitocondria. Dessa forma, cuprizona induz déficit de cobre que prejudica a função mitocondrial no cérebro, provocando alterações de metabolismo energético em células de oligodendrócitos levando à desmielinização (GAO et al., 2000).

A cuprizona causa intoxicação no SNC, afetando especificamente micróglia e astrócitos (ativando-os) e provocando perda da mielina e oligodendrócitos durante as primeiras semanas de ingestão da cuprizona (administrada na ração) (FRANCO-PONS et al., 2007). O modelo de desmielinização por cuprizona induz astrocitose hipertrófica e hiperplásica e não ativa células imunes periféricas (GROEBE et al., 2009), fornecendo um importante modelo para análise da reação inflamatória restrita às células gliais.

A desmielinização cortical pode ser observada após 3 semanas de exposição ao cuprizona sendo quase completa após 6 semanas de tratamento (GUDI et al., 2009; SKRIPULETZ et al., 2008). Além disso, a desmielinização também pode ser detectada em outras regiões do cérebro tais como o hipocampo, gânglios basais e cerebelo

(KOUTSOUDAKI et al., 2009; POTT et al., 2009). Após a fase crônica da desmielinização, cujo pico ocorre após quatro semanas de administração da cuprizona, os animais podem ser recuperados por remoção da cuprizona da dieta. Dentro das quatro semanas seguintes à retirada da cuprizona da ração, há remielinização substancial (BENETTI et al., 2010).

Figura 9 - Curso de des – remielinização em corpo caloso após a administração de cuprizona.



Fonte : Adaptado de GUDI et al., 2014.

Durante desmielinização induzida por cuprizona, oligodendrócitos começar a sofrer apoptose depois de 3-7 dias de exposição cuprizona (HESSE et al., 2010; MASON et al., 2000). A expressão de RNAm de genes de proteínas de mielina, tais como a proteína proteolítica da mielina (PLP), glicoproteína associada à mielina (MAG) e a proteína básica da mielina (MBP) é drasticamente regulada negativamente na semana 1 (MATSUSHIMA; MORELL, 2001; SKRIPULETZ et al., 2011) e com consequente ativação de astrócitos e micróglias (GUDI et al., 2014).

Esse agente causa, portanto, neuroinflamação e desmielinização e tem sido usado como um modelo de estudo desses dois fenômenos. A neuroinflamação induzida pela cuprizona é limitada às células residentes do SNC, sem nenhum envolvimento de células imunes periféricas. Uma vez que a neuroinflamação residente mediada por células da glia (astrócitos e micróglia) parece ser o principal fenômeno que perpetua os danos neurais presentes na EM, esse modelo torna-se muito útil para estudar o papel de fármacos em EM.

Recentemente tem sido reportado na literatura que o Sildenafiltem papel neuroprotetor sobre as celulas gliais (PIFARRE et al., 2011; PIFARRÉ et al., 2014) e atua protegendo as celulas neurais contra danos do estresse oxidativo (LIU et al., 2010). O presente estudo visa contribuir para a caracterização do efeito neuroprotetor do Sildenafil e esclarecer o mecanismo de ação desse fármaco nos processos neuroinflamatórios.

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CAPÍTULO 2

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Research Article

Sildenafil (Viagra) Protective Effects on Neuroinflammation: The Role of iNOS/NO System in an Inflammatory Demyelination Model

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We recently demonstrated that sildenafil reduces the expression of cytokines, COX-2, and GFAP in a demyelinating model induced in wild-type (WT) mice. Herein, the understandings of the neuroprotective effect of sildenafil and the mediation of iNOS/NO system on inflammatory demyelination induced by cuprizone were investigated. The cerebella of iNOS$^{-/-}$ mice were examined after four weeks of treatment with cuprizone alone or combined with sildenafil. Cuprizone increased GFAP, Iba-1, TNF-α, COX-2, IL-1β, and IFN-γ expression, decreased expression of glutathione S-transferase pi (GSTpi), and damaged myelin in iNOS$^{-/-}$ mice. Sildenafil reduced Iba-1, IFN-γ, and IL-1β levels but had no effect on the expression of GFAP, TNF-α, and COX-2 compared to the cuprizone group. Sildenafil elevated GSTpi levels and improved the myelin structure/ultrastructure. iNOS$^{-/-}$ mice suffered from severe inflammation following treatment with cuprizone, while WT mice had milder inflammation, as found in the previous study. It is possible that inflammatory regulation through iNOS-feedback is absent in iNOS$^{-/-}$ mice, making them more susceptible to inflammation. Sildenafil has at least a partial anti-inflammatory effect through iNOS inhibition, as its effect on iNOS$^{-/-}$ mice was limited. Further studies are required to explain the underlying mechanism of the sildenafil effects.

1. Background

Nitric oxide (NO) is a highly reactive molecule with a range of physiological functions [1, 2]. This messenger plays an important role in the modulation of vascular tone [3], neurotransmission [4, 5], and immune system [6–8]. NO is produced from L-arginine by NO synthases (NOSs). In addition to the constitutive forms of the enzyme (endothelial (eNOS or NOS3) and neuronal (nNOS or NOS1)), there is also an inducible form (iNOS or NOS2). This last is most commonly associated with inflammatory conditions in which NO is produced in large amounts.

There is strong evidence to suggest the involvement of iNOS in the development of neurodegenerative disease. Induction of iNOS, NO, and NO byproducts has been found in multiple sclerosis (MS) patients and animal models and correlates with disease severity and level of inflammatory infiltrate [9–12]. Interestingly, however, iNOS-deficient mice developed a more severe MS model [13, 14]. It was found that the elimination of iNOS does not improve, and may actually aggravate, demyelination in the cuprizone-demyelinating model [15], which suggests that the iNOS/NO system may be neuroprotective. More information is required to understand

the role of iNOS/NO in inflammatory response, oligodendrocyte cell death, and myelin damage/loss.

The cyclic guanosine 3′,5′-monophosphate (cGMP) signaling pathway is an important NO-signaling molecule. NO binds to soluble guanylyl cyclase (sGC) and increases concentration of cGMP, activating signaling cascades and leading to cGMP-dependent responses [16, 17]. The cGMP signal can be terminated by the action of several phosphodiesterases (PDE) [18]. The cGMP-selective PDE5 is expressed in the cardiovascular, neural, and immune systems [18]. Studies have shown that selective PDE5 inhibitors, widely used in the treatment of erectile dysfunction in humans, such as sildenafil (Viagra; Pfizer) and vardenafil (Levitra; Bayer), raise cGMP levels in the brain and offer protective effects, improve cognition and memory [19], reduce neuronal cell death following ischemic cerebrovascular injury [20], decrease white matter damage, and regulate inflammatory responses in MS models [21, 22]. Recent studies by the authors of an MS model induced in wild-type C57BL6 mice found that sildenafil has an anti-inflammatory action, reducing levels of proinflammatory cytokines and cyclooxygenase-2 (COX-2) and protecting the myelin structure [22]. However, the mechanism of sildenafil neuroprotection remains unknown.

In the present study, inflammatory demyelination was induced in $iNOS^{-/-}$ mice, and sildenafil was administered for four weeks. The focus of this study was to identify the role of a potent inflammation-associated molecule, iNOS-derived NO, in protective mechanisms related to sildenafil. The present study also aimed to clarify the role of NO protective and/or deleterious mechanisms in the demyelinating model.

2. Materials and Methods

2.1. Experimental Design. Five iNOS knockout (B 6.129 P2-Nos2) mice, aged 7 to 10 weeks, weighing 15 to 20 g, were used per group. The mice were examined for health status, acclimated to the laboratory environment at 25°C and 12 h light/dark photoperiod, and housed in metal cages. The control group received standard laboratory diet and pure water. Over a four-week period, the experimental groups received either 0.2% cuprizone (oxalic-bis-cyclohexylidenehydrazide Sigma-Aldrich Inc., St. Louis, MO, USA) mixed into the chow and pure water or 0.2% cuprizone in the chow and 25 mg/kg of body weight of sildenafil (Viagra; Pfizer Inc., New York, NY, USA) administered through the drinking water [22–24]. Body weight was accessed every week and the drug concentration in the water was adjusted to maintain the dose. All experiments were carried out in compliance with ethical guidelines for animal experimentation (L-10/2010-CEUA; 05/10-CIBIO FIOCRUZ). After treatment, the animals were anaesthetized (i.m.) with ketamine (115 mg/kg) and xylazine (10 mg/kg) (Sespo Comércio e Indústria Ltda., São Paulo, SP, Brazil).

2.2. Immunofluorescence (IF). After anesthesia, the animals were transcardially perfused with physiological saline (20 mL), followed by 4% paraformaldehyde (Sigma-Aldrich) (40 mL) in 0.1 M phosphate (sodium phosphate monobasic and dibasic heptahydrate, Sigma-Aldrich) buffered saline (PBS), pH 7.2. Cerebella were dissected and immersed in 15% sucrose overnight, followed by 30% sucrose for a second night (36 hours total). The specimens were then embedded in OCT-Tissue Tek compound (Sakura Finetek, Torrance, CA, USA) and frozen in n-hexane (Dinâmica, São Paulo, SP, Brazil) cooled with liquid nitrogen. Cryosections (8 μm thick) were permeabilized (0.3% Triton X-100) and incubated for 1 h with blocking solution (3% BSA plus 0.2% Tween 20 in Tris buffered saline). Subsequently, the sections were incubated with antibodies for Glial Fibrillar Acidic Protein (GFAP) (DakoCytomation, cat. no. ZO 334) and Iba-1 (Wako, Osaka, Japan, cat. no. 019-19741) (both 1 : 100). Sections were incubated with primary antibodies overnight and then incubated with polyclonal Cy3-conjugated secondary antibodies (Jackson, cat. no. 705-165-147) against rabbit immunoglobulin (1 : 200) for 1 h. The slides were washed and mounted in fluorescent Prolong Gold Antifade medium (Life Technologies, cat. no. P36930) for observation under an inverted fluorescence microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and the Release 4.7.2 image analysis software.

2.3. Immunohistochemistry (IH). After perfusion as described for IF, cerebella were immediately removed and postfixed in the same fixative overnight. The samples were dehydrated in an ethanol series (Isofar Chemical Co., RJ, Brazil), cleared in xylene, and embedded in paraffin (Merck, catalog no. 1071642504). Sections (5 μm thick) were cut on an RM 2035 microtome (Reichert S, Leica), rehydrated, washed in 0.05 M PBS, and incubated in this buffer with 1% bovine serum albumin (BSA, fraction V) (Miles, Naperville, IL, USA) for one hour. Endogenous peroxidase was blocked and antigen retrieval was performed, pretreating the sections with 20 mM citrate buffer, pH 6.0, at 100°C, for 30 min. All groups were incubated with the rabbit polyclonal anti-COX-2 (Abcam, Canada/US, cat. no. ab15191) (1 : 100, overnight at 4°C). After washing, the sections were overlaid for 1 h with a biotin-conjugated secondary antibody using an HRP kit (DakoCytomation, CA, USA, Biotinylated Link Universal HRP; cat. no. K0690) and visualized with 3′-3-diaminobenzidine (DAB) as the chromogen. The sections were then weakly counterstained with Harris' hematoxylin and mounted in entellan (Merck, cat. no. 1079610100).

2.4. Western Blotting (WB). Cerebella were quickly dissected, and each group was homogenized in an extraction cocktail (10 mM EDTA, Amresco, Solon, USA; 2 mM phenylmethane sulfonyl-fluoride, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 10 μg of aprotinin/mL, and 100 mM Tris, pH 7.4). Cerebella of five animals were mixed and homogenized to form a pool from each group. The WB was done in accord with Nunes et al. [22]. Briefly, the proteins (40 μg total) were separated on 6.5% (IFN-γ), 10% (TNF-α, IL-1β), or 12% (GFAP, eNOS) acrylamide gel. After overnight in 5% nonfat milk, the primary antibodies against GFAP (1 : 10,000, DakoCytomation, cat. no. ZO 334), COX-2 (1 : 1000, Abcam, cat. no. ab15191), TNF-α (1 : 1000,

Peprotech, NJ, USA, cat. no. 500-P64), IFN-γ (1 : 2500, Peprotech cat. no. 500P119), IL-1β (1 : 2500, GenWay Biotech, Inc., CA/USA, cat. no. 18-732-292194), GST3/GSTpi (1 : 250, Abcam, Canada, cat. no. ab53943), and eNOS (1 : 1000, BD Biosciences, cat. no. 610299) were incubated for four hours followed by horseradish peroxidase-conjugated antibodies anti-rabbit (1 : 80,000, Sigma-Aldrich, cat. no. A9169), anti-mouse (1 : 1,000, Sigma-Aldrich, cat. no. A0168), or anti-goat secondary antibody (1 : 100,000, Sigma-Aldrich, cat. no. A5420). For quantification, the pixel density of each band was determined using the Image J 1.38 software (http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD, USA). For each protein investigated, the results were confirmed in three sets of experiments. Immunoblotting for β-actin (1 : 1,000, Sigma-Aldrich, cat. no. A2228) was performed as a control.

2.5. Luxol Fast-Blue (LFB). After perfusion as described for IF, the samples were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections (5 μm thick) were cut on an RM 2035 microtome (Reichert S, Leica). Myelin was detected using Luxol-Fast Blue (LFB) staining (Solvent Blue 38; Sigma-Aldrich) in accord with Nunes et al. [22]. The sections were observed under an inverted microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and Release 4.7.2 image analysis software (Zeiss).

2.6. Transmission Electron Microscopy (TEM). After anesthesia, the animals were sacrificed by transcardial perfusion with physiological saline (20 mL), followed by 40 mL of fixative—2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate acid (Sigma-Aldrich) buffer, pH 7.2. Cerebella were quickly dissected and postfixed in the same fixative overnight. Next, cerebellum fragments were washed twice in the same buffer and postfixed in a solution containing 1% osmium tetroxide (Sigma-Aldrich), 2 mM calcium chloride, and 0.8% potassium ferricyanide (Sigma-Aldrich) in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone and embedded in SPIN-PON resin (Embed 812-Electron Microscopy Science, Washington, PA, USA). Resin polymerization was performed at 60°C for 3 days. Semithin sections (0.5 μm in thickness) were placed on glass slides, stained with toluidine blue. Ultrathin sections (70 nm in thickness) were placed on 300-mesh nickel grids, counterstained with 5% uranyl acetate (Electron Microscopy Science) and lead citrate (Sigma-Aldrich), and examined using a FEI Morgagni 268D transmission electron microscope.

2.7. Statistical Analysis. The densitometric values of the immunoreactive bands (immunoblotting) were analyzed using the GraphPad Prism software package (San Diego, CA, USA). One-way analysis of variance (ANOVA), followed by Dunnett's and/or Tukey's posttest, was used to compare groups. The results were expressed as means ± SE, when appropriate. A P value < 0.05 indicated statistical significance.

3. Results

Clinical analysis revealed that cuprizone-treated animals suffered from motor limitations, such as tremors, and abnormal walking and posture. The group treated with sildenafil (25 mg/kg) exhibited normal walking and posture, and tremors were either mild or nonexistent.

The clinical signs were observed and recorded by three observers. The iNOS$^{-/-}$ control animals exhibited normal motor function and posture and explored their environment normally. This group was classified as score 0 (no sign). The mice treated with cuprizone exhibited arched (shortened) posture and tremors and had difficulty in exploring the environment. This group was classified as score 2. Mice treated with 25 mg/kg of sildenafil both walked and were able to explore the environment normally, with no or mild tremors, and were classified as score 1.

Cuprizone increased GFAP, TNF-α, and COX-2 expression in cerebellum, indicating astrocyte activation (reactive gliosis) and neuroinflammation. Sildenafil treatment did not reduce the levels of these proteins in mice without iNOS.

Western Blotting (WB) analysis showed that GFAP, a marker of astrocyte activation (reactive gliosis), was present in the cerebellum of untreated iNOS$^{-/-}$ animals (control) (Figure 1(a)). Treatment with 0.2% cuprizone for four weeks significantly increased expression of this protein (Figures 1(a) and 1(c); $P < 0.001$). Animals that concomitantly received sildenafil (25 mg/Kg) and cuprizone also exhibited a high level of GFAP in comparison to control (Figures 1(a) and 1(c); $P < 0.001$).

Immunofluorescence (IF) analysis of GFAP in the cerebellum revealed the expression and location of this cytoskeletal intermediate filament protein in the animals. In the molecular layer of the cerebellum, GFAP labeling revealed long astrocytic processes with a typical arrangement that was perpendicular to the pia mater membrane (Bergmann glia) (Figure 2(e)). Astrocytic processes were also seen around other cells and vessels (arrowheads in Figures 2(b), 2(d), and 2(e)). The control group showed basal expression of GFAP localized normally (Figures 2(a) and 2(d)). Treatment with CPZ induced astrogliosis, increasing the intensity of labeling in the astrocytes (Figures 2(b) and 2(e)). In the sildenafil group, GFAP labeling was more intense than for control (Figures 2(c) and 2(f)).

The iNOS$^{-/-}$ control group had a basal level of TNF-α (Figure 3(a)). WB analysis showed that cuprizone induced a significant increase of this cytokine, indicating neuroinflammation (Figures 3(a) and 3(b); $P < 0.05$, compared to control group). Animals that received cuprizone plus sildenafil also had a significant increase of TNF-α, compared to control ($P < 0.01$).

COX-2 was analyzed by WB and immunohistochemistry (IH) tests. This enzyme was expressed in a minimal amount in the cerebellum of the iNOS$^{-/-}$ control group (Figures 3(c) and 4(a)). CPZ administration induced a significant increase in COX-2 expression, compared to control (Figures 3(c) and 3(d); $P < 0.01$). Sildenafil treatment did not decrease COX-2, which remained high in comparison with the control group

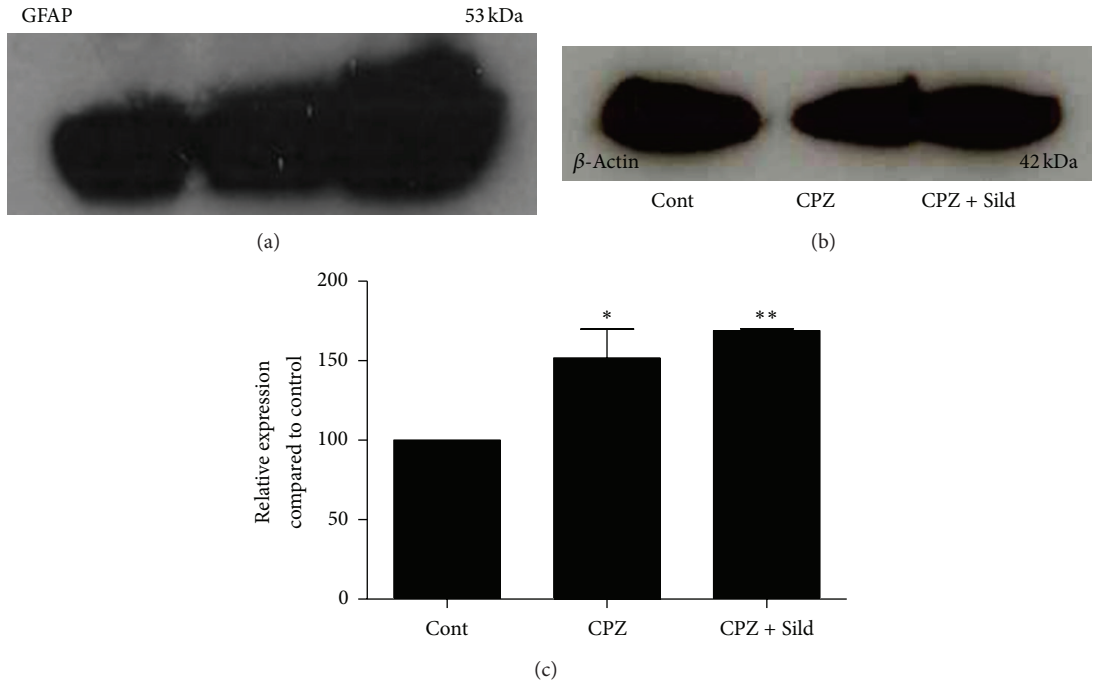

Figure 1: Western blotting for GFAP. (a) GFAP immunoblot of iNOS$^{-/-}$ control (Cont), cuprizone (CPZ), and cuprizone plus sildenafil (CPZ + Sild) groups. (b) β-actin immunoblot. (c) Graph represents quantification and statistical analysis. The control group showed basal expression of GFAP. CPZ treatment induced a significant increase of this protein and sildenafil plus CPZ did not reduce GFAP expression, which remained higher in relation to the control group. The experiment was performed in triplicate ($n = 5$ animals/group). The results were expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

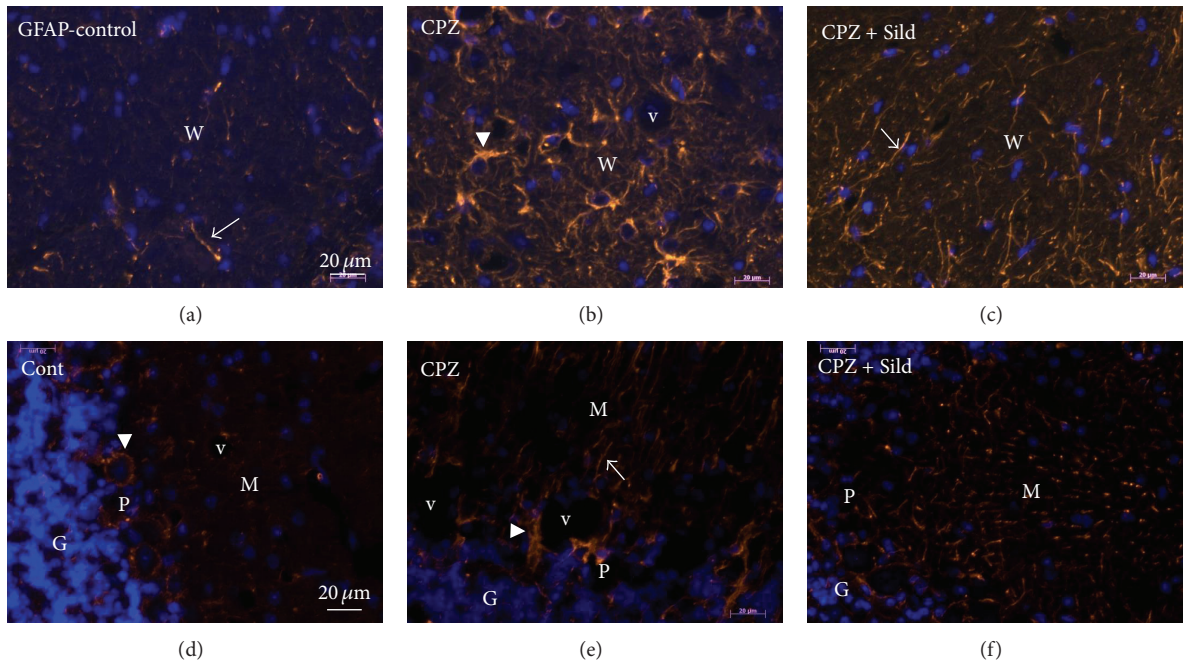

Figure 2: Immunofluorescence for GFAP. (a) and (d) show expression and physiological locations of GFAP in mice without iNOS and without treatment. CPZ administration ((b), (e)) induced reactive gliosis, with thicker and more numerous astrocytic processes, compared to control. GFAP remained high in relation to control, after application of sildenafil plus CPZ ((c), (f)). Arrows show astrocytic processes and arrowheads point to processes around vessels and other cells. W: white matter, M: molecular layer, P: purkinje layer, G: granular layer, and v: vessel. Bars: 20 μm.

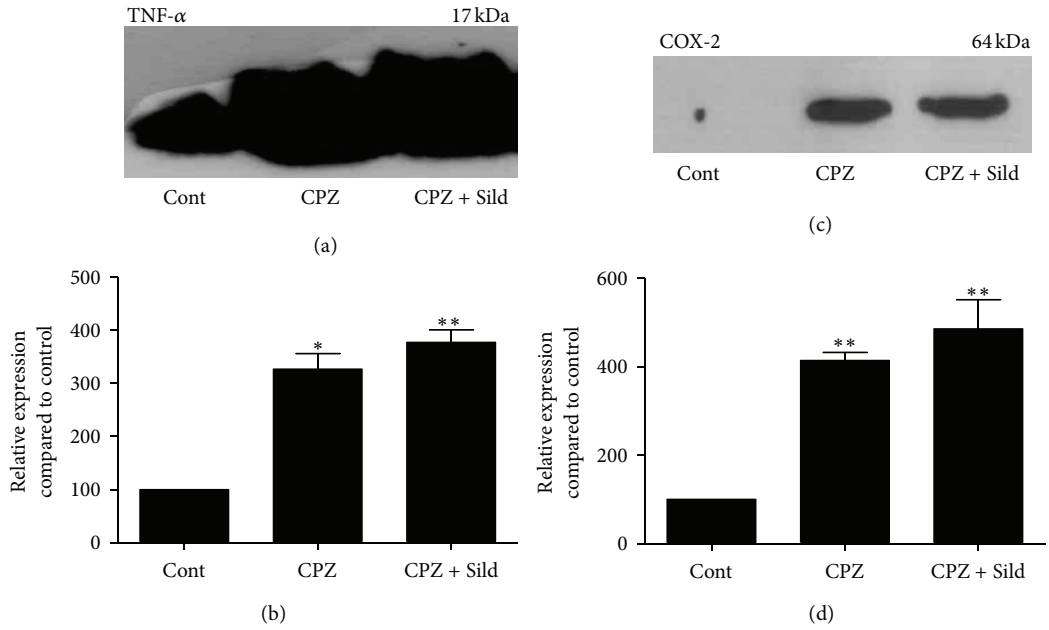

Figure 3: Western blotting for TNF-α and COX-2. ((a), (c)) Immunoblots of iNOS$^{-/-}$ control (Cont), cuprizone (CPZ), and cuprizone plus sildenafil (CPZ + Sild) groups. ((b), (d)) Graphs represent quantification and statistical analysis. The control group showed basal expression of TNF-α. CPZ treatment caused a significant increase of this cytokine, and sildenafil plus CPZ did not decrease its expression, which remained higher in relation to the control group. Only minimum amounts of COX-2 were present in the control group. CPZ and CPZ + Sild caused a significant increase of this enzyme, compared to control. The experiment was performed in triplicate ($n = 5$ animals/group). The results were expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

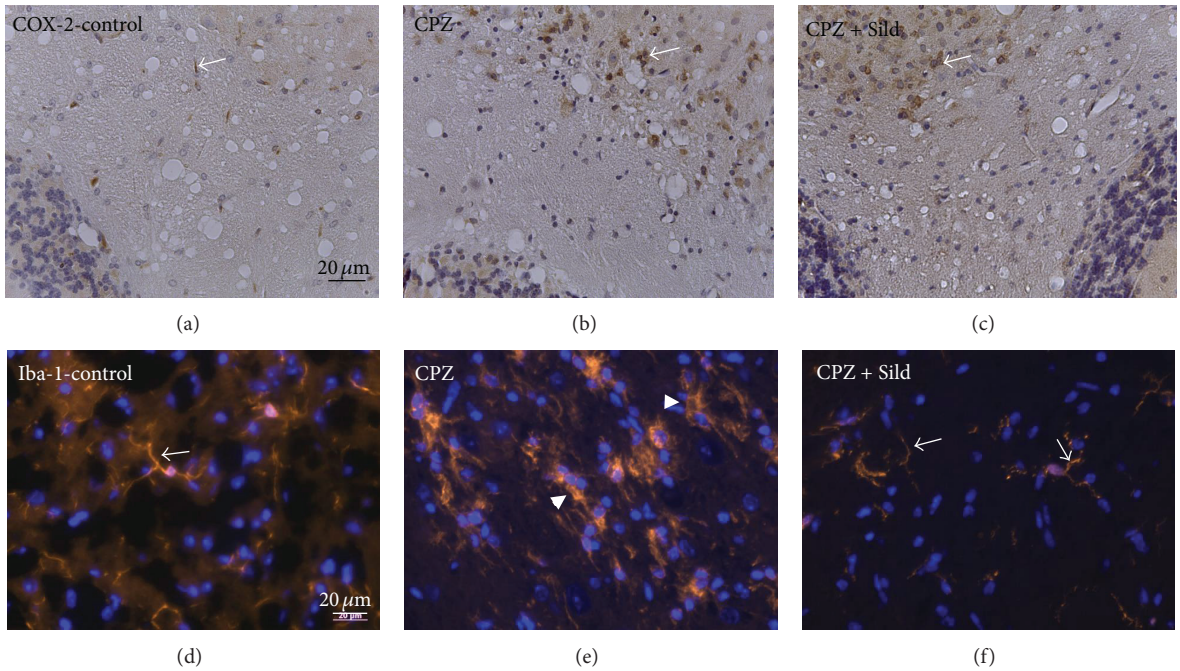

Figure 4: Immunohistochemistry for COX-2 ((a)–(c)) and immunofluorescence for Iba-1 ((d)–(f)). iNOS$^{-/-}$ control (a) showed very low COX-2 expression (arrow). After CPZ treatment (b), COX-2 labeling significantly increased, mainly in white matter, in relation to control. Animals treated with CPZ + Sild (c) also increased COX-2, comparing to the control group. A basal expression of Iba-1 was seen in control animals without iNOS (d). CPZ increased Iba-1 and induced an activated phenotype (arrowheads) of microglia (e). Sildenafil plus CPZ decreased Iba-1 and induced latent phenotype (arrows) of microglia (f).

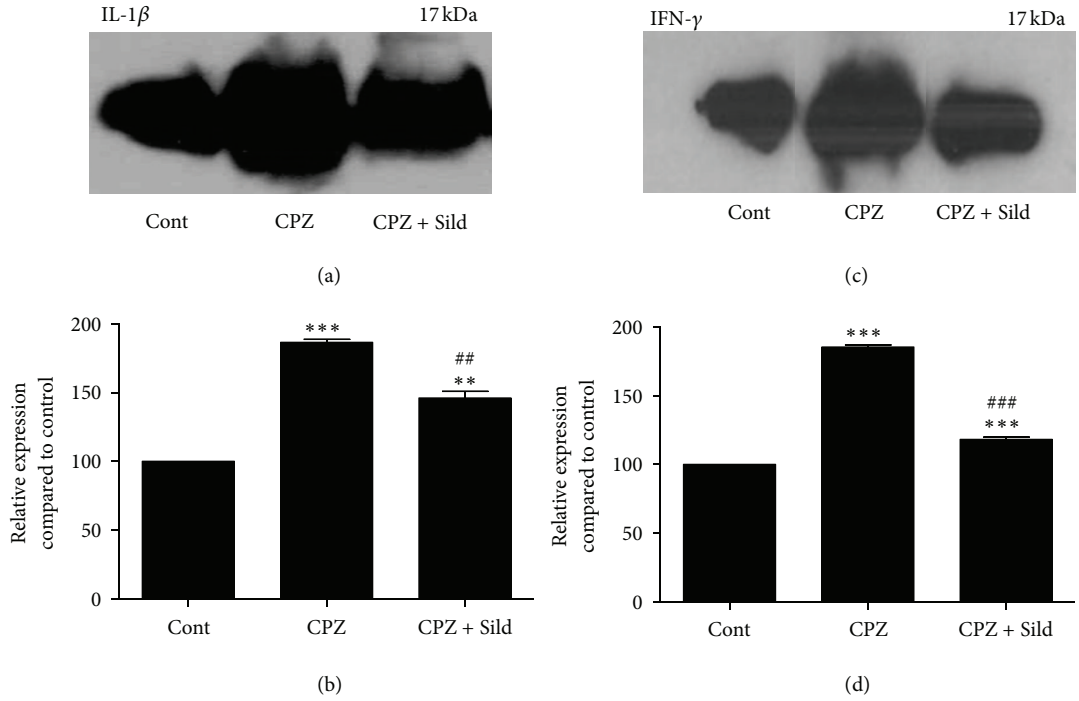

Figure 5: Western blotting for IL-1β and IFN-γ. ((a), (c)) Immunoblots of iNOS$^{-/-}$ control (Cont), cuprizone (CPZ), and cuprizone plus sildenafil (CPZ + Sild) groups. ((b), (d)) Graphs represent quantification and statistical analysis. The control group showed basal expression of IL-1β and IFN-γ. CPZ treatment induced a significant increase of these cytokines, compared to control. Sildenafil plus CPZ significantly decreased IL-1β and IFN-γ expression, in relation to the CPZ group. The experiment was performed in triplicate. The results were expressed as mean ± SE. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with CPZ.

(Figures 3(c) and 3(d); $P < 0.01$). IH labeling revealed the expression and location of COX-2 (Figures 4(a)–4(c)), which was mainly found in the white matter of the cerebellum. The control group had a very low expression of this enzyme (Figure 4(a)), while treated animals had a high expression of COX-2 in white matter (Figures 4(b) and 4(c)).

Immunoblot control with β-actin is shown in Figure 1(b).

Cuprizone increased Iba-1, IL-1β, and IFN-γ expression in the cerebellum of iNOS$^{-/-}$ mice. While sildenafil decreased the expression of these proteins, the level of expression remained above that of control animals.

The microglial marker, Iba-1, was analyzed by IF. There was physiological expression of Iba-1 in the cerebellum of iNOS$^{-/-}$ control animals, with cells with processes that were typically branched, thin and weakly labeled (arrow, Figure 4(d)). Cuprizone treatment induced a stronger expression of Iba-1 (Figure 4(e)), compared to the control group, with thicker and more intensely labeled microglial processes. These processes lost their typical thin branched appearance, indicating that the cell phenotype had acquired activated characteristics. Sildenafil together with CPZ decreased Iba-1 expression, and the microglia exhibited thin, highly branched processes, typical of a latent state (Figure 4(f)).

Immunoblotting for IL-1β and IFN-γ revealed basal expression of these cytokines in the iNOS$^{-/-}$ control group (Figures 5(a) and 5(c)). Cuprizone strongly increased the expression of these cytokines (both $P < 0.001$), compared to the control group (Figures 5(a)–5(d)), which indicates neuroinflammation. Sildenafil treatment resulted in a significant decrease of IL-1β and IFN-γ compared to the cuprizone group ($P < 0.01$). However, the levels of IL-1β and IFN-γ remained higher in the sildenafil-treated group when compared with baseline levels of the control group ($P < 0.01$ and $P < 0.001$, resp.).

Mice without iNOS underwent a significant increase in eNOS expression, compared to wild-type animals.

Untreated mice iNOS$^{-/-}$ (control group; $P < 0.01$) and iNOS$^{-/-}$ animals treated with CPZ or CPZ + Sild (both $P < 0.001$) showed a significant increase in eNOS levels, compared with wild-type mice that did not undergo treatment. The expression of eNOS in iNOS$^{-/-}$ mice treated with CPZ and CPZ plus sildenafil was not significantly higher when compared to iNOS$^{-/-}$ control animals but showed a propensity to increase (Figures 6(a) and 6(b)).

Cuprizone decreased GSTpi, indicating the depletion of mature oligodendrocytes. Sildenafil increased GSTpi expression in the cerebellum of iNOS$^{-/-}$ mice.

GSTpi, a marker of myelinating oligodendrocytes, was expressed in the cerebellum of the control group (Figure 7(a)). After treatment with CPZ, this marker decreased significantly ($P < 0.001$) compared to the control group, suggesting an impairment of mature oligodendrocytes and consequent myelin damage. Sildenafil treatment together with CPZ induced partial recovery of the GSTpi marker, indicating that sildenafil had a protective effect on mature

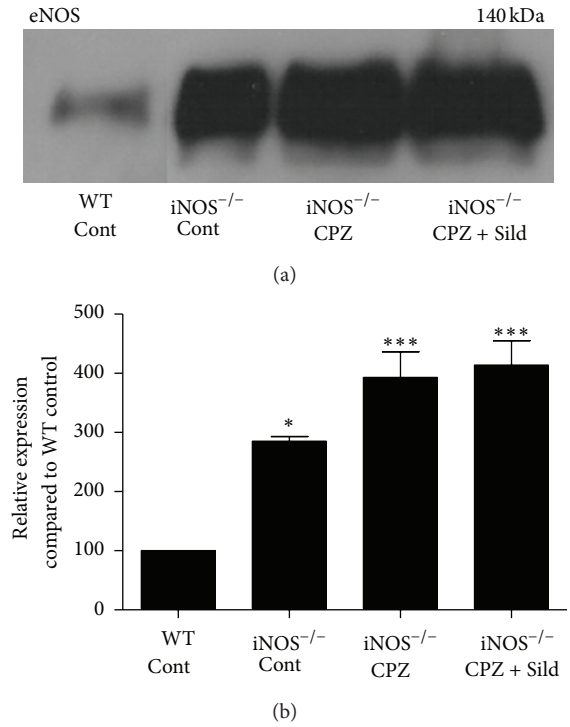

Figure 6: Western blotting for eNOS. (a) Immunoblot of wild-type mice control (WT cont), iNOS$^{-/-}$ mice control (iNOS$^{-/-}$ cont), iNOS$^{-/-}$ animals treated with cuprizone (iNOS$^{-/-}$ CPZ), and iNOS$^{-/-}$ animals treated with cuprizone plus sildenafil (iNOS$^{-/-}$ CPZ + Sild). (b) Graph represents quantification and statistical analysis. eNOS was physiologically expressed in WT mice. Animals without iNOS without treatment showed a significant increase of this enzyme, compared to WT control. After CPZ and CPZ plus sildenafil, iNOS$^{-/-}$ animals also showed a significant increase of eNOS, compared to WT animals, but no significant difference was identified between iNOS$^{-/-}$ control and treated animals. The experiment was performed in triplicate. The results were expressed as mean ± SE. $^{*}P < 0.05$, $^{***}P < 0.001$, compared to control.

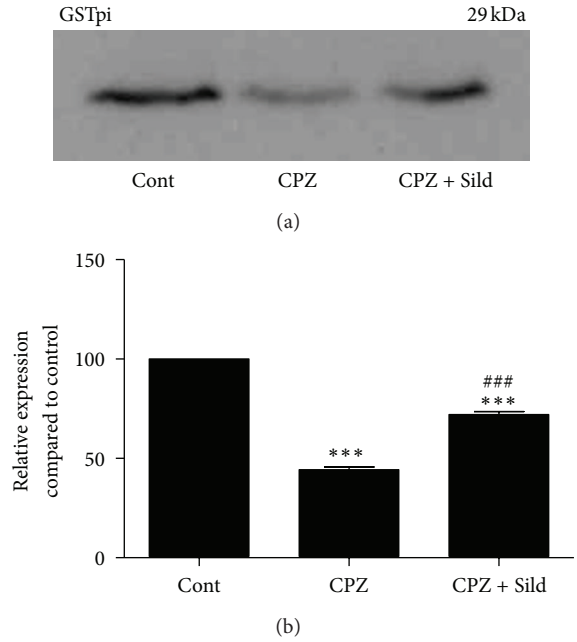

Figure 7: Western blotting for GSTpi. (a) Immunoblot of iNOS$^{-/-}$ control (Cont), cuprizone (CPZ), and cuprizone plus sildenafil (CPZ + Sild) groups. (b) Graph represents quantification and statistical analysis. iNOS$^{-/-}$ control showed basal expression of GSTpi. After CPZ, GSTpi decreased significantly, compared to iNOS$^{-/-}$ cont. CPZ + Sild treatment increased GSTpi expression in relation to the CPZ group, but the levels of this protein remained significantly decreased compared to control. The experiment was performed in triplicate. $^{***}P < 0.001$ compared to control; $^{###}P < 0.001$ compared to CPZ.

oligodendrocytes. In the sildenafil group, GSTpi decreased in comparison with control ($P < 0.001$) but increased in comparison to the cuprizone group ($P < 0.001$; Figures 7(a) and 7(b)).

Myelin sheath structure was disorganized in animals without iNOS which did not undergo treatment (control). Cuprizone induced more severe myelin disruption. Sildenafil improved myelin structure and ultrastructure.

Interestingly, iNOS$^{-/-}$ animals without treatment (control) had moderate disorganization of the myelin structure and ultrastructure (Figures 8(a), 8(d), and 8(g)). Standard LFB staining showed that the control group iNOS$^{-/-}$ had vacuoles in white matter (arrow in Figure 8(a)), resulting in inhomogeneous and disorganized tissue. Qualitative analysis of ultrathin cerebellum sections by TEM revealed that the myelin sheath was often shredded, with blanks, and without the characteristic lamellar pattern (arrows in Figures 8(d) and 8(g)).

Cuprizone-treated animals exhibited more severe damage to myelin structure and ultrastructure (Figures 8(b), 8(e), and 8(h)). LFB staining (Figure 8(b)) showed vacuoles (arrows) and spaces (arrowhead) as slits in white matter, characteristic of highly disorganized tissue. TEM revealed that CPZ caused serious damage to the myelin sheath ultrastructure, which had numerous spaces between the shreds in practically all fibers (represented by arrows in Figures 8(e) and 8(h)). The typical lamellar pattern was entirely absent.

Simultaneous treatment with sildenafil and CPZ resulted in a noticeable improvement of myelin organization (Figures 8(c), 8(f), and 8(i)). White matter was more homogeneous, presenting fewer and, in general, smaller vacuoles and slit-like spaces (Figure 8(c)). Ultrathin section analysis revealed a more preserved myelin sheath which rarely showed signs of shredding.

4. Discussion

The cuprizone model is characterized by primary and reversible demyelination, due to peripheral immune system-independent myelin injury [25]. In this model, demyelination is accompanied by a well-characterized sequence of events involving the depletion of mature oligodendrocytes, microglia activation, and astrocyte proliferation [26]. Therefore, demyelination and resident neuroinflammation induced

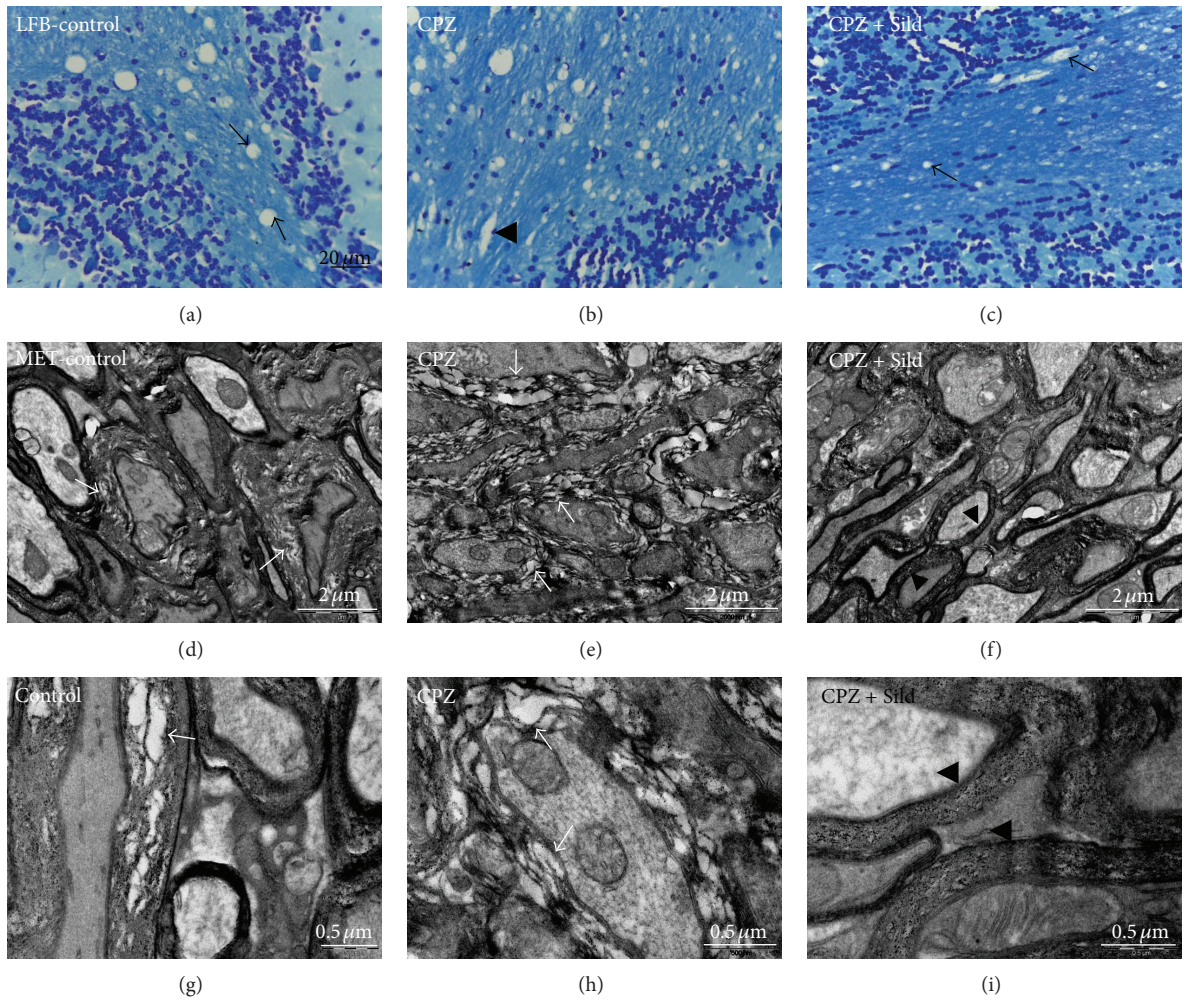

Figure 8: Luxol Fast Blue (LFB) staining ((a)–(c)) and electron micrographs ((d)–(i)). (a), (d), and (g) represent iNOS$^{-/-}$ control group; (b), (e), and (h) represent CPZ-treated animals; (c), (f), and (i) represent CPZ + Sild-treated animals. Arrows in (a), (c): vacuoles in the white matter; arrowhead in (b): spaces between fibers; arrows in (d), (e), (g), and (h): damaged myelin sheath; arrowheads in (f), (i): preserved myelin. Bars = 20 μm ((a)–(c)); 2 μm ((d)–(f)); 0.5 μm ((g)–(i)).

by cuprizone in rodents have been widely used as a model for MS [20, 27]. In the present study, the usefulness of the cuprizone model is considerable as it allows evaluation of the role of the iNOS/NO-sGC-cGMP pathway in the inflammation and demyelination mediated by resident CNS cells.

The cerebellum was chosen for analysis, as it is an important CNS affected region in MS patients, revealing severe white matter atrophy [28, 29]. Furthermore, the authors of the present study had recently investigated this region [22]. The present data, relating to iNOS$^{-/-}$ animals, will be discussed with reference to this previous study. Nunes et al. (2012) [22] showed that in C57BL/6 wild-type (WT) mice cuprizone induced tissue damage, increased GFAP, Iba-1, and COX-2 expression, and caused demyelination, in comparison to the control group. However, cuprizone did not affect the expression of cytokines (TNF-α, IFN-γ, IL-1β, and IL-2) in WT mice. In the previous study by the authors, sildenafil reduced GFAP and Iba-1 expression in comparison to the cuprizone group, preserved myelin and axon ultrastructure, and significantly downregulated IFN-γ, TNF-α, IL-1β, IL-2, and COX-2 expression in comparison to the control and/or cuprizone groups. The previous results demonstrated the protective effect of sildenafil on the cerebellum. Sildenafil promotes the accumulation of cGMP, which is the main NO signaling molecule. It was considered important to investigate the role of NO in the MS-model and the effects of sildenafil more profoundly. Therefore, in the present study, the relationship between iNOSnull mice, the MS-model, and the effects of sildenafil in the cerebellum was investigated.

In the absence of iNOS/NO, cuprizone significantly increased expression of GFAP, TNF-α, COX-2, Iba-1, IL-1β, and IFN-γ. In addition, cuprizone intoxication decreased GSTpi, a marker for myelinating oligodendrocytes, damaged myelin, and induced tremors, abnormal walking, and posture. This data indicates that cuprizone intoxication occurred

even without the iNOS-NO system and was stronger than in WT mice, where cuprizone did not increase cytokine expression [22]. Interestingly, $iNOS^{-/-}$ control animals showed an altered myelin structure.

It was hypothesized that, in the absence of iNOS, eNOS may be overexpressed as a compensatory mechanism. Bernardini et al. [30] showed that treatment with endotoxin influenced NOS expression, upregulating iNOS and, simultaneously, downregulating eNOS. There appeared to be a regulatory relationship between the expression of iNOS and eNOS. In fact, it was found here that eNOS was strongly expressed in $iNOS^{-/-}$ animals in comparison with WT mice. The eNOS levels remained high after the administration of cuprizone and cuprizone plus sildenafil. In normal conditions, low levels of NO produced by both eNOS and nNOS participate in cell signaling and regulate physiologic processes [31]. However, eNOS overexpression (and consequently, constant high concentration of NO) can be responsible for myelin changes and proinflammatory susceptibility in $iNOS^{-/-}$ animals.

On the other hand, iNOS possesses an important feedback mechanism in inflammatory conditions, when the increase of this enzyme is self-regulated, and induces a reduction of some proinflammatory proteins [32–35]. The inhibition of iNOS activity induces enhancement of IL-1β, IL-6, and TNF-α levels [36] and, subsequently, a persistent increase of iNOS expression, downregulating the TNF receptor [35]. In the present study, absence of iNOS may explain increased TNF-α, IFN-γ, and IL-1 after cuprizone treatment in $iNOS^{-/-}$ mice, while cuprizone did not increase these cytokines in WT mice [22]. This feedback mechanism is coregulated by a high concentration of cGMP [32]. Therefore, another hypothesis for explaining the more severe inflammation induced by cuprizone in mice without iNOS is the absence of iNOS feedback mechanism.

Interestingly, although sildenafil had a low anti-inflammatory effect on $iNOS^{-/-}$ mice, it considerably improved the myelin structure of mice without iNOS. Cuprizone is a copper chelator which leads to direct oligodendrocyte death with subsequent demyelination [37]. In this model, oligodendrocyte death and demyelination are independent of immune and inflammatory response. It was found that cGMP analog (8-Br-cGMP) protects differentiated oligodendrocytes from death initiated by staurosporine, thapsigargin, or kainate [38]. It is possible that sildenafil, through the accumulation of cGMP, has a direct beneficial effect on oligodendrocytes, protecting these cells and improving myelination, independent of its anti-inflammatory effects.

In conclusion, the findings of the present study show that $iNOS^{-/-}$ mice are more susceptible to cuprizone intoxication due to the potential involvement of two mechanisms: (1) iNOS-negative feedback mechanism in inflammatory conditions is absent and, consequently, proinflammatory proteins, such as cytokines and COX-2, are excessively increased; (2) eNOS is overexpressed by a compensatory mechanism and generates chronically high levels of NO, damaging the tissue. Also, the results of the present study suggest that sildenafil may exert its anti-inflammatory effects mainly through iNOS inhibition, by cGMP-iNOS feedback. In addition, sildenafil may have a direct protective effect on oligodendrocytes. Further studies are required to explain the molecular mechanism of sildenafil protection in the central nervous system.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

Catarina Raposo and Ana Karolina Santana Nunes were involved in the treatment of animals, development of WB, IF and MET testing procedures, analysis and interpretation of results, and the drafting of the paper. Catarina Raposo and Ana Karolina Santana Nunes contributed equally to the development of this study. Rayana Leal de Almeida Luna and Shyrlene Meiry da Rocha Araújo contributed to the treatment of animals, development of IH and LFB testing procedures, and analysis and interpretation of results. Maria Alice da Cruz-Höfling contributed to the analysis and interpretation of data and paper revision. Christina Alves Peixoto contributed to the supervision of technical procedures, the analysis and interpretation of data, and paper revision and gave final approval of the version to be published.

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CAPÍTULO 3

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Research Report

Involvement of AMPK, IKβα-NFκB and eNOS in the sildenafil anti-inflammatory mechanism in a demyelination model

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ABSTRACT

Sildenafil (Viagra®) has recently been found to have a neuroprotective effect, which occurs through the inhibition of inflammation and demyelination in the cerebellum. However, the mechanism of action of sildenafil remains unknown. AMPK, the regulatory protein of the lipid and glucose metabolism, plays a protective role by activating the eNOS enzyme. The production of a nanomolar concentration of NO by eNOS has an anti-inflammatory effect through the cGMP signaling pathway and plays an important role in the regulation of the nuclear transcription factor (NFkB), preventing the expression of inflammatory genes. The present study investigated whether AMPK–eNOS–NO–cGMP–IKβα–NFkB is involved in the mechanism of action of sildenafil in a cuprizone-demyelination model. Neuroinflammation and demyelination induced by cuprizone in rodents have been widely used as a model of MS. In the present study, five male C57BL/6 mice (7–10 weeks old) were used. Over a four week period, the groups received: cuprizone (CPZ) 0.2% mixed in feed; CPZ in the diet, combined with the administration of sildenafil (Viagra®, Pfizer, 25 mg/kg) orally in drinking water, starting concurrently (sild-T0) or 15 days (sild-T15) after the start of CPZ. Control animals received pure food and water. The cerebella of the mice were dissected and processed for immunohistochemistry, immunofluorescence (frozen), western blotting and dosage of cytokines (Elisa). CPZ induced an increase in the expression of GFAP, IL-1β TNF-α, total NFkB and inactive AMPK, and prompt microglia activation. CPZ also induced a reduction of IKβα. The administration of sildenafil reduced the expression of the pro-inflammatory cytokines IL-1β and TNF-α and increased the expression of the anti-

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inflammatory cytokine IL-10. In addition, the administration of sildenafil reduced expression of GFAP, NFkB, inactive AMPK and iNOS, and increased IKβα. Interestingly, sildenafil also reduced levels of NGF. In general, the sild-T0 group was more effective than sild-T15 in improving clinical status and promoting the control of neuroinflammation. The present study offers evidence that sildenafil has anti-inflammatory and neuroprotective effects, which are probably achieved through modulation of AMPK–IKβα–NFκB signaling. In addition, eNOS may play a role in the sildenafil neuroprotective mechanism, contributing to the activation of AMPK. However, other pathways such as MAPK–NFkB and the downstream proteins AMPK (AMPK–SIRT1–NFκB) should also be further investigated. An understanding of these mechanisms of action is critical for the clinical use of sildenafil to control neuroinflammation in neurodegenerative diseases such as MS.

1. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory disorder characterized by demyelination of the nerve cells, which leads to severe psychomotor impairment. Although the autoimmune/inflammatory nature of MS has already been described, the control of the disease through the use of immunosuppressant and immunomodulators has been shown to be unsatisfactory. Therefore, the identification of new drugs with the potential to interfere in the pathogenesis of this disease is of major importance.

Sildenafil citrate (Viagra®, Pfizer), a phosphodiesterase (PDE)-5 inhibitor, is widely used to treat erectile dysfunction and pulmonary hypertension (Derchi et al., 2014; McMurray et al., 2007). It has been shown that Sildenafil reduces apoptosis in the brain and weakens the effect of learning and memory impairments in different mouse aging models (Puzzo et al., 2014; Orejana et al., 2015), elevates the pain threshold level of diabetic neuropathic mice (Wang et al., 2011), improves neurogenesis (Zhang et al., 2002) and recovers the brain plasticity of rats after a stroke (Zhang et al., 2005). The protective effects of sildenafil have also been demonstrated in MS animal models. Sildenafil inhibits demyelination, protects mature and immature oligodendrocytes, decreases microgliosis and astrogliosis and expression of proinflammatory cytokines in cuprizone and experimental autoimmune encephalomyelitis (EAE) MS-models (Pifarre et al., 2011, 2014; Nunes et al., 2012; Raposo et al., 2013). Regarding the mechanism of the drug, it has been demonstrated that brain-derived neurotrophic factor (BDNF), a recognized neuroprotectant in EAE, is up-regulated by sildenafil in immune and neural cells. Microarray analysis has also shown a notable up-regulation of several members of the granzyme B cluster (GrBs). Immunostaining revealed expression of GrBs in Foxp3+-T regulatory cells (Tregs) suggesting that these proteases have a role in sildenafil-induced suppression of T effector cells (Teffs) (Pifarre et al., 2014). Despite this comprehensive range of positive effects, however, the mechanism underlying the benefits of sildenafil in the central nervous system (CNS) are not yet fully understood. Furthermore, sildenafil has previously been tested in MS–animal models simultaneous to the onset of induced clinical disease, something that is clearly not possible in human patients, where diagnosis is often a lengthy process. The early administration of the drug prevents disease progression, but it has not been demonstrated if sildenafil protects the CNS after the disease has already been installed. A more detailed understanding of the mechanism of action of sildenafil, and whether the drug has a protective role even after the onset of MS, are therefore important points to be clarified.

AMP-activated protein kinase (AMPK) is an intracellular energy sensor that plays a central role in the regulation of glucose and lipid metabolism (Jing et al., 2008; Ronnett et al., 2009). AMPK activators down-regulate inflammation *in vitro* and *in vivo* in various animal models (Davis et al., 2006; Łabuzek et al., 2010; Nath et al., 2009a) and the loss of AMPK exacerbates the effects of experimental autoimmune encephalomyelitis (EAE) (Nath et al., 2009c). In addition, studies have shown that AMPK is up-regulated in activated astrocytes during reactive gliosis (Turnley et al., 1999) and has a role in the prevention of astrocyte apoptosis (Blázquez et al., 2001). On the other hand, the anti-inflammatory activity of AMPK is exerted through multiple signaling pathways, including the phosphorylation and activation of endothelial nitric oxide synthase (eNOS) and production of nitric oxide (NO). NO may act as an endogenous activator of AMPK, suggesting a reciprocal relationship between AMPK and eNOS (Zhang et al., 2008). Also, NO can directly inhibit the activity of NFκB, through the phosphorylation of IKβα, a NFκB inhibitory protein (Aizawa et al., 2003; Grumbach et al., 2005; Lee et al., 2012).

Therefore, as sildenafil has been indicated as a drug with potential application in the treatment of MS through modulation of the inflammatory phenomena, the present study aimed to contribute to the understanding of the mechanism by which this drug acts in the control of neuroinflammation in a cuprizone demyelination model, investigating the involvement of the AMPK–IKβα–NFκB signaling pathway and the eNOS/NO system. The hypothesis to be evaluated was whether the AMPK and IKβα–NFκB signaling participates in the protective effect of sildenafil against inflammation caused by cuprizone demyelination. The influence of the constitutive eNOS-derived NO will be also analyzed. In addition, two periods of oral treatment (15 and 30 days) were evaluated, to provide information about the time course of the effects of sildenafil, important for the possible use of this

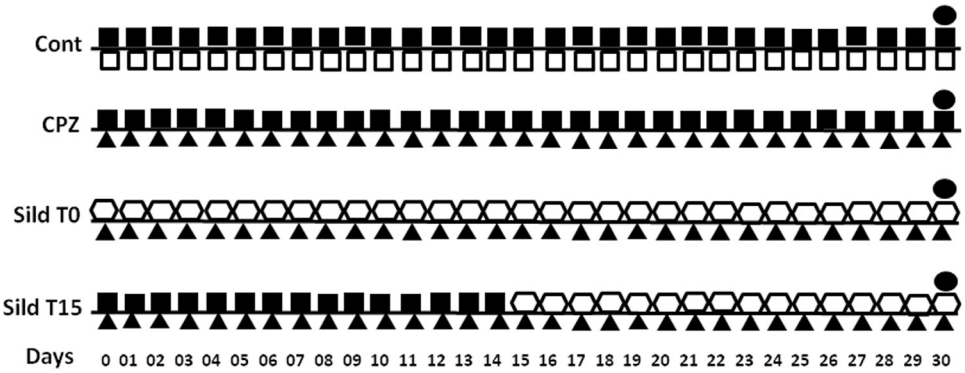

Fig. 1 – Schematic representation of experimental design. Horizontal lines show the treatments in each group. Solid squares represent pure water; unfilled squares represent standard food; solid triangles represent cuprizone (0.2%) mixed into the food; unfilled lozenges indicate sildenafil (25 mg/kg) in the water; solid circles indicate euthanasia. Group One (Cont) received standard food and pure water only and no treatment. Group Two (CPZ) received 0.2% cuprizone mixed into food over a period of 30 days starting on day 0 and pure water. Group Three (Sild T0) received cuprizone in food and sildenafil in water, starting on day 0 and continuing to day 30. Group Four (Sild T15) received cuprizone in food and sildenafil from day 15 to day 30 ($n=5$ mice/group).

treatment in MS patients. Understanding sildenafil targets in a cuprizone demyelination model can improve clinical intervention protocols for the treatment of demyelinating diseases.

2. Results

2.1. Sildenafil improves clinical score

The clinical signs were recorded by three observers and scored accordingly. Five animals from each treatment group were observed. The experimental design is demonstrated schematically in Fig. 1. The control group exhibited typical exploratory behavior and motor function, tail posture and tone were normal; this group scored 0 (no abnormal signals). The cuprizone-treated mice exhibited arched (shortened) posture, tail tone down and tremors, and demonstrated difficulty in exploring their environment; this group was classified as grade 2 (1.8 ± 0.12). Animals that received 25 mg/kg sildenafil treatment starting on day 0 (T0 group) displayed no or mild tremors, and their capacity to explore their environment appeared close to normal; these mice were classified with a score of 0.5 (0.4 ± 0.24). In this group, however, one mouse showed no improvement in clinical signs. Mice receiving 25 mg/kg sildenafil treatment starting on day 15 (T15 group) showed tremors and an arched posture, inferior clinical improvement to group (T0), and were classified with a score of 1 (1.3 ± 0.12). The clinical scores for each group are shown in Fig. 2.

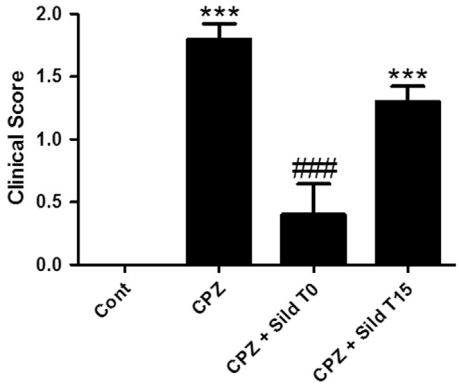

Fig. 2 – Clinical score measured on day 30th. The clinical signs of each animal ($n=5$/group) were scored and recorded by three independent observers. An arbitrary scale was provided in accordance with the evolution of illness and aggravation of clinical signs at the end of treatment: 0 – no sign of illness; 1 – tremors; 2 – shortened posture and ataxia. Intermediate values were acceptable. $^{***}p < 0.001$, compared to control; $^{###}p < 0.001$, compared to CPZ.

2.2. Sildenafil attenuates reactive astrogliosis

Immunohistochemical data revealed astrocytes of control mice with normal phenotype and basal expression of GFAP in the molecular layer (Bergman glia), among neurons of the Purkinje and in the granular layers (Fig. 3A). After treatment with cuprizone (CPZ), the astrocytic processes were thicker and more intensely labeled for GFAP, indicating reactive gliosis (Fig. 3B). Conversely, CPZ-treated mice from the T0 and T15 groups displayed reduced GFAP labeling when compared to the CPZ group untreated with sildenafil. Moreover, the astrocyte phenotype of the T0 and T15 groups (Fig. 3C) was similar to the phenotype of the control group (Fig. 3D).

WB data showed that treatment with CPZ for 30 days significantly increased expression of GFAP, compared with the basal level. In contrast, mice from the T0 and T15 groups exhibited a significant reduction in GFAP levels, when compared with the CPZ group (Fig. 3E and F).

2.3. Sildenafil decreases NFkB expression and increases IKβα, its inhibitory protein, in parallel with attenuation of microglia activation

Iba-1 (microglia marker) and NFκB (latent gene regulatory protein) double immunolabeling in control animals showed

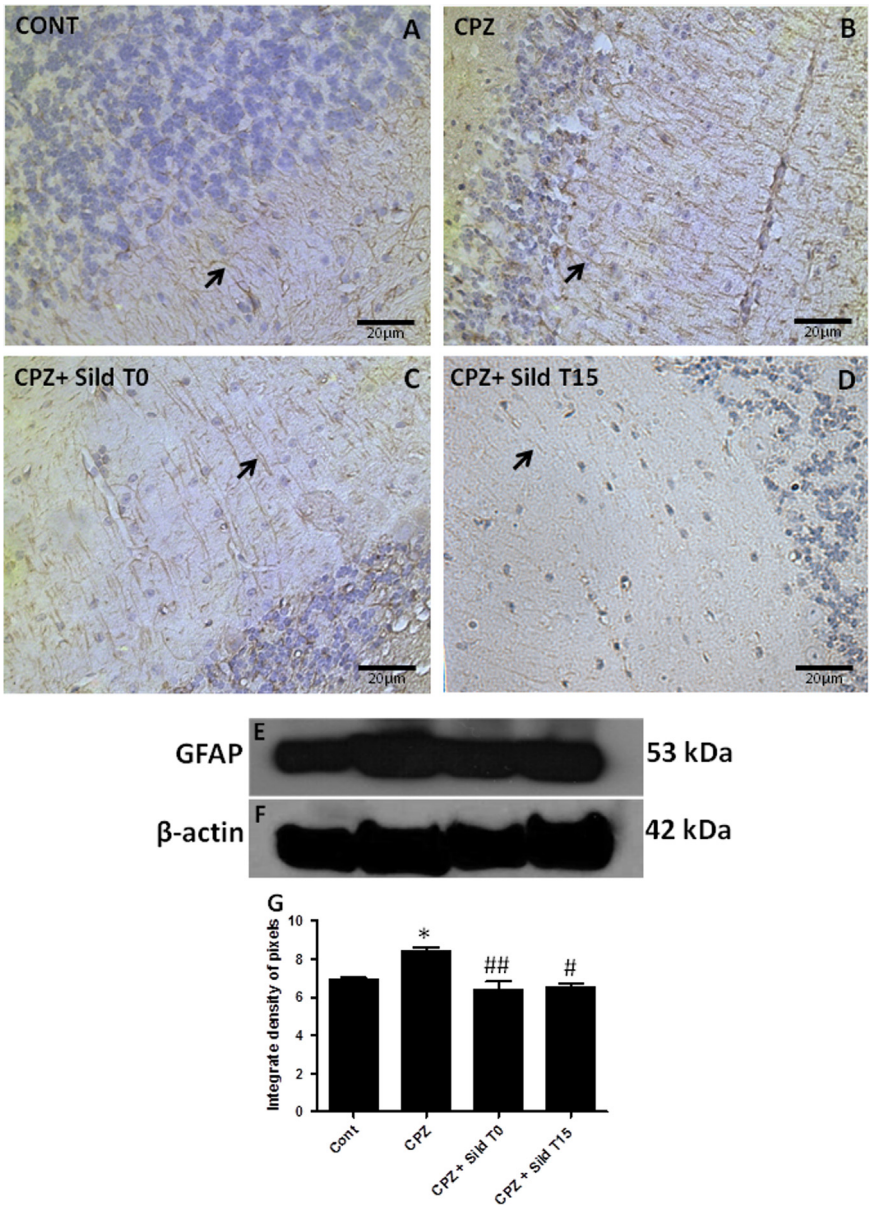

Fig. 3 – Immunohistochemistry (A–D) and immunoblotting (E–G) for GFAP in cerebellum. (A) Controls showed the physiological expression of GFAP, located in the Bergmann glia in the molecular layer and between neurons in the Purkinje and Granular layers. (B) CPZ induced astrogliosis, increasing both GFAP expression and the thickness of the astrocytic processes in all layers of the cerebellum. (C and D) Administration of sildenafil (T0 and T15) recovered the physiological expression of this intermediate filament. (E) Immunoblotting showed that CPZ treatment increased GFAP, compared with the control animals ($*p<0.05$). Treatment with sildenafil T0 and T15 decreased the expression of GFAP in relation to the CPZ group ($^{\#}p<0.05$, $^{\#\#}p<0.01$). β-acti immunolabeling was performed as a control for equal loading. Bars=20 mm.

basal expression of Iba-1 and an apparent phenotype of vigilant microglia (latent), with few cytoplasmic processes (Fig. 4A). Likewise, no labeling was found for the nuclear transcription factor, NFκB, suggesting inactivated microglia (Fig. 4B; merge in Fig. 4C). In contrast, after CPZ treatment, the microglia displayed an activated phenotype, characterized by a number of processes, as well as stronger Iba-1 labeling, compared to the control group (Fig. 4D). Iba1 is a microglia/macrophage-specific calcium-binding protein, which displays actin-bundling activity and participates in membrane ruffling and phagocytosis in activated microglia (Ohsawa et al., 2004). It can be presumed that Iba1 positive microglia in CPZ animals are activated and phagocytic. Furthermore, intense NFκB labeling was detected and the merge panel with Iba and NFκB double labeling indicated that the nuclear transcription factor was expressed by microglia, as there is an overlap in the labeling of both (Fig. 4E; merge in Fig. 4F). The microglia of the sildenafil (T0) group showed few cytoplasmatic processes and decreased Iba-1 labeling, compared to the CPZ group (Fig. 4G) while NFκB labeling was not observed (Fig. 4H; merge in Fig. 4I). The microglia of the sildenafil T15 group showed many cytoplasmic processes, and the expression of both

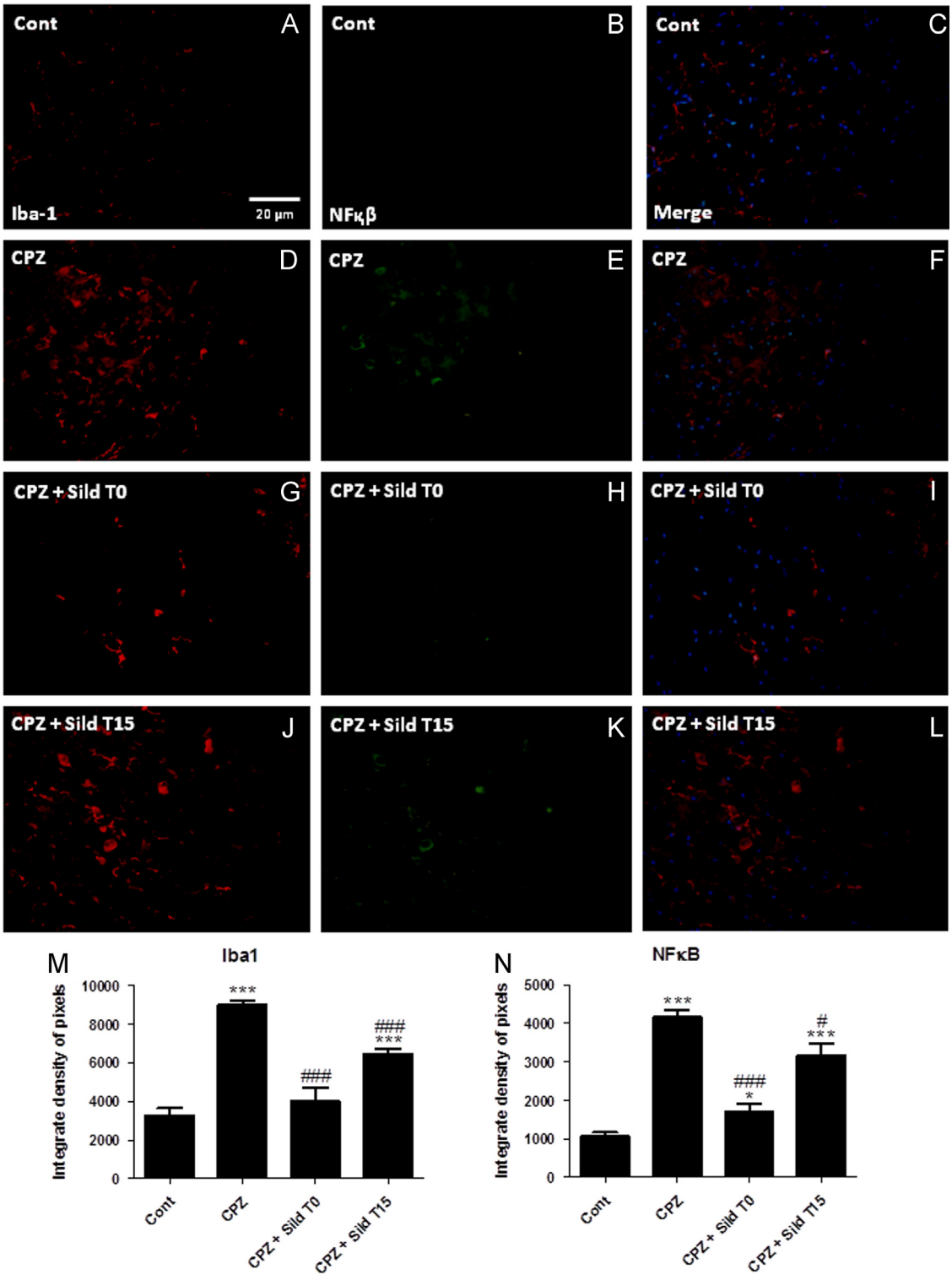

Fig. 4 – Immunofluorescence for Iba-1 (A, D, G, J), NFkB (B, E, H, K) and merge (C, F, I, L) in cerebellum. (A–C) The control group displayed a low expression of Iba-1 and NFκB. (D–F) Treatment with cuprizone (CPZ) increased Iba-1 and NFκB expression, suggesting activation and inflammatory response of microglia. Merge (F) shows overlapping of Iba-1 and NFκB. Administration of sildenafil reduced the expression of Iba-1 and NFκB, compared with the CPZ group, with this effect more visible when starting treatment concomitantly with CPZ (T0) (G–I), than starting 15 days after CPZ (T15) (J–L). Bars = 20 μm. Quantification of Iba-1 (M) and NFκB (N) expression is shown. $^{*}p < 0.05$, $^{***}p < 0.001$, compared with control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, compared with CPZ.

Iba-1 and NFκB (Fig. 4J) decreased, in comparison with the CPZ group, but was greater than the control animals (Fig. 4K; merge in Fig. 4L). The Iba1 and NFκB quantitative comparison and statistical analysis between the groups are shown in the graphs (Fig. 4M and N).

In agreement with immunohistochemistry data, concentrations of the nuclear transcription factor NFκB and the NFκB inhibitor IKβα in the cerebella lysates (WB data) of control mice displayed very slow expression (Fig. 5A–D). In contrast, in cerebella lysates of mice receiving CPZ, NFκB expression increased significantly, whereas IKβα expression decreased, suggesting activation of NFκB and gene regulation. Treatment with sildenafil (T0) significantly decreased NFκB levels and strongly increased IKBα levels in comparison with the CPZ group, whereas treatment with sildenafil (T15) decreased NFκB and maintained an unchanged IKBα level compared to baseline.

2.4. Sildenafil changes the markers of metabolic regulation and inflammation

A possible regulatory role of sildenafil on metabolic and inflammation, induced by a CPZ demyelinating effect, was assessed by measuring AMP-activated Protein Kinase (AMPK) levels. The likely participation of the NOS/NO system in such a sildenafil mechanism was also evaluated. The WB results showed basal levels of iNOS, eNOS, and total AMPK (inactive) in the cerebella lysates of control animals (Fig. 6A–F). Immunohistochemistry also showed total AMPK (Fig. 7A–E) and phosphoAMPK (active) in the control group (Fig. 7F–J). Treatment with CPZ did not change iNOS and eNOS levels, comparing to the basal level of the control group. However, CPZ promoted significant increases in the expression of total AMPK and reduced phosphoAMPK, indicating glucose and metabolic imbalance. AMPK may also be involved in the regulation of inflammation induced by CPZ. Treatment with sildenafil for 30 days (T0 group) significantly decreased the expression of iNOS and total AMPK and increased phosphoAMPK, in comparison with the CPZ group. The eNOS levels were significantly elevated in T0 animals, possibly indicating feedback between this enzyme and AMPK. Feedback may also occur between iNOS and eNOS. Sildenafil treatment starting on day 15 (T15 group) significantly decreased total AMPK and iNOS content in cerebella lysates and tissue sections, compared to the control and/or CPZ groups, whereas the eNOS level remained steady. PhosphoAMPK decreased in T15 animals, compared to the control group.

2.5. Sildenafil decreased serum levels of cytokines

Control mice showed minimum levels of IL1β, and low levels of IL-2, TNF-α and NO (nitric oxide). Levels of IL-1β and TNF-α increased significantly in CPZ-treated mice compared to control, but IL-2 and NO remained unchanged. Treatment with sildenafil (T0) and (T15) decreased levels of IL-1β and TNF-α, compared to the CPZ group, but did not affect IL-2. Expression of NO was increased significantly in animals treated with sildenafil (T0), in relation to the control group (Fig. 8A–D).

2.6. Sildenafil increases levels of IL-10 and decreases NGF levels

To evaluate whether sildenafil has a neuronal protective effect, western blotting analysis of the anti-inflammatory regulators IL-10 (Fig. 9A and B) and nerve growth factor (NGF) (Fig. 9C and D) was performed. Control animals displayed physiological levels of both proteins. Mice treated with CPZ showed a significant increase in NGF whereas IL-10

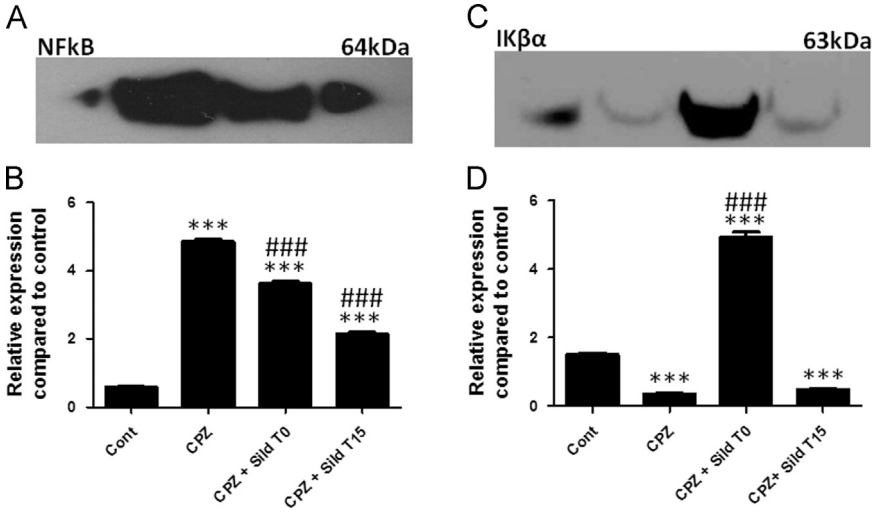

Fig. 5 – Immunoblotting for NFkB (A and B) and its inhibitory protein IKβα (C and D) in cerebellum. The control group showed basal expression of both proteins. NFκB was detected in minimum amounts. CPZ induced an increase in NFκB expression, concomitantly with a decrease in IKβα levels. Treatment with sildenafil starting on day 0 (T0) reduced the expression of NFκB and increased IKβα, compared with the CPZ group. Sildenafil treatment starting on day 15 (T15) decreased NFκB expression, but did not change IKβα levels, compared with the CPZ group. β-actin immunolabeling was performed as a control for equal loading (not shown). $^{***}p<0.001$, compared with control; $^{###}p<0.001$, compared with CPZ.

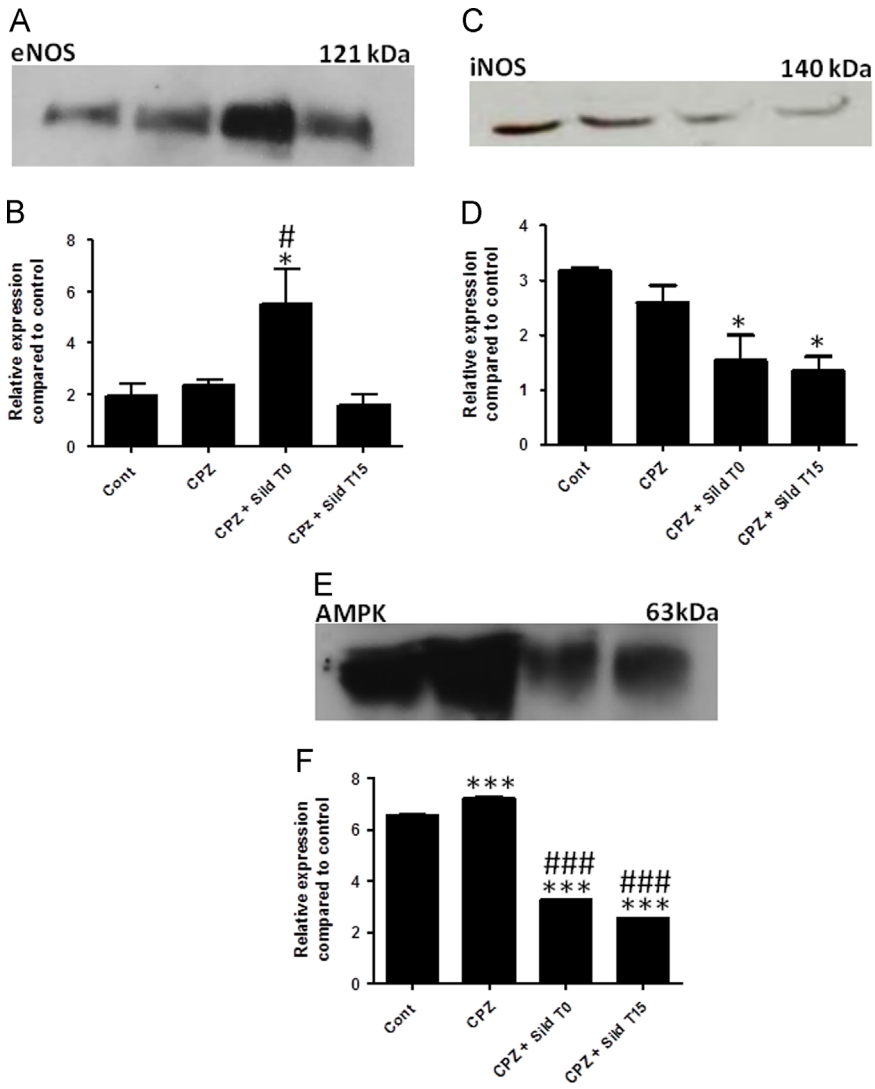

Fig. 6 – Immunoblotting for eNOS (A and B), iNOS (C and D) and AMPK (E and F) in cerebellum. Controls showed a basal expression of all enzymes. CPZ treatment did not change significantly the expression of eNOS and iNOS, however increased AMPK. The treatment with sildenafil significantly increased eNOS (T0, but not T15) and decreased iNOS and AMPK (T0 and T15), compared to CPZ and/or to control. β-actin immunolabeling was done as a control for equal loading (not showed). $^{*}p<0.05$, $^{***}p<0.001$, comparing with control; $^{###}p<0.001$, comparing with CPZ.

expression remained stable. This may indicate a reaction of the damaged tissue as it tries to recover the injured cells. Treatment with Sildenafil significantly increased levels of IL-10 at T0 but not T15, indicating an anti-inflammatory effect. Interestingly, sildenafil treatment (T15) significantly decreased NGF expression, compared with the CPZ and control groups.

3. Discussion

By examining eNOS/NO, AMPK and/or IKβα–NFκB, the present study intended to investigate the mechanisms by which sildenafil reduces microglial and astroglial reactive gliosis and neuroinflammation in a CPZ-demyelinating mouse model. The study was performed in the cerebellum, as previous studies by the group of the authors of the study have shown the anti-inflammatory effects of sildenafil in the cerebellum of mice-MS model induced by cuprizone (Nunes et al., 2012; Rapôso et al., 2013) and it is important to clarify the mechanism of protection. The action of sildenafil in improving the clinical status of patients with MS was initially attributed to the induction of neurogenesis; however, recent data has suggested that sildenafil is a modulator of inflammation in the central and peripheral nervous system and protects the myelin sheath both in pathological and healthy conditions (Nunes et al., 2012; Pifarre et al., 2011; Raposo et al., 2013, 2014, Garcia et al., 2014). Furthermore, intracellular accumulation of cGMP in different models of inflammation reduces production of pro-inflammatory cytokines such as IFN-γ, TNF-α and interleukins (ILs), and reduces oxidative stress, modulating inflammatory response (Scheiblich et al., 2014). In addition, inhibition of PDEs appears to block the inflammatory response of microglia, reducing changes to the

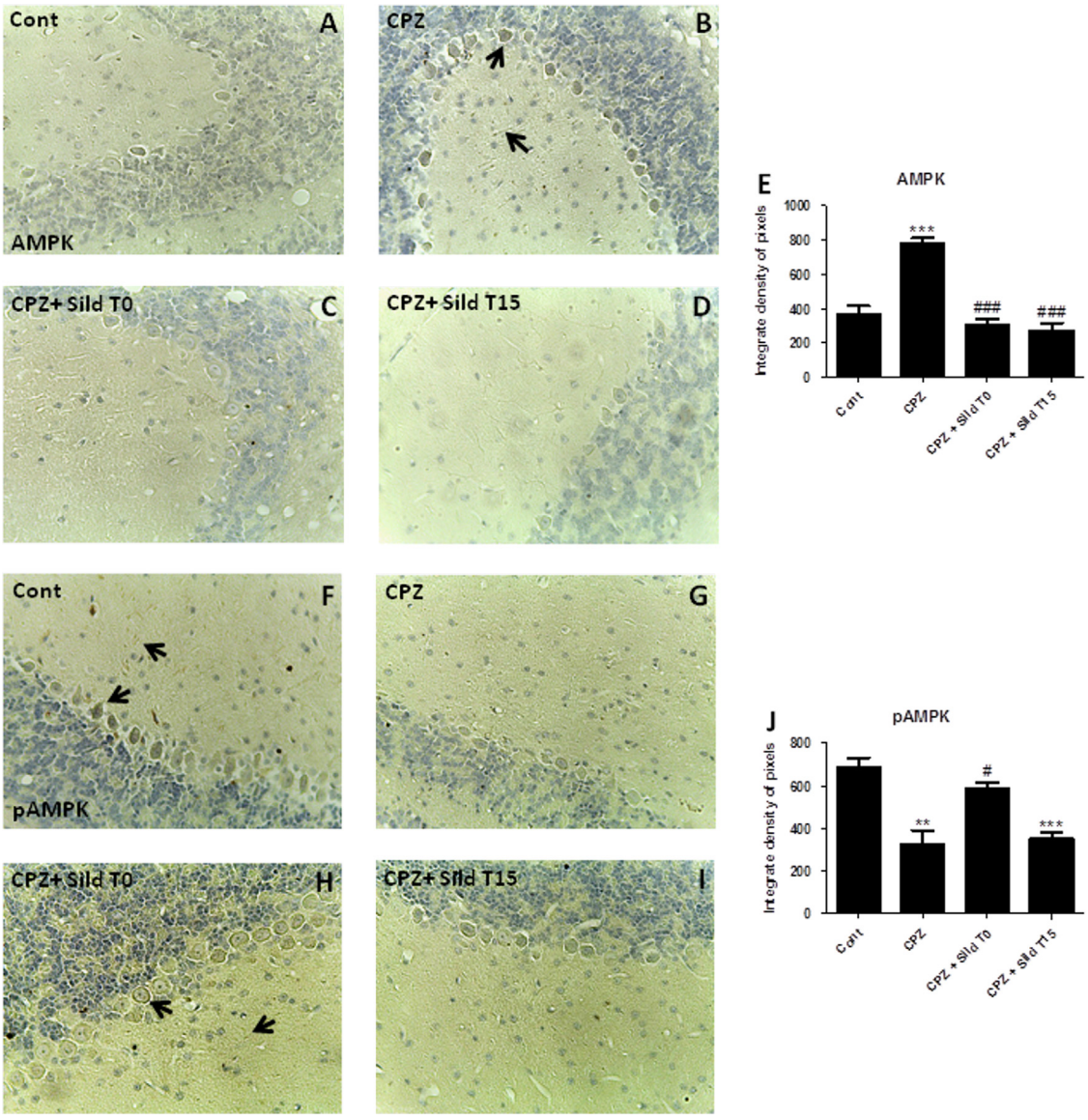

Fig. 7 – Immunohistochemistry for AMPK (A–E) and pAMPK (F–J) in cerebellum. Controls (A and F) showed basal expression of both proteins, located in the Purkinje cells and in processes and nuclei at molecular layer (arrows in F). Treatment with CPZ increased AMPK (arrows in B) and reduced pAMPK immunolabeling (G), suggesting regulation of glucose and lipid metabolism. Administration of sildenafil reduced AMPK (T0 and T15) (C and D) and increased pAMPK (T0, but not T15) (H and I) immunolabeling, in comparison with the CPZ group. Bars = 20 μm. Quantification of AMPK (E) and pAMPK (J) immunolabeling is shown. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, compared with CPZ.

myelin sheath (Paris et al., 2000; Wakita et al., 2003). Corroborating this data, the results of the present study found that sildenafil reduced the inflammatory process in a CPZ-demyelinating mouse model, suggesting that more effective results are attained through earlier and longer sildenafil intervention. The results of the present study also suggest that the mechanism of sildenafil in the CNS involves modulation of AMPK, IKβα–NFκB and/or eNOS/NO. These findings are innovative, as to the best of the authors' knowledge no other work has yet described the involvement of AMPK, IKβα–NFκB or eNOS/NO in the central nervous system of cuprizone in an *in vivo* mouse model. In addition, the therapeutic strategy used here (oral treatment with sildenafil in two periods) is new and provides relevant information for the possible use of this drug in patients.

AMP-activated protein kinase (AMPK), an enzyme involved in energy homeostasis, regulates inflammatory responses, yet its precise mechanisms are not fully understood. Several studies have reported that the activation of AMPK by 5-aminoimidazole-4-carboxamide riboside (AICAR) can inhibit acute and chronic colitis (Bai et al., 2010), inflammation in cystic fibrosis (Myerburg et al., 2010), proinflammatory effects after lung injury (Zhao et al., 2008), and is therapeutically efficient in an autoimmune encephalomyelitis (EAE) animal model of MS (Nath et al., 2005). Recent evidence suggests that activation of AMPK may suppress the activation of NFκB, thus contributing to inflammation regulation (Zhao et al., 2011).

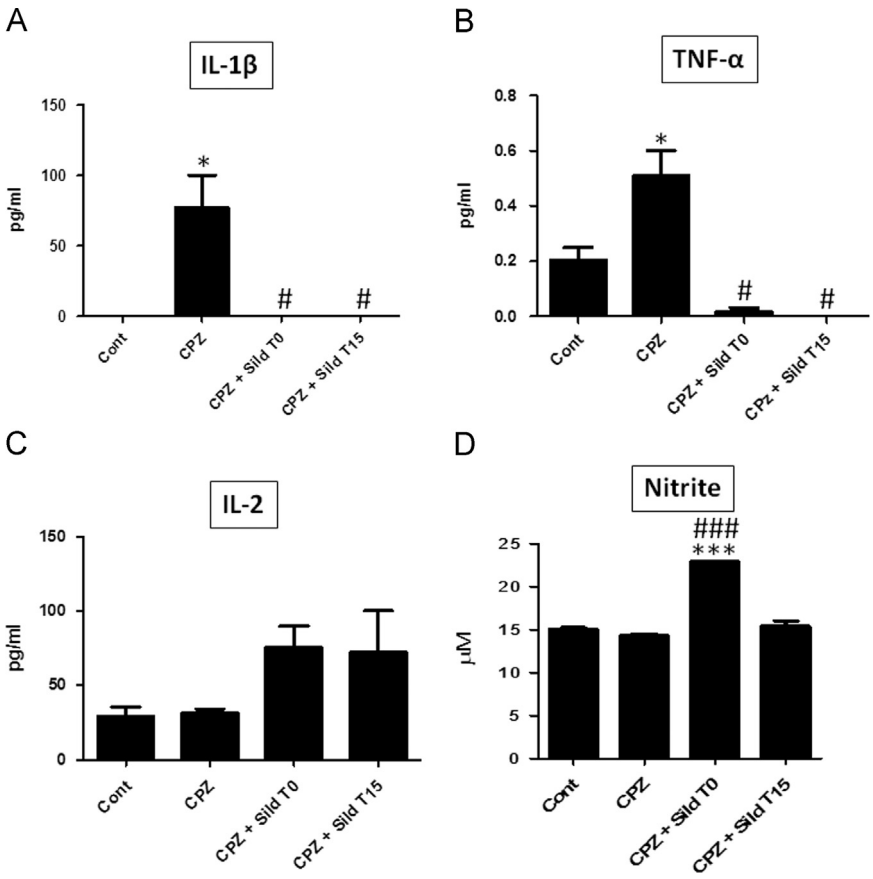

Fig. 8 – Cytokines levels measured by ELISA in serum samples. Controls showed low or undetectable levels of cytokines and basal level of nitrite. CPZ significantly increased IL-1β (A) and TNF-α (B), but did not change IL-2 (C) and nitrite levels (D). Sildenafil treatment (T0 and T15) decreased IL-1β and TNF-α, compared with the CPZ group, but did not change IL-2. Interestingly, sildenafil (T0 but not T15) increased nitrite levels, compared to control and CPZ groups. $^{*}p<0.05$, $^{***}p<0.001$, compared with control; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, compared with CPZ.

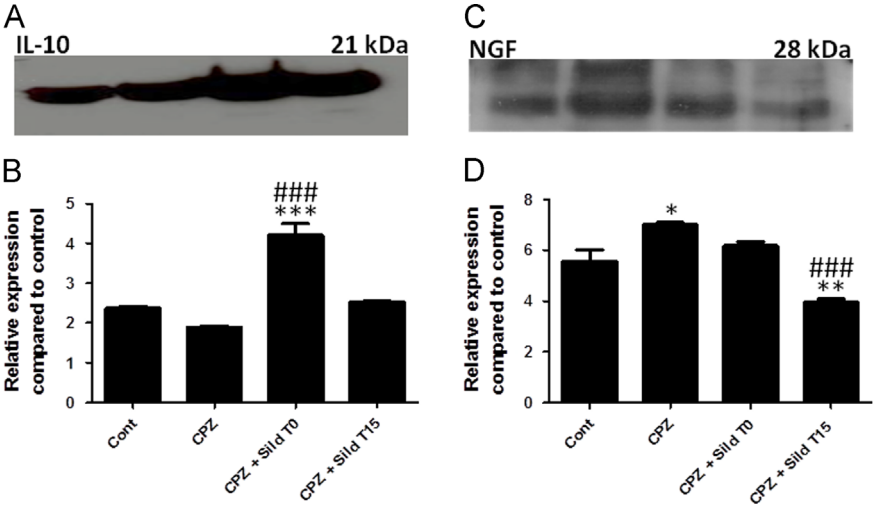

Fig. 9 – Immunoblotting for anti-inflammatory and protective markers, IL-10 (A and B) and NGF (C and D) in the cerebellum. Control animals showed basal levels of IL-10 and NGF. CPZ administration did not change IL-10, however it significantly increased NGF. Sildenafil treatment (T0) increased IL-10, compared to the control and CPZ groups. Interestingly, sildenafil (T15) significantly reduced NGF. β-actin immunolabeling was performed as a control for equal loading. $^{*}p<0.05$, $^{**}p<0.05$, $^{***}p<0.001$, compared with control; $^{\#\#\#}p<0.001$, compared with CPZ.

However, the NFκB subunits are not direct phosphorylation targets of AMPK, but instead the inhibition of NFκB signaling is mediated by several downstream targets of AMPK, such as SIRT1 (silent information regulator 1) (Schug et al., 2010; Yang et al., 2007), p53 (Kawauchi et al., 2008, 2009) and Forkhead box O (FoxO) factors (Zhou et al., 2009). According to the results of the present study, sildenafil treatment starting either at day 0 or 15 from the beginning of CPZ ingestion reduced non-phosphorylated (inactive) AMPK and, starting at day 0 only, increased phospho-AMPK (active). In addition, sildenafil decreased NFκB expression and increased its inhibitory protein, IKβα. However, whether AMPK induces NFκB inhibition, and what downstream targets may be involved in this inhibition, require further investigation.

It has been established that NO can stimulate NFκB activation. However, evidence also suggests that NO produced by eNOS inhibits NFκB and acts as an endogenous activator of AMPK, suggesting a reciprocal relationship between AMPK and eNOS. This interaction between NFκB activation, NO production and NFκB inhibition represents a classical negative feedback loop, which prevents sustained activation of NFκB (Zhang et al., 2008). Also, the activation of AMPK by NO represents a positive feedback loop, which contributes to the control of the inflammatory process. Furthermore, NO–cGMP can directly inhibit the activity of NFkB by IKβα activation (its inhibitory protein), or indirectly by activating Protein Kinase A (PKA). Moreover, eNOS gene transcription can directly inhibit NFkB, limiting the local inflammatory process (Castier et al., 2009; Grumbach et al., 2005; Lee, 2013; Verhelst et al., 2011). The data of the present study showed that while inactive AMPK was reduced by sildenafil and phosphoAMPK was activated, eNOS levels were significantly elevated suggesting reciprocity between AMPK and eNOS. In addition, treatment with sildenafil reduced NFκB and increased IKβα. This data indicates that sildenafil activates AMPK, which in turn induces increased eNOS expression and NO production. The hypothesis is that NO starts the feedback loop, further activating AMPK and inhibiting NFκB. On the other hand, the cGMP increase induced by sildenafil can directly or indirectly (by PKA) inhibit the activity of NFkB by IKβα activation (its inhibitory protein), independent of AMPK.

Activators of AMPK such as AICAR and metformin exert down-regulation in the inflammatory parameters in different animal models (Fryer et al., 2002; Giri et al., 2008; Hawley et al., 2002; Kubota et al., 2011; Meares et al., 2013). Metformin can activate AMPK, subsequently inducing the decrease of inflammatory cytokines in an EAE model (Nath et al., 2009b). The data of the present study suggested that the neuroprotective effects of sildenafil may involve NFκB inhibition by AMPK and that eNOS–NO feedback may also be involved. A recent study has shown that sildenafil attenuates the LPS-induced inflammatory response in cultured microglia via protein kinase activated by MAPK–NFκB (Zhao et al., 2011). The involvement of other pathways in a CPZ-demyelinating model needs to be considered.

NFκB is essential for both innate and adaptive immunity (Feng et al., 2014). The factor is an inducible latent gene regulatory protein which is detected in most cell types and is involved in a range of inflammatory processes. It consists of homo- or heterodimers of different subunits and structurally related proteins (Rel/NFkB proteins). The transcriptional activation of the NFκB pathway is controlled by the NFkB inhibitory protein, IKβα. IKβα is phosphorylated by IKβα kinase (IKK), a complex that is composed of the regulatory subunit IKK-α. Besides the involvement of NFκB in T-cell proliferation and activation (Mc Guire et al., 2013), it is also a key element in the coordinate control of gene expression during monocyte/macrophage activation. In particular, the macrophage-derived cytokines, IL-1β and TNF-α, are potent direct activators of NFκB through their own receptors. The expression of IL-1β and TNF-α is in turn controlled by NFκB, thus resulting in a positive feedback loop (Aktan, 2004; Schins et al., 2000). Also, Steinberg et al. (2006) observed that TNF-α suppresses AMPK activity by up-regulating the expression of protein phosphatase 2C, an inhibitor of AMPK signaling, thus contributing to the inflammatory process. In addition to its central mediatory function in cytokine expression, NFkB in myeloid cells may be induced by physical as well as oxidative stress of cells, or in other words via the iNOS (Carling et al., 2012; Zhang et al., 2008) or cyclooxygenase- 2 (COX-2) (Yi et al., 2011). The present study found that sildenafil reduces IL-1β, TNF-α and iNOS expression. This can directly contribute to the reduction of NFκB.

Over-activated microglia and astrocytes can cause overproduction of NO and various pro-inflammatory cytokines, including TNF-α and IL-1β, a factor which is believed to contribute to progressive neuronal damage in inflammation-mediated neurological disorders, such as traumatic injury, stroke, Alzheimer's, Parkinson's and Huntington's diseases and multiple sclerosis (Hroudová et al., 2014; Parent, 2003; Tran et al., 2011). In MS, the breakdown of the blood brain barrier (BBB) following CNS inflammation allows immune mediators from the peripheral immune system to cross the barrier. In this condition NGF, which is mainly produced by astrocytes and stored in the extracellular matrix, can inhibit the inflammatory infiltrates by preventing perivascular macrophages and mast cells from crossing the BBB. This interaction protects CNS integrity and aids recovery after inflammation (Flügel and Bradl, 2001). Also, levels of NGF increase in the cerebrospinal fluid (Laudiero et al., 1992) and optic nerves (Micera et al., 1999) of patients affected by MS. This may represent a mechanism of response to promote tissue repair and protect against CNS tissue inflammation. The efficacy of recombinant NGF in promoting myelin repair was evaluated using the EAE model in the common marmoset (Colafrancesco and Villoslada, 2011). This model has many similarities with MS, including a chronic relapsing course, primary inflammatory demyelination, and changes in magnetic resonance imaging brain scans. This study showed that the continuous intra cerebroventricular infusion of recombinant human NGF prevented the full development of EAE lesions. The brain of the marmoset treated with NGF showed a reduction of inflammation and demyelination. These effects were attributed to the capacity of NGF to modify the CNS microenvironment by inducing an anti-inflammatory effect (down-regulation of IFN-γ production by infiltrating T-cells and up-regulation of IL-10 by glial cells). Indeed, the infusion of NGF led to reduced BBB reduced breakdown. Given these results, initial treatment with sildenafil proved beneficial, as it increased the levels of

the anti-inflammatory cytokine IL-10, important for the repair of neuronal tissue damage. Interestingly, sildenafil reduced NGF expression. As in the CPZ group NGF increased (and is increased during EAE and in MS patients), it is conceivable that the increase of this growth factor is a mechanism for the control of the inflammatory environment, but, once inflammation is controlled, levels of NGF quickly decrease.

4. Conclusion

In summary, it was found that better results were attained when sildenafil intervention began earlier and continued for longer. This finding is relevant for the orientation of clinical trials. The present study also provides evidence that sildenafil treatment has an anti-inflammatory and neuroprotective action, probably through the modulation of AMPK, NFkB and eNOS/NO signaling. In addition, reduction of IL-1β and TNF-α levels can contribute to the control of neuroinflammation, as these cytokines, by their own receptors, directly activate NFκB. The modulation of IL-10 and NGF seems also to be involved in neuroprotective effects of sildenafil. Other pathways such as MAPK–NFκB and AMPK–SIRT1–NFκB or AMPK–FoxO or p53–NFκB need to be further investigated, as the understanding of their mechanisms of action is critical to the clinical use of sildenafil for the control of neuroinflammation in neurodegenerative diseases such as in MS.

5. Experimental procedure

5.1. *Experimental design*

Four groups of C57BL/6 mice aged 7–10 weeks and weighing 15–20 g were used ($n=5$ per group). The mice were examined for health status, acclimated to the laboratory environment at 25 °C and 12-h light/dark photoperiod and housed in metal cages. The control group ("Group One") received a standard laboratory diet and pure water. Over a period of 30 days, the experimental groups received 0.2% cuprizone (oxalic bis-cyclohexylidenehydrazide – Sigma-Aldrich Inc., St. Louis, MO, USA) mixed into their food. The second group ("Group Two") received no treatment; a third group ("Group Three") received 25 mg/kg of sildenafil (Pfizer Inc., New York, NY, USA) from day 0 to day 30 (T0) through drinking water in accordance with previously described standard methods (Ramamurthy and Ronnett, 2006; Saraiva et al., 2009; Zhang et al., 2002), and a fourth group ("Group Four") received 25 mg/kg of sildenafil from day 15 to day 30 (T15) through the drinking water. The experimental design is demonstrated schematically in Fig. 1. Body weight was measured every week and the drug concentration in the water was adjusted daily to maintain the dose. The clinical signs of each animal were measured on day 30 of the experiment by three independent observers. An arbitrary scale was provided in accordance with the evolution of the illness and aggravation of clinical signs at the end of treatment: 0 – no sign of illness; 1 – tremors; 2 – shortened posture and ataxia. Intermediate values were acceptable. All experiments were carried out in compliance with ethical guidelines for animal experimentation (L-10/2010-CEUA/FIOCRUZ). After treatment, the experimental and control animals were anaesthetized with a mixture of ketamine (115 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) 1:1 (Sespo Comércio e Indústria Ltda., São Paulo, SP, Brazil) and euthanized as described below.

5.2. *Immunohistochemistry*

After anesthesia, the animals were transcardially perfused with physiological saline (20 ml), followed by 40 ml of 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate (sodium phosphate monobasic and dibasic heptahydrate – Sigma-Aldrich) buffered saline (PBS), pH 7.2. The cerebella were immediately removed and post-fixed in the same fixative overnight. The samples were dehydrated in an ethanol series (Isofar Chemical Co., RJ, Brazil), cleared in xylene and embedded in paraffin (Merck, #1071642504). Sections (5 μm thickness) were cut with a RM 2035 microtome (Reichert S, Leica), re-hydrated, washed in 0.05 M PBS and incubated in PBS with 1% bovine serum albumin (BSA, fraction V) (Miles, Naperville, IL, USA) for one hour. Endogenous peroxidase was blocked and antigen retrieval was performed by pre-treating the sections with 20 mM citrate buffer, pH 6.0, at 100 °C, for 30 min. Sections of the cerebella of all groups were incubated with rabbit polyclonal primary antibody anti-GFAP (glial fibrillary acidic protein) or anti-phosphoAMPK overnight at 4 °C. After washing, sections were overlaid for 1 h with a biotin-conjugated secondary antibody, using horseradish peroxidase (HRP) – kit (DakoCytomation, CA, USA, Biotinylated Link Universal HRP; #K0690) and visualized with 3′-3-diaminobenzidine as a chromogen. Slices were then weakly counter-stained with Harris' hematoxylin and mounted in entellan (Merck, #1079610100). Details of all antibodies are provided in Table 1. Reaction was performed in three slices per animal ($n=5$ per group) and analysis was performed in five panels per reaction ($5 \times 3 = 15$ panels per animal).

5.3. *Immunofluorescence (IF)*

After anesthesia, the animals were transcardially perfused with physiological saline (20 ml), followed by 4% paraformaldehyde (Sigma-Aldrich) (40 ml) in 0.1 M PBS, pH 7.2. The cerebella were immersed overnight in 15% sucrose, and then in 30% sucrose for a second night (36 h total). The specimens were then embedded in OCT-TissueTek (Sakura Finetek, Torrance, CA, USA) and frozen in n-hexane (Dinâmica, São Paulo, SP, Brazil) and cooled with liquid nitrogen. Cryo-sections, 8 μm thick, were permeabilized with 0.3% Triton X-100 and incubated for 1 h with a blocking solution (3% BSA plus 0.2% Tween 20), in Tris buffered saline, pH 7.4. Subsequently, the same sections were double labeled with anti-rabbit Iba-1 (Ionized calcium binding adapter molecule 1) followed by anti-mouse NFkB primary antibodies overnight, and then incubated with polyclonal Cy2- and Cy3-conjugated secondary antibodies (Jackson, #715-225-150, and #705-165-147, respectively) against rabbit and mouse immunoglobulin (1:200) for 1 h. The sections were rinsed in PBS solution and mounted in fluorescent Prolong Gold Antifade medium (Life Technologies #P36930) for

Table 1 – Antibodies used for immunofluorescence and western blot.

Primary antibody	Commercial source	Catalog number	Species	Antibody type	Working concentration
GFAP	Dako Company Ltd.	Z0334	Rabbit	Polyclonal	IHC 1:100 WB 1: 5000
Iba-1	Wako Pure Chemical Industries, Ltd.	019-19741	Rabbit	Polyclonal	IHC 1:250
NFκB	Santa Cruz Biotechnology	sc109	Rabbit	Polyclonal	IHC 1:100 WB 1:250
IκB-α	Santa Cruz Biotechnology	sc-371	Rabbit	Polyclonal	WB 1: 1000
AMPK-α1	Abcam	ab3759	Rabbit	Polyclonal	WB 1:1000
eNOS	BD Biosciences	610299	Rabbit	Polyclonal	WB 1:1000
iNOS	BD Biosciences	610332	Rabbit	Polyclonal	WB 1:1000
NGF	Abcam	ab6199	Rabbit	Polyclonal	WB 1:1000
IL-10	Abcam	ab33471	Rabbit	Polyclonal	WB 1:1000

observation under an inverted fluorescence microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and using Release 4.7.2 image analysis software. Reaction was performed in three slices per animal ($n=5$ per group) and analysis was performed in five panels per reaction ($5\times3=15$ panels per animal).

5.4. *Western blotting (WB)*

After anesthesia, animals were euthanized and the cerebella were quickly dissected and homogenized in an extraction cocktail (10 mM EDTA, Amresco, Solon, USA; 2 mM phenylmethane sulfonyl-fluoride, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 10 μg of aprotinin/ml and 100 mM Tris, pH 7.4). The cerebella of five animals were mixed and homogenized to form a pool from each group. WB was performed in accordance with Nunes et al. (2012). Briefly, proteins (40 μg total) were separated on 12% (GFAP, eNOS, iNOS, IKβα, IL-10, NGF) or 14% (AMPK, NFkB,) acrylamide gel. After overnight incubation with 5% non-fat milk, primary antibodies against NFkB p65, IKβα, AMPK, GFAP, eNOS, iNOS, IL-10 and NGF were incubated for 4 h followed by anti-rabbit HRP-conjugated (1:80,000, #A9169), anti-mouse (1:1000, #A0168) or anti-goat secondary antibodies (1:100,000, #A5420), all from Sigma-Aldrich. For quantification, the pixel density of each immunoblot was determined using Image J 1.38 software (http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD, USA). For each protein, the results were confirmed in three sets of experiments. Immunoblotting for β-actin (1:1000, Sigma-Aldrich, #A2228) was performed as a control for equal loading.

5.5. *Dosage of IL-1β, IL- 2 and TNF-alpha (ELISA)*

The quantification of IL-1β, IL-2 and TNF-alpha in serum samples was performed using R&D Systems Quantikine Mouse TNF-alpha, IL-2 or IL-1β immunoassay kits (#MTA00B) in accordance with the manufacturer's instructions. Monoclonal antibodies specific for mouse TNF-α, IL-2 or IL-1β were pre-coated on the microplate. Standards, controls and samples were pipetted into the wells and any TNF-alpha, IL-2 or IL-1β present was bound to the immobilized antibody. After washing away any unbound substance, an enzyme linked polyclonal antibody specific for mouse TNF-alpha, IL-2 or IL-1β was added to the wells. Following washing, a substrate solution was added. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of mouse TNF-alpha, IL-2 or IL-1β bound at the initial steps. The sample values were then read off the standard curve. All dosages were performed in triplicate.

5.6. *Measurement of NO*

Measurement of nitric oxide production in the serum used Griess colorimetric assay (Green et al., 1982) to detectnitrite (NO_2^-) resulting from oxidation of NO. Samples were diluted fourfold with distilled water and deproteinized by adding 1/20 volume of a zinc sulfate solution (300 g/L), to give a final concentration of 15 g/L. After 3.500 g centrifugation for 10 min, 100 μL of samples were added to an ELISA plate (96 wells) in duplicate, followed by the same volume of Griess reagent. To prepare a standard curve, a solution of sodium nitriteat an initial concentration of 100 μM was serially diluted in PBS. After incubation for 10 min in the dark, the absorbance of the reaction product was read at 490 nm to allow information of nitrite concentration to be obtained. The absorbance of different samples was compared with the standard curve.

5.7. *Statistic analysis*

Values were analyzed using the GraphPad Prism software package (San Diego, CA, USA; v. 5.0). One-way analysis of variance (ANOVA) followed by Dunnett's and/or Tukey's post-test were used to compare groups. The results were expressed as mean±S.E., where appropriate. A p-value <0.05 indicated statistical significance.

Conflict of interest statement

The author(s) declared that no conflicts of interest exist with respect to the authorship and/or publication of this article.

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CAPÍTULO 4

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Research paper

Phosphodiesterase-5 inhibition promotes remyelination by MCP-1/CCR-2 and MMP-9 regulation in a cuprizone-induced demyelination model

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ABSTRACT

While it has recently been shown that sildenafil (Viagra®) has a protective effect on myelination/remyelination, the mechanism of this protection is still unknown. In general, cytokines, chemokines and metalloproteinases have a pro-inflammatory action, but can also exert a role in modulating glial cell activation, contributing to the balance of cell response. Investigating these molecules can contribute to clarifying the mechanisms of sildenafil neuroprotection. In addition, it is not known whether sildenafil is able to restore an already installed neurodegenerative process or if the treatment period is critical for its action. The aim of the present study was to evaluate, in a cuprizone (CPZ)-induced demyelination model, the effects and mechanisms of time-dependent treatment with sildenafil (beginning 15 days after neurodegeneration and continuing for 15 days, or starting concomitantly with neurodegeneration and continuing for 30 days) on neuroinflammation and remyelination. Neuroinflammation and demyelination induced by CPZ in rodents has been widely used as a model of multiple sclerosis (MS). In the present study, five male C57BL/6 mice aged 7–10 weeks were used per group. For four weeks, the groups received either cuprizone (CPZ) 0.2% mixed in feed or CPZ combined with the administration of sildenafil (Viagra®, Pfizer, 25 mg/kg) orally in drinking water, starting concurrently with (sild-T0) or 15 days (sild-T15) after the start of CPZ treatment. Control animals received pure food and water. The cerebella were dissected and processed for immunohistochemistry, immunofluorescence (frozen), Western blotting, Luxol fast blue staining and transmission electron microscopy. Magnetic resonance was performed for live animals, after the same treatment, using CPZ 0.3%. CPZ induced an increase in the expression of IL-1β and a decrease in MCP-1, CCR-2, MBP and GST-pi, as well as promoting damage in the structure and ultra-structure of the myelin sheath. Interestingly, the administering of sild-T0 promoted a further increase of MMP-9, MCP-1, and CCR-2, possibly contributing to changes in the microglia phenotype, which becomes more phagocytic, cleaning myelin debris. It was also observed that, after sild-T0 treatment, the expression of GST-pi and MBP increased and the myelin structure was improved. However, sild-T15 was not efficient in all aspects, probably due to the short treatment period and to starting after the installation of the degenerative process. Therefore, the present study shows that sildenafil modulates inflammation, with the involvement of MMP-9, MCP-1, and CCR-2, and also contributes to myelin repair. These protective effects were dependent on the therapeutic strategy used. This clarification can strengthen research proposals into the mechanism of action of sildenafil and contribute to the control of neurodegenerative diseases such as MS.

1. Introduction

Multiple sclerosis (MS) is a chronic immune-inflammatory disease of the central nervous system (CNS) characterized by demyelination of the white matter and axonal injury. The cerebellum has been described as an important region of the CNS that is affected by neurodegenerative diseases, such as MS, revealing severe white matter atrophy and inflammation (Riccitelli et al., 2012; Shields et al., 2012). In demyelinating diseases, important functions such as electrical conduction, connectivity and axolemal organization are compromised. Consequently, the injured axons become unable to exercise their functions efficiently, leading to severe psychomotor deficits (Bénardais et al., 2013). Therefore, research into new drugs with a

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possible remyelinating or neuroprotective role has been the subject of studies by many research groups.

Various studies have shown, for example, that the inhibition of phosphodiesterase-5 (PDE-5) by sildenafil (Viagra®) reduces neurologic deficits and increases neurogenesis and functional recovery after a stroke in rats (Charriaut-Marlangue et al., 2014; Chen et al., 2014). In terms of myelination, sildenafil has been shown to promote efficient reconstitution of the myelin sheath and control the inflammatory processes involved in demyelination models, including EAE (Experimental Autoimmune Encephalopathy) and CPZ (Cuprizone) (Nunes et al., 2012; Pifarre et al., 2011; Rapôso et al., 2013; Zhou et al., 2009). Furthermore, it has been observed that patients suffering from erectile dysfunction and, in parallel, multiple sclerosis showed an improvement in clinical status for both pathologies (Xiao et al., 2012). Thus, the beneficial implications of this drug may be associated with several factors, including cGMP accumulation in the brain.

The demyelination process is accompanied by the activation of microglial cells and astrocytes, which may assume beneficial or deleterious functions (Allan et al., 2005). The activation of these cells is associated with phenotypic changes, compatible with inflammation. Microglia and astrocytes produce pro-inflammatory cytokines (IL-1β, TNF-α), chemokines and their receptors (MCP-1/CCR-2) (Conductier et al., 2010), as well as metalloproteinase-9 (MMP-9) (Castier et al., 2009). Generally, the excessive expression of these inflammatory mediators is associated with detrimental effects on the tissue, promoting axonal injury (Kim et al., 2012).

During the remyelination process, microglia changes from a pro-inflammatory (M1) to an anti-inflammatory or immunoregulatory (M2) phenotype. Studies in vitro have observed an increase in the differentiation of oligodendrocytes when exposed to microglia M2, leading to a remyelinating state (Miron et al., 2013). The expression of MCP-1/CCR-2 by glial cells may promote this change in the phenotypes of microglia in an attempt to repair the injured environment (Low et al., 2001). Besides the regulation of these chemokines, matrix metalloproteinase (MMP-9) has an important role in controlling demyelinating diseases during the remyelination phase. MMP-9 is involved in tissue and vascular remodeling, oligodendrocyte differentiation and synaptic plasticity (Hsieh et al., 2008; Morello et al., 2011; Oh et al., 1999). A study assessing myelination by Schwann cells in the peripheral nervous system demonstrated that MMP-9 regulates the differentiation of these cells, contributing to remyelination (Kim et al., 2012).

Borán et al. (2008) showed that the cGMP–PKG pathway stimulated the regulation of microglial cell morphology, inducing a dramatic reorganization in the actin cytoskeleton compatible with a protective phenotype, which is more effective at removing dead cells. This data supports the hypothesis that cGMP–PKG signaling can modulate the pathways involved in inflammation, playing a role in the regulation of glial cell activation. This effect can contribute to the resolution of neuroinflammation. However, this mechanism remains unclear; it is possible that MMP-9 and MCP-1/CCR-2 are involved.

Therefore, the aim of the present study was to evaluate the effects and mechanisms of time-dependent treatment with sildenafil (for 15 or 30 days) on the restoration of myelin and balancing of neuroinflammation. The expression of MBP (myelin basic protein), GST-pi (mature oligodendrocytes marker), MCP-1/CCR-2, MMP-9, and IL-1β were evaluated, and the tissue structure and ultrastructure were analyzed by Luxol fast blue staining, transmission electron microscopy and magnetic resonance.

2. Materials and methods

Four groups of C57BL/6 mice aged 7 to 10 weeks and weighing 15 to 20 g were used (n = 5 per group; 20 animals total) in each processing method (80 animals for all experiments). The mice were examined for health status, acclimated to the laboratory environment at 25 °C and a 12-h light/dark photoperiod, and housed in metal cages. All experiments were carried out in compliance with the ethical guidelines for animal experimentation (L-10/2010-CEUA/FIOCRUZ).

2.1. Experiment design

The control group received a standard laboratory diet and pure water. Over a period of 30 days, the experimental groups received 0.2% cuprizone (CPZ) (oxalic bis-cyclohexylidenehydrazide — Sigma-Aldrich Inc., St. Louis, MO, USA) mixed in food. One group received no sildenafil treatment; while a second group received 25 mg/kg of sildenafil (Pfizer Inc., New York, NY, USA) from the first day of CPZ treatment (day 0) until the 30th day (day 30). This group was named T0. A third group received 25 mg/kg of sildenafil from day 15 to day 30 (T15) of CPZ treatment. Sildenafil was administered via drinking water, as previously described by Ramamurthy and Ronnett (2006), Saraiva et al. (2009), and Zhang et al. (2002). Body weight was measured every week and the drug concentration in the water was adjusted daily to maintain the dose (Fig. 1). Following treatment, the experimental and control animals were anesthetized with a mixture of ketamine (115 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) 1:1 (Sespo Comércio e Indústria Ltda., São Paulo, SP, Brazil) and euthanized as described below.

2.1.1. Immunohistochemistry (IH)

After anesthesia, the animals were transcardially perfused with physiological saline (20 ml), followed by 40 ml of 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate (sodium phosphate monobasic and dibasic heptahydrate — Sigma-Aldrich) buffered saline (PBS), pH 7.2. The cerebella were immediately removed and post-fixed overnight in the same fixative. The samples were dehydrated in an ethanol series (Isofar Chemical Co., RJ, Brazil), cleared in xylene and embedded in paraffin (Merck, #1071642504). Sections (5 μm thickness) were cut on an RM 2035 microtome (Reichert S, Leica), re-hydrated, washed in 0.05 M PBS and incubated in PBS with 1% bovine serum albumin (BSA, fraction V) (Miles, Naperville, IL, USA) for 1 h. The endogenous peroxidase was blocked with 10% hydrogen peroxide (10 min), and antigen retrieval was performed by pre-treating the sections with 20 mM citrate buffer, pH 6.0, at 100 °C, for 30 min. Cerebellum sections of all groups were incubated overnight at 4 °C with the rabbit polyclonal primary antibody anti-MBP (Abcam Cambridge, USA; ab40390). After washing, sections were overlaid for 1 h with a biotin-conjugated secondary antibody, using a horseradish peroxidase (HRP) kit (DakoCytomation, CA, USA, Biotinylated Link Universal HRP; #K0690) and visualized using 3′-3-diaminobenzidine as a chromogen. Slices were then weakly counter-stained with Harris hematoxylin and mounted in Entellan (Merck, #1079610100). Reaction was performed in three slices per animal and analysis was performed in five panels per reaction (5 × 3 = 15 panels per animal). The Image Manipulation Program Gimp 2.8 (freely distributed, http://www.gimp.org/) was used in analysis.

2.1.2. Western blotting (WB)

After anesthesia, animals were euthanized and the cerebella were quickly dissected and homogenized in an extraction cocktail (10 mM EDTA, Amresco, Solon, USA; 2 mM phenylmethane sulfonyl-fluoride, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 10 μg of aprotinin/ml and 100 mM Tris, pH 7.4). The cerebella of five animals were mixed and homogenized to form a pool from each group. WB was performed in accordance with Nunes et al. (2012). Briefly, proteins (40 μg total) were separated with 12% (CCR-2, MCP-1, and MMP-9) or 14% (GST-pi and MBP) acrylamide gel. After overnight incubation with 5% non-fat milk, the membranes were incubated for 4 h with primary antibodies against CCR-2 (Ab10396), MCP-1 (Ab9669), MMP-9 (Ab38898), GST-pi (Ab47709) and MBP (Ab7349) (all from Abcam, Canada/US), followed by HRP-conjugated anti-rabbit (1:80,000, #A9169), anti-mouse (1:1000, #A0168) or anti-goat (1:100,000, #A5420) secondary antibodies (all from Sigma-Aldrich). For quantification, the pixel density of each immunoblot was determined using the Image J 1.38 software program (http://rsbweb.

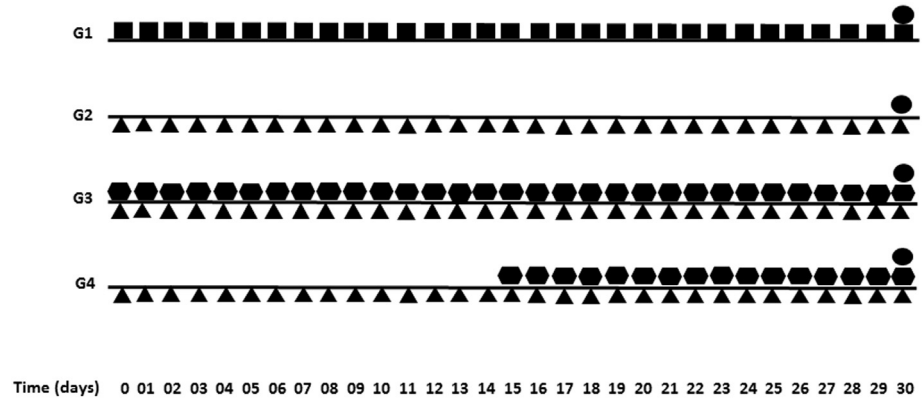

Fig. 1. Experimental design. Black horizontal lines show the sequence of treatments in each group; treatments carried out on the same day are displayed sequentially from the top down. Group 01 (G1) — solid squares represent the control group treated with drinking water and standard diet for 30 days; Group 02 (G2) — solid triangles represent treatment with cuprizone (CPZ 0.2%) mixed into the feed for 30 days and drinking water; Group 03 (G3) — solid lozenge, is concomitant treatment with sildenafil 25 mg/kg in drinking water and CPZ 0.2% mixed in feed on day 0 until day 30; Group 04 (G4) — solid triangles represent treatment with CPZ 0.2% and the solid lozenge treatment with sildenafil 25 mg/kg started on the 15th, concomitant with the CPZ until day 30. Solid circle is euthanasia of animals.

nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD, USA). The results for each protein were confirmed in three sets of experiments. Immunoblotting for β-actin (1:1000, Sigma-Aldrich, #A2228) was performed as a loading control.

2.1.3. Luxol fast blue (LFB)

After anesthesia, the animals were transcardially perfused with physiological saline (20 ml) followed by 4% paraformaldehyde (40 ml) in 0.1 M phosphate-buffered saline (PBS), pH 7.2. Then, the cerebella

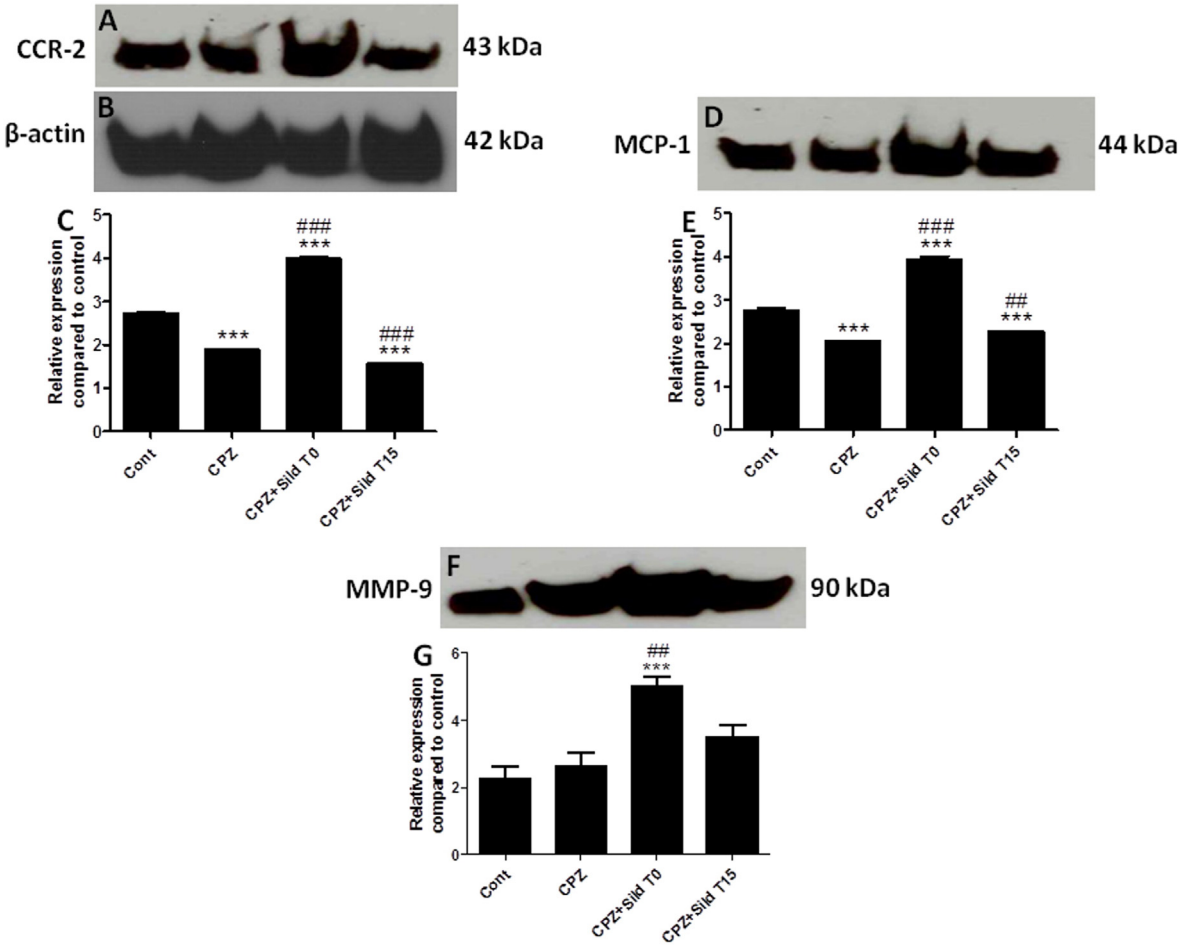

Fig. 2. Immunoblot for CCR-2, MCP-1 and MMP-9. The control group exhibited basal expression of all proteins. Cuprizone treatment significantly decreased the expression of CCR-2 (A, C) and MCP-1 (D, E) and did not change the expression of MMP-9 (F, G). Treatment with sildenafil (T0) significantly increased the expression of CCR-2, MCP-1 and MMP-9 in comparison with the control and cuprizone groups. Sildenafil (T15) did not restore CPZ effects, with the expression of CCR-2 and MCP-1 increasing. The control loaded with β-actin is represented in B. Statistical comparison was performed using one-way ANOVA for parametric measurements, combined with Dunnett's and Tukey's post hoc tests. Unpaired Student's *t*-tests were used to compare each treatment with control. ***$p < 0.001$ compared with control and ###$p < 0.001$ compared with the cuprizone group.

were immediately removed and post-fixed overnight in the same fixative. The samples were dehydrated in a graded ethanol series, cleared in xylol, and embedded in Paraffin (Merck, Rio de Janeiro, RJ, Brazil). Sections 5 μm thick were cut on an RM 2035 microtome (Reichert S, Leica). Myelin was detected using the Luxol fast blue (LFB) stain (Solvent Blue 38, Sigma Chemical Co., St. Louis, MO, USA). Sections were deparaffinized and incubated overnight in 0.1% LFB solution at 56 °C. The following day, slides were cooled at 4 °C and stained sections were differentiated in LI_2CO_3 for 5 min, then briefly in 70% ethanol, followed by counterstaining with eosin. Dehydration was performed in graded alcohol baths and xylene and the sections were cover slipped with Entellan. The sections were observed using a light microscope (Zeiss). Reaction was performed using three slices per animal and analysis was performed in five panels per reaction ($5 \times 3 = 15$ panels per animal). The Image Manipulation Program Gimp 2.8 (freely distributed, http://www.gimp.org/) was used for quantification. In addition, blood vessel diameter was measured, with two diameters of five randomly chosen vessels, using 15 panels per animal, calculated using the Image J 1.38 software program (http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD, USA).

2.1.4. Transmission electron microscopy (TEM)

After anesthesia, the animals were sacrificed by transcardial perfusion with physiological saline (20 ml), followed by 40 ml fixative (2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2). Then, the cerebella were quickly dissected and post-fixed overnight in the same fixative. For routine procedures, after fixation, fragments of the cerebellum were washed twice in the same buffer, post-fixed for 2 h in a solution containing 1% osmium tetroxide, 2 mM calcium chloride and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone, and embedded in Embed 812. Polymerization was performed at 60 °C for 3 days

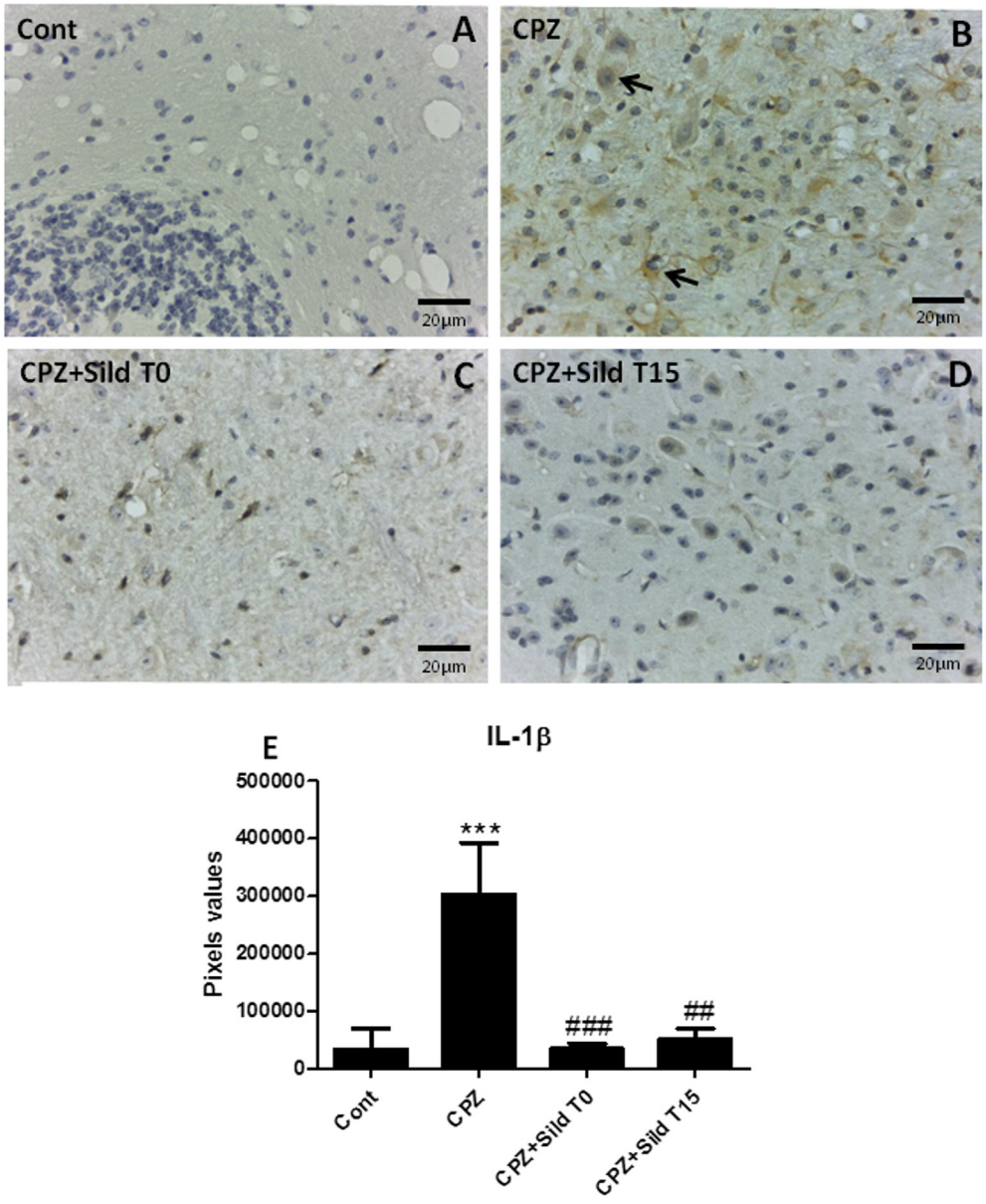

Fig. 3. Immunohistochemistry for IL-1β in the cerebellum. A — Control animals showed minimum amounts of this cytokine. B — Cuprizone treatment induced a significant increase in the expression of IL-1β. C, D — The administration of sildenafil induced a significant decrease for both treatment periods. Arrows indicate the labeling of cellular bodies. Quantification (fifteen arbitrarily selected areas per animal) was performed using the GIMP 2.8 image analysis software (E). n = 5 animals/group. Statistical comparison was performed using one-way ANOVA for parametric measurements, combined with Dunnett's and Tukey's post hoc tests. Unpaired Student's *t*-tests were used to compare each treatment with the control. ***$p < 0.001$ compared with control, ###$p < 0.001$ and ##$p < 0.01$ compared with the cuprizone group. Bars = 20 μm.

(Saraiva et al., 2006). Semi-thin sections (0.5 μm thick) were collected in glass slides, stained with toluidine blue, and used for morphometric analysis of the myelin. Ultrathin sections (70 nm thick) were collected on 300-mesh nickel grids, counterstained with 5% uranyl acetate and lead citrate and examined using a FEI Morgani 268D transmission electron microscope. Semi-quantitative analysis of myelin ultrastructure was performed in ten image fields, by two observers, using an arbitrary scale (3 = no damage, with typical lamellar pattern and preserved axons; 2 = two of structural characteristics of damage, namely collapsed myelin layers, reduced thickness, loss of myelin sheath, swollen axons, detached axon from the sheath; 1 = three of the aforementioned characteristics; 0 = four of the aforementioned characteristics).

2.2. Magnetic resonance image (MRI)

For MRI, the CPZ group received 0.3% cuprizone. This concentration was chosen for MRI only to better demonstrate tissue damage. It is, however, a highly lethal concentration and was not necessary for the other methods. The sildenafil treatments were the same as described

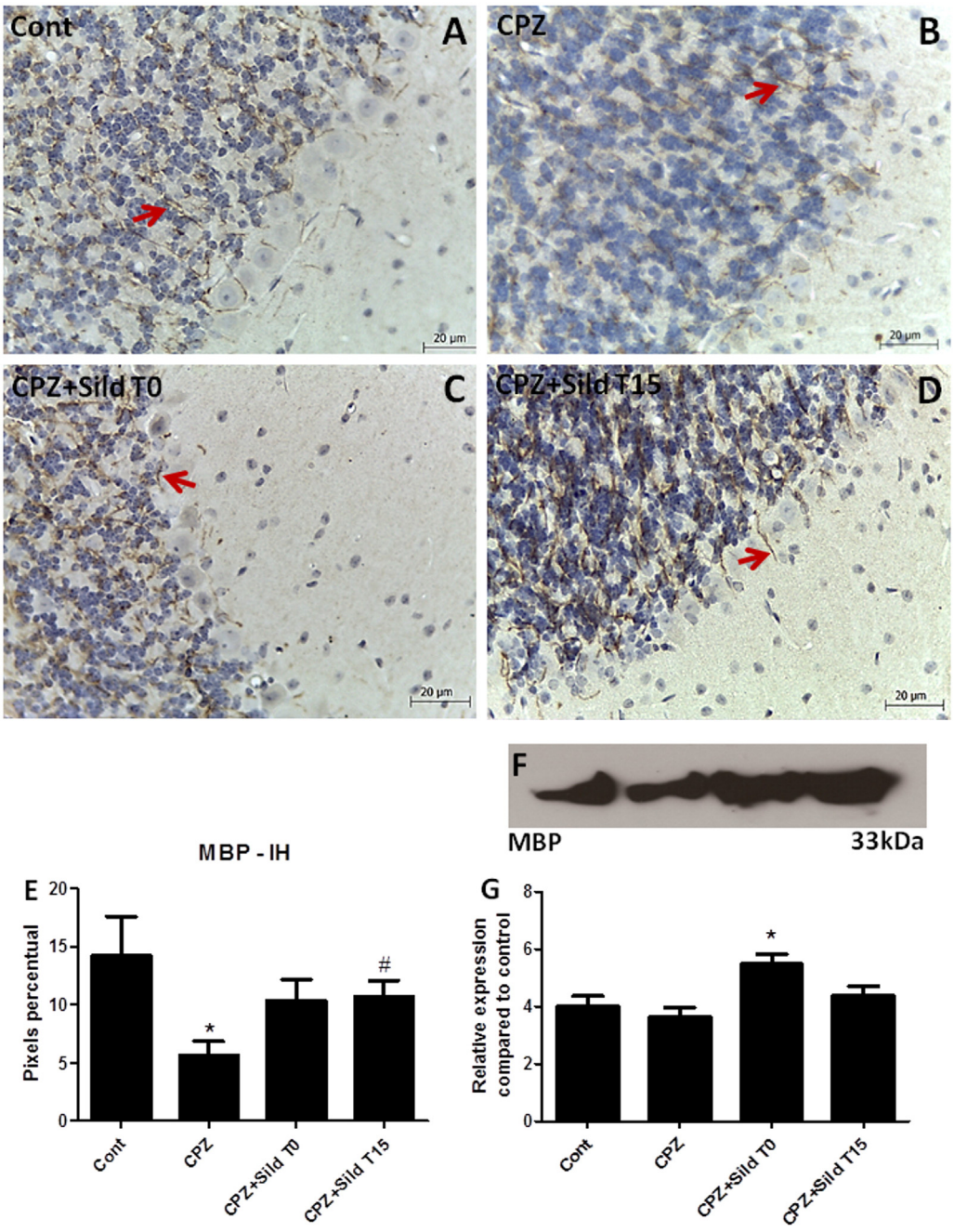

Fig. 4. Immunohistochemistry and immunoblot for MBP protein. A — Location and physiological expression of this protein in the cerebellum of the control animals. B — Cuprizone treatment induced a significant decrease in the expression of MBP. C, D — The administration of sildenafil (T0 and T15) increased the expression of MBP to levels close to the physiological state. Immunohistochemistry quantification (fifteen arbitrarily selected areas per animal) was performed using the GIMP 2.8 image analysis software (E). n = 5 animals/group. F, G — In immunoblotting analyses, the control group exhibited basal expression of the protein. Cuprizone treatment decreased the expression of MBP (not significant). Sildenafil (T0) significantly increased the expression of MBP in comparison with the control group. Treatment with sildenafil (T15) promoted an increase of this protein, but not significant. Arrows indicate the labeling of myelin fibers. Bars = 20 μm. Statistical comparison was performed using one-way ANOVA for parametric measurements, combined with Dunnett's and Tukey's post hoc tests. Unpaired Student's *t*-tests were used to compare each treatment with the control. *$p < 0.05$ compared with control, #$p < 0.05$, comparing with CPZ.

for the other methods (sildenafil 25 mg/kg; T0 and T15 groups; n = 5 per group). After treatments, MRI measurements of the brain were performed using a 3 T scanner (Signa Excite HDx, GE Healthcare, Milwaukee, WI, USA). Image acquisition was performed in accordance with Tovar-Mol, 2005. Ten slices per animal were quantified using the Image Manipulation Program Gimp 2.8 (freely distributed, http://www.gimp.org/).

2.3. Statistical analysis

Values were analyzed using the GraphPad Prism software package (San Diego, CA, USA; v. 5.0). One-way analysis of variance (ANOVA) followed by Dunnett's and/or Tukey's post-test were used to compare groups. Unpaired Student's *t*-tests were used to compare each treatment with the control. The results were expressed as means ± S.E., where appropriate. A p-value <0.05 indicated statistical significance.

3. Results

3.1. Sildenafil promoted regulation of the inflammatory process by increasing levels of the chemokine MCP-1 and its receptor, CCR-2, metalloproteinase MMP-9 and decreasing cytokine IL-1β

To examine the effects of sildenafil as an inflammation modulator, three markers were evaluated: IL-1β cytokine, chemokine MCP-1 and its receptor CCR-2, and metalloproteinase MMP-9. Immunoblotting for MCP-1 and CCR-2 revealed basal expression of this chemokine and its receptor in the control group. CPZ significantly decreased levels of MCP-1 and CCR-1 in comparison with control. Interestingly, sildenafil treatment starting on day zero (T0), for 30 days, induced a significant increase of MCP-1 and CCR-2, compared to the control and CPZ groups. However, treatment with sildenafil starting on day 15 (T15) after CPZ administration, and continued for 15 days, resulted in a significant decrease in the levels of MCP-1 and CCR-2, compared to the control group (Fig. 2A–E).

Immunoblotting for MMP-9 revealed basal expression of this metalloproteinase in the control group. Animals treated with CPZ exhibited an increase in MMP-9 levels. During sildenafil treatment for 30 days (T0), MMP-9 was significantly increased in comparison with the control and CPZ groups. Treatment with sildenafil for 15 days (T15) did not induce significant changes in MMP-9 levels, compared to the control and CPZ groups (Fig. 2F,G).

Immunohistochemistry for IL-1β in the cerebellum revealed that this cytokine was absent in the control group (Fig. 3A). However, treatment with cuprizone induced an intense expression of IL-1β in the molecular layer (Fig. 3B). Treatment with sildenafil for 30 (T0) and 15 days (T15) promoted a significant reduction in the labeling of this cytokine, compared to the CPZ group (Fig. 3 C,D; quantifications in the graph, Fig. 3E).

3.2. Expression of the proteins MBP and GST-pi was restored following sildenafil treatment

The results show the action of sildenafil in response to a demyelination process. IH and WB of MBP were performed. The control group had a pattern of this protein, mainly in the granule layer. WB analysis showed no significant change in MPB expression in the CPZ group, compared with control. The quantification of IH showed a significant decrease of this protein in CPZ animals, compared to control. The panels of IH revealed that CPZ treatment resulted in fewer, but thicker and poorly defined MBP positive fibers. This may indicate abnormal MBP distribution and myelination. The animals that underwent sildenafil treatment starting on day 0 (T0) had significantly increased MBP expression (by WB analysis) and exhibited thinner and better distributed myelinated fibers, compared to the CPZ group. This can be interpreted as improved myelination and MPB distribution, as the fibers are similar to the control group. The quantification of IH showed an increase, which was not significant, of MBP in this group, compared to CPZ. Shorter treatment with sildenafil which began later (T15) induced an increase in the expression of MBP which was statistically significant in IH, but not in WB analysis (see IH in panels A–D of Fig. 4 and graph in Fig. 4F; WB in Fig. 4F,G).

Immunoblotting for GST-pi, a marker of mature oligodendrocytes, revealed basal levels of this protein in the control group. Animals treated with CPZ had significantly lower levels of GST-pi (an absence of mature oligodendrocytes) than the control group. However, treatment with sildenafil for 30 (T0) and for 15 days (T15) demonstrated a significant increase in GST-pi expression in comparison to the CPZ group, indicating the recovery of mature oligodendrocytes (Fig. 5A,B).

3.3. Beneficial effect of sildenafil on white matter injury — improved myelin structure and ultrastructure

To observe the morphological aspects of the myelin sheath, Luxol fast blue (LFB) staining and transmission electron microscopy (TEM) were performed. LFB staining (Fig. 6A–D) revealed a normal myelin structure in the control group (Fig. 6A). The animals treated with cuprizone exhibited a significant decrease in LFB staining (graph in Fig. 6E), characterized by lighter areas indicative of myelin disorganization and the presence of vacuoles (Fig. 6B). In animals treated with sildenafil starting on day 0 (T0), myelin organization was characterized by oriented fibers and LFB staining was more uniform and significantly more intense than in the CPZ group, and similar to that found in the control group (Fig. 6C). In animals where sildenafil treatment started later, on day 15 (T15), LFB staining showed partial improvement in myelin structure, with some lighter areas and vacuoles similar to the CPZ group. The intensity of the staining remained lower than in the control group (Fig. 6D and graph E).

To examine if myelin protection by sildenafil was due to better vascularization, induced by this drug, blood vessel diameter was measured (Fig. 6F). The results showed that in CPZ animals vessel diameter was significantly increased, compared to control. In the groups treated with sildenafil T0 and T15, vessel diameter remained greater than in

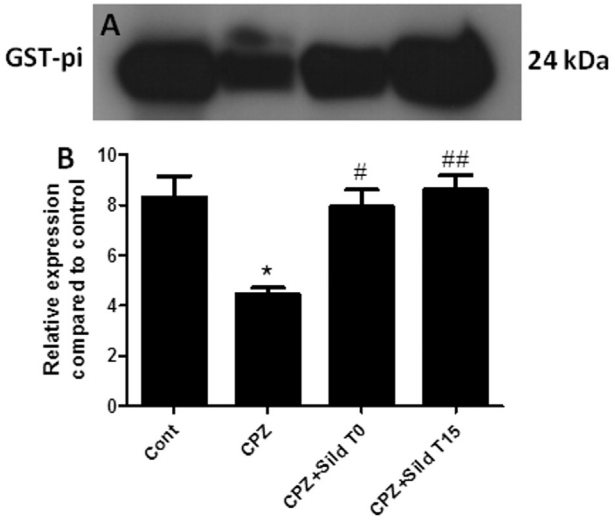

Fig. 5. Immunoblot for GST-pi. A — The control group exhibited basal expression of the protein. Cuprizone treatment significantly decreased the expression of GST-pi in comparison with the control group. Treatment with sildenafil (T0 and T15) significantly increased the expression of GST-pi in comparison with the cuprizone group. B — Bands were evaluated through integrated density of pixels and statistical comparison was performed using one-way ANOVA for parametric measurement, combined with Dunnett's and Tukey's post hoc tests. Unpaired Student's *t*-tests were used to compare each treatment with the control. *p < 0.05 compared with control and #p < 0.05 and ##p < 0.001 compared with the cuprizone group.

the control group. In the T15 group this increase was significant compared to CPZ animals.

Semi-qualitative analysis of ultrathin cerebellum sections revealed normal myelin in the control group, with a typical lamellar pattern and preserved axons (Fig. 7A). The cuprizone-treated animals exhibited significant ultrastructural alterations in myelin and axons: collapsed myelin layers, reduced thickness or loss of the myelin sheath, absence of myelin lamellar pattern, swollen axons and/or axons detached from the sheath (Fig. 7B; scores in Fig. 7E). Animals treated with sildenafil starting on day 0 (T0) exhibited a significant improvement in myelin ultrastructure, with typical myelin lamellar arrangement, rare fibers with reduced myelin thickness, and normal axons (Fig. 7C), indicating that sildenafil treatment was efficient in protecting the myelin from cuprizone-induced injury. Animals treated with sildenafil starting on day 15 (T15) exhibited significant protection of myelin in comparison with CPZ animals, yet remained significantly damaged, compared to control. Irregular myelin with abnormal lamellar arrangements, were found in this group (Fig. 7D).

3.4. Recovery of degenerated areas after treatment with sildenafil

Magnetic resonance imaging (MRI) allows the demyelinating focus in the brain to be diagnosed. Through this technique, it was analyzed whether treatment with sildenafil using two different therapeutic strategies (starting on day 0 — T0), or on day 15 — T15) would effectively reverse demyelination processes. The control animals showed normal

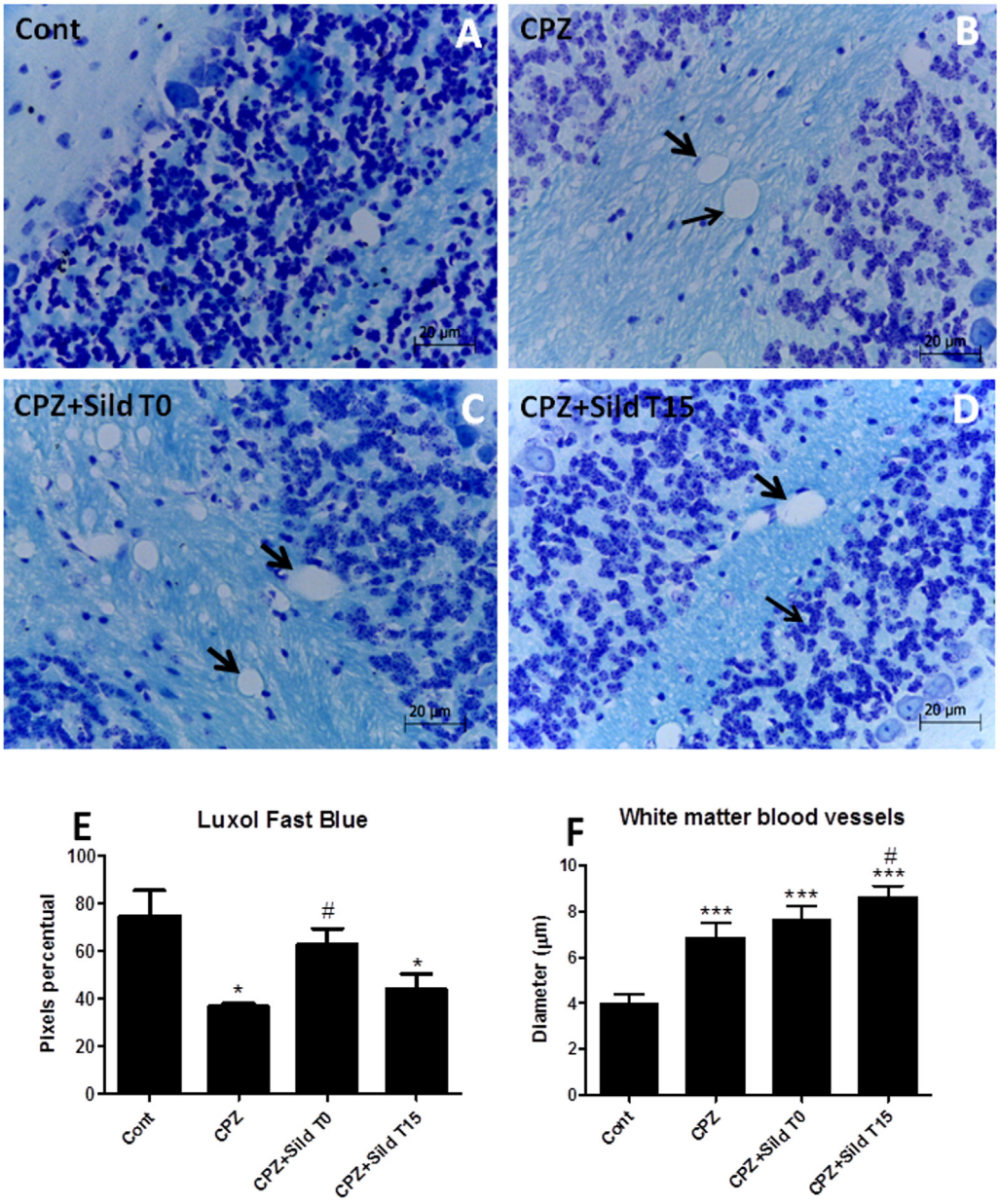

Fig. 6. Luxol fast blue (LFB). A — The control group exhibited labeling intensity and characteristic of normal myelination. B — LFB staining was lighter and there were many vacuoles in the groups treated with cuprizone. C — The animals treated with sildenafil (T0) exhibited uniform myelin organization and LFB staining intensity similar to the control group. D — Treatment with sildenafil (T15) exhibited lighter LFB staining and some vacuoles were detected. Immunohistochemistry quantification (fifteen arbitrarily selected areas per animal) was performed using the Image Manipulation Program Gimp 2.8 (E). n = 5 animals/group. In CPZ animals the vessel diameter (F) was significantly increased, compared to control. In the groups treated with sildenafil T0 and T15, the vessels remained with increased diameters, comparing to control group. Bar = 20 µm. Statistical comparison was performed using one-way ANOVA for parametric measurements, combined with Dunnett's and Tukey's post hoc tests. Unpaired Student's *t*-tests were used to compare each treatment with the control. *$p < 0.05$ and ***$p < 0.001$ compared with control, #$p < 0.05$, comparing with CPZ.

brain structure, with corpus callosum integrity (the region with the highest amount of myelin) and the morphology of the ventricles having a normal appearance (Fig. 8A,B). After ingestion of CPZ, the animals showed a significant structural loss of the corpus callosum and exaggerated dilation of the lateral ventricles (Fig. 8C,D), indicating an absence of tissue around the ventricles (degeneration). These alterations were measured as a quantification of white areas (Fig. 8I). The animals treated with sildenafil T0 showed a significant recovery, with more preserved corpus callosum structure as well as a reduction of ventricle dilatation (Fig. 8E,F). However, treatment with sildenafil T15 was not effective at promoting the recovery of areas affected by CPZ, which continued to have more degenerated areas than control animals (Fig. 8G,H).

4. Discussion

The beneficial effects of sildenafil on inflammation and demyelination have been the subject of studies by various research groups (Nunes et al., 2012; Pifarre et al., 2011; Rapôso et al., 2013, 2014). However, further detailed studies are necessary to better understand the mechanisms of the neuroprotective role of this drug.

In the present study, the effect of treatment with sildenafil 25 mg/kg in two therapeutic designs, one starting concomitantly with cuprizone administration (on day 0) and lasting for 30 days (group T0), and the other starting 15 days after cuprizone administration and lasting for 15 days (group T15), were investigated. The results showed that sildenafil increased expression of myelination related proteins (GST-pi and

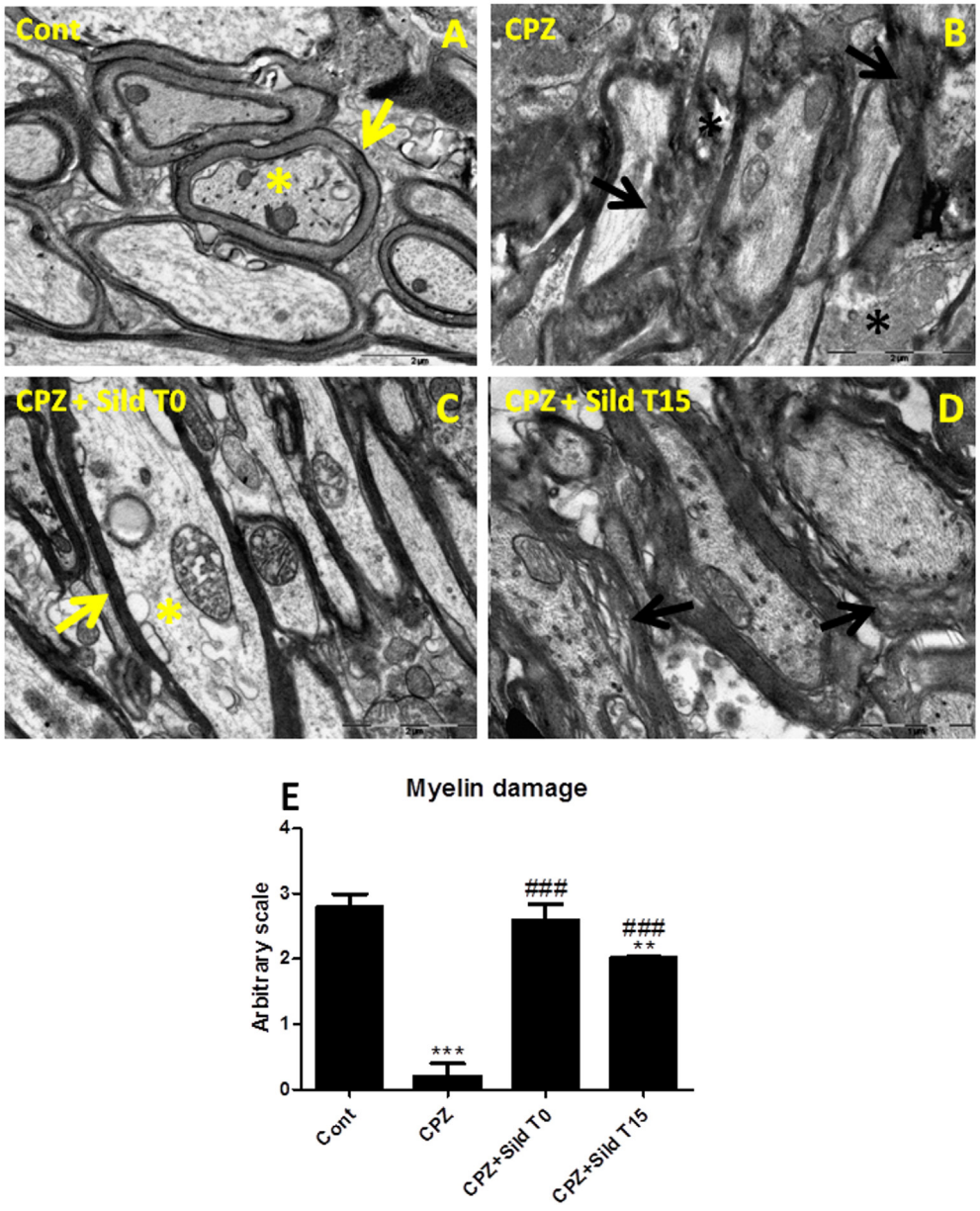

Fig. 7. Cerebellum ultrathin sections. A — Standard normal myelin (yellow arrow) and intact axons (yellow asterisk). B — After treatment with cuprizone, the animals exhibited shredded and collapsed myelin (black arrows) and reduced myelin sheath thickness. Axons were damaged (black asterisks). C — Sildenafil (T0) promoted the recovery of the ultrastructural pattern of myelin, no shredded or collapsed myelin and restoring normal thickness (arrow). Axons look normal (yellow asterisk). D — Treatment with sildenafil (T15) totally recovered myelin ultrastructure and remained with damaged myelin (black arrows). E — Semi-quantitative analysis of myelin ultrastructure, using an arbitrary scale (3 = no damage, with typical lamellar pattern and preserved axons; 2 = two of these structural characteristics of damage: collapsed myelin layers, reduced thickness, loss of myelin sheath, swollen axons, detached axon from the sheath; 1 = three of those mentioned characteristics; 0 = four of those mentioned characteristics) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

MBP), decreased the labeling of the cytokine IL-1β, increased expression of the chemokine MCP-1 and its receptor CCR-2, and increased MMP-9 expression. Furthermore, treatment with sildenafil promoted myelin repair, demonstrated by structural (LFB staining) and ultrastructural analysis (TEM). MRI also showed more preserved tissue in animals treated with sildenafil (T0), than those treated with sildenafil T15 or CPZ only. As expected, the blood vessel diameter increased in animals treated with sildenafil T0 and T15, although, dilatation of vessels was also observed in the CPZ group, suggesting that CNS protection by sildenafil is not a non-specific effect due to improvement of blood support, but has a specific mechanism. In addition, it was demonstrated in the present study that the time of treatment is important in order for the drug to be most effective, since the T0 group showed more significant anti-inflammatory and protective effects than the T15 group.

The cuprizone-demyelination model, used in the present study, is accepted for the study of multiple sclerosis-related lesions and is characterized by oligodendrocyte loss and consequent myelin degeneration (see revision in Kipp et al., 2009). Cuprizone [oxalic acid bis

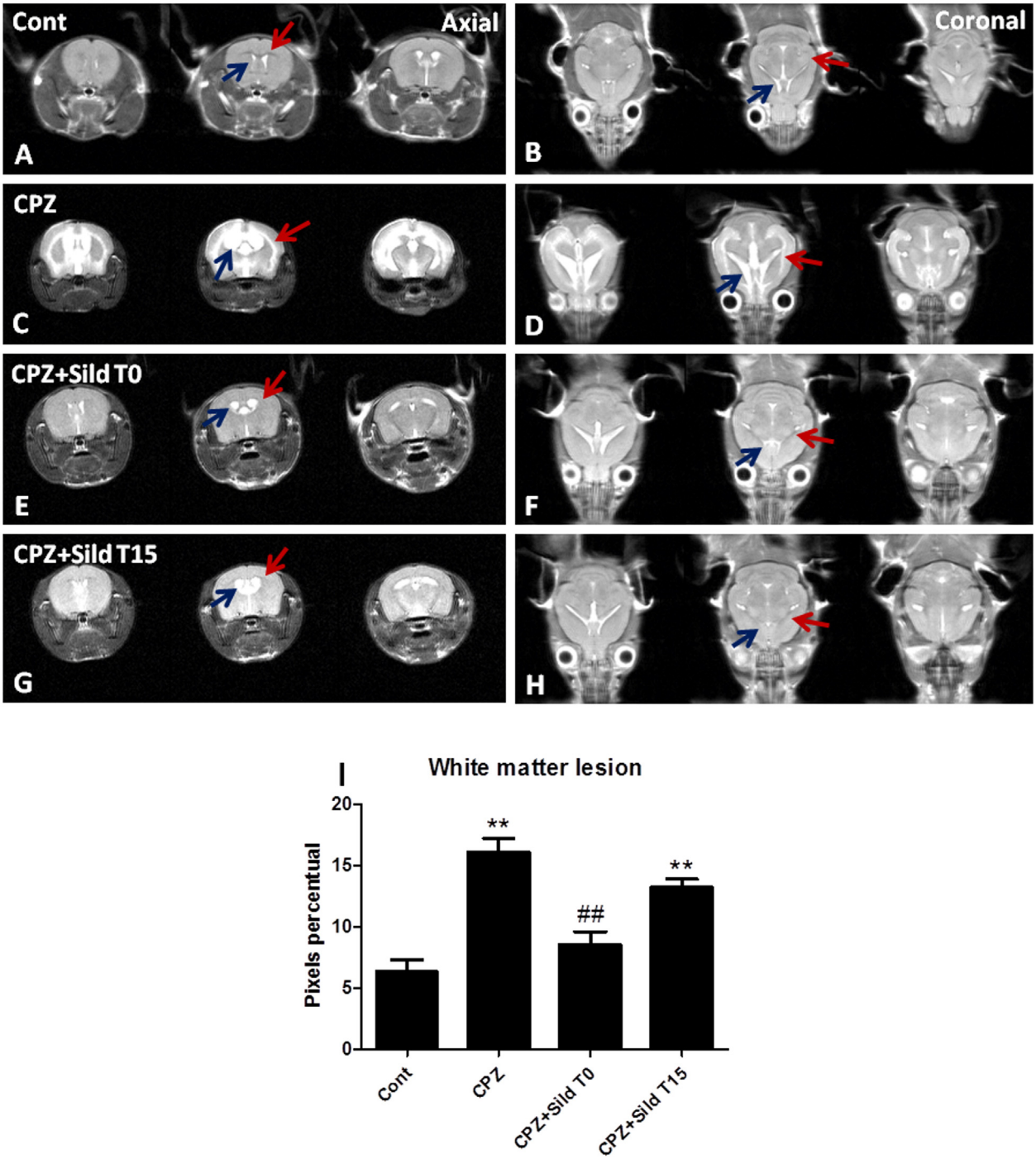

Fig. 8. Coronal and axial sections of magnetic resonance imaging (MRI) in the brain after contrast with gadolinium. A, B — Control animals showed normal brain structure, with corpus callosum integrity (the region with the highest amount of myelin) and morphology of the ventricles having a normal appearance. C, D — After treatment with CPZ, the animals showed structural loss of the corpus callosum and exaggerated dilation of the ventricles, indicating tissue degeneration. E, F — Animals treated with sildenafil (T0) showed partial recovery of the corpus callosum structure as well as reduction of ventricle dilatation. G, H — Treatment with sildenafil (T15) did not totally restore the normal aspect of the corpus callosum. Red arrows point to the corpus callosum and blue arrows point to the ventricle. Ten slices per animal were quantified (I). The quantification was done using the Image Manipulation Program Gimp 2.8.

(cyclohexylidene hydrazide)] is a copper-chelating agent, which has unique neurotoxic properties and is a useful tool for studying the demyelination of the CNS (Salinas Tejedor et al., 2015; Acs et al., 2009). The mechanisms of oligodendrocyte death have not to date been elucidated, although it has been demonstrated that these probably include mitochondrial/cellular respiration disturbance (Arnold and Beyer, 2009) and enzymatic changes (Komoly et al., 2009, Cammer et al., 1995). In addition, the inhibition of oligodendrocyte precursor differentiation (Cammer, 1999; Salinas Tejedor et al., 2015) and secretion of pro-inflammatory cytokines from activated microglia/macrophages are also described (Pasquini et al., 2007).

The characterization of CCR-2 as a chemokine closely related to the pathology of MS and MS-animal models (EAE and cuprizone) has been well-established. In general, during the first weeks of cuprizone exposure, it undergoes a typical chemokine upregulation, and both microglia and astrocytes produce CCR-2 (Harkness et al., 2003). However, CCR-2$^{-/-}$ mice were seen to experience a delay in the process of reepithelization and angiogenesis in a model of focal brain injury (Low et al., 2001). It is possible that CCR-2 has a protective role, and that its increase in MS and in MS-models may be related to an attempt to restore balance and to control the injury. Corroborating this hypothesis, an increase of CCR-2 may be associated with a reduction of macrophage infiltrates after a stroke, showing the neuroprotective effects of CCR-2 (Rawji and Yong, 2013). Moreover, there is ample evidence to indicate the role of CCR-2 in tissue repair after injury, increasing neurogenesis due to its chemotactic property in neural precursor cells (Semple et al., 2010). Liu et al. (2007) showed that cerebral artery occlusion induced upregulation of CCL2 (a CCR-2 ligant) in the subventricular zone and promoted neural progenitor cell motility and neuron differentiation, suggesting the neurogenic function of this chemokine. It has been proposed that treatment with sildenafil induces angiogenesis, improving blood flow in the cerebral ischemia model (Zhang et al., 2002), and inducing neurogenesis (Chen et al., 2014). The present results showed that sildenafil treatment increased the expression of CCR2 and its chemokine MCP-1. Concomitantly, sildenafil had protective effects, decreasing the pro-inflammatory marker, IL-1β, and improving tissue structure and the ultrastructure of myelin. This data suggests that the protective role of sildenafil in the demyelinating condition may be the result of an increase in CCR2/MCP1, which induces angiogenesis and neurogenesis and reduces inflammatory infiltrate. However, it is important to consider other protection mechanisms, such as the increase of neuronal growth factor (NGF), which can contribute to the proliferation and differentiation of neurons (Nunes et al., in press).

In addition to angiogenesis and neurogenesis, the cleaning of damaged myelin is an essential mechanism in repairing tissue in demyelinating diseases. It has also been demonstrated that the absence of activated microglia/macrophages promotes accumulation of myelin debris, preventing remyelination (Kotter et al., 2006). In particular, it has been identified that myelin debris can inhibit the differentiation of oligodendrocyte precursor cells in myelinating (mature) oligodendrocytes (Tanaka and Yoshida, 2014). Mediators in the microenvironment and the extent of the injury define at what time microglia/macrophages may assume an active and phagocytic phenotype (Rawji and Yong, 2013). The expression of MCP-1/CCR-2 by the glial cells may promote this change in the phenotype of microglia in an attempt to repair the injured environment (Low et al., 2001). In addition, Borán et al. (2008) estimated that stimulation of the cGMP–PKG pathway acts beneficially in microglia, inducing the phagocytic phenotype (M2) and decreasing expression of inflammatory genes, in detriment to the proinflammatory phenotype (M1). Therefore it is suggested that the increase of CCR-2/MCP-1, associated with the stimulation of cGMP–PKG signaling, both induced by sildenafil, can stimulate the M2 phenotype of microglia, contributing to the resolution of neuroinflammation and to remyelination. The decrease in IL-1β found here corroborates this hypothesis.

Another mechanism by which sildenafil can protect the neural tissue is through MMP-9. The present study has demonstrated that expression of this metalloproteinase increases after sildenafil treatment. In general, metalloproteinase families are highly involved in the spread of inflammation, myelin degradation and extracellular matrix remodeling. However, it has also been proposed that MMPs have a role in modulating different physiological processes, such as development, growth, apoptosis, and cell migration (Mccawley and Matrisian, 2001; Sekton, 2010). Jiang et al. (2013) demonstrated increased expression of several MMPs in an MS-model, especially during the remyelination phase, demonstrating the importance of the regulatory role of MMP in tissue regeneration. Parallel in vitro and in vivo studies showed that MMP-9 facilitates vascular remodeling in the white matter, and that oligodendrocytes are the major source of this metalloproteinase (Morello et al., 2011; Oh et al., 1999). An in vivo study using a focal lesion model demonstrated that oligodendrocytes respond to MMP-9 signaling, improving myelination (Pham et al., 2012). In addition, recent studies indicate that blocking MMP-9 mRNA expression causes a reduction in myelin basic protein (MBP) in mice treated with CPZ (Liu et al., 2014). Therefore, it is possible that the increase in MMP-9 induced by sildenafil is part of the protective mechanism of this drug, facilitating vascular remodeling. In addition, a greater number of mature oligodendrocytes may produce MMP-9 after sildenafil treatment, as was shown here by the increase of GST-pi (mature oligodendrocyte marker) after treatment with sildenafil. Studies using in vitro oligodendrocytes are being developed to clarify sildenafil mechanisms on myelination.

It can therefore be concluded that treatment with sildenafil for 30 days was efficient at recovering the myelin sheath, neuronal environment and the integrity of damaged areas such as the cerebellum and corpus callosum. This effect may be associated with interaction between the chemokine MCP-1 and its receptor CCR-2 and the metalloproteinase MMP-9, involved in the control of inflammation, tissue repair process and the organization of myelin. Therefore, the present study suggests that the use of sildenafil may have significant therapeutic potential for the treatment of multiple sclerosis pathogenesis.

Conflict of interest statement

The author(s) declare there are no conflicts of interest with respect to the authorship and/or publication of this article.

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CAPÍTULO 5

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Review Article

Phosphodiesterase-5 Inhibitors: Action on the Signaling Pathways of Neuroinflammation, Neurodegeneration, and Cognition

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Phosphodiesterase type 5 inhibitors (PDE5-Is) have recently emerged as a potential therapeutic strategy for neuroinflammatory, neurodegenerative, and memory loss diseases. Mechanistically, PDE5-Is produce an anti-inflammatory and neuroprotection effect by increasing expression of nitric oxide synthases and accumulation of cGMP and activating protein kinase G (PKG), the signaling pathway of which is thought to play an important role in the development of several neurodiseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). The aim of this paper was to review present knowledge of the signaling pathways that underlie the use of PDE5-Is in neuroinflammation, neurogenesis, learning, and memory.

1. Introduction

The PDE superfamily consists of 11 subtypes (PDE1–PDE11), the classification of which is based largely on their sequence homology. PDE1, PDE2, PDE3, PDE10, and PDE11 hydrolyse cGMP and cAMP, PDE4, PDE7, and PDE 8 preferentially cleave cAMP, while PDE5, PDE6, and PDE9 cleave cGMP. PDE5 is highly expressed in the cerebellar Purkinje cells [1] and is also expressed in other brain areas such as the hippocampus, caudate, substantia nigra, and cerebellum [2, 3]. There are indications that PDEs can affect neuronal cell survival and when functioning incorrectly they may play a part in neurodegenerative diseases, such as Alzheimer's disease, major depression disorder, multiple sclerosis, Huntington's disease (HD), and Parkinson's disease [4].

Inhibition of specific PDE5s (PDE5-Is) and accumulation of cGMP may inhibit neuroinflammation and improve synaptic plasticity and memory [5, 6]. Among the compounds synthesized and screened against PDE5, the most utilized drugs in neuroinflammation/neurodegeneration assay are the cGMP based sildenafil (VIAGRA, Pfizer) and vardenafil (Levitra, Bayer HealthCare Pharmaceuticals) and the β-carboline derived tadalafil (Cialis, Eli Lilly Laboratories). The possible use of these drugs in the central nervous system (CNS) is related to their ability to cross the blood-brain barrier (BBB). Sildenafil has been described as clearly crossing the BBB (FDA 1998) and there is also evidence of the ability of vardenafil to do the same [7, 8]. According to Akkerman et al. [9] the neuropharmacokinetic (plasma and brain) profile of vardenafil after oral administration is detected within 4 min after dose [9]. Until recently it was considered that tadalafil was unable to cross the blood-brain barrier (BBB) [10], but the most recent results have shown that tadalafil reaches the brain in sufficient concentrations to potently inhibit PDE5 [11].

In recent years there has been tremendous interest in the potential therapeutical use of PDE5-Is in the treatment of several diseases. There is mounting evidence from clinical and experimental trials that indicates that NO-cGMP-PKG is the central mechanism of a network of signaling pathways

that interconnects neuroinflammation, neurodegeneration, and cognitive disorders, resulting in increased pharmaceutical interest in PDE5-Is as promising therapeutic targets for neurodiseases.

2. Neuroinflammation/Neurodegeneration

2.1. Blood-Brain Barrier. Neuroinflammation is an inflammation of the nervous tissue that can be initiated in response to a variety of stimuli, including infection, head injury, toxic metabolites, or autoimmunity, and has a pivotal role involved in the progression of neurodegenerative disorders. Until recently the CNS was considered immunologically privileged, as many antibodies and peripheral immune cells are blocked by the blood-brain barrier (BBB), a highly specialized brain endothelial structure composed of pericytes, astrocytes, and microglia. The BBB maintains the chemical composition of the neuronal environment, which is required for the proper functioning of the neuronal circuits, synaptic transmission, synaptic remodeling, angiogenesis, and neurogenesis [12]. The immune system influences the functioning of the BBB, which, in turn, affects the functioning of the CNS under both physiological and pathological conditions. In some cases, the BBB separates the CNS from the immune system; in others it acts as a mediator in neuroimmune interaction, and still in others it may act as an immune system attack target [13]. Under physiological conditions, immune cells cross the BBB at a very low rate through specific interactions [14, 15]. In neurodegenerative disorders, however, the BBB is impaired. The inflammatory process is initiated when the glial cells are activated, with a consequent change in the transendothelial transport of monocytes and T lymphocytes, the secretion of cytokines, and finally neuronal damage and death. Inflammatory cytokines such as TNF-α and IL1-β can upregulate the expression of adhesion molecules on endothelial cells, which bind to the leukocyte ligands and allow activated leukocyte entry into the CNS [16, 17]. Upon inflammation, endothelial cells also produce chemokines which recruit leukocytes into the CNS [18]. The traffic of immune cells through the BBB may initiate and/or contribute to a "vicious circle" in the disease process, resulting in progressive synaptic and neuronal dysfunction and neuronal loss in disorders such as Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and neuroAIDS [19, 20].

2.2. Astrocytes. Astrocytes are a highly heterogeneous population of cells which regulate pH, the extracellular levels of neurotransmitters and ions, and energy metabolism. They are also involved in the formation and functioning of the blood-brain barrier (BBB) [21] and participate actively in neurotransmission [22]. In the small arteries, astrocytes contribute to neurovascular coupling, which synchronizes levels of neuronal metabolic demand with local cerebral blood flow regulation [23]. Abnormal astrocytic activity coupled with vascular instability has been observed in AD models [24].

In CNS insult situations, the typical response is a degree of reactive gliosis [25], an astrocytic response involving the positive gene regulation of cytoskeletal proteins such as glial fibrillary acidic protein (GFAP). The phenotypic changes include crumpled and bushy projections and large nuclei, culminating in hypertrophy of the cell body, hyperplasia, and functional alterations, in some cases resulting in glial scars [26]. Activated astrocytes also increase expression of the calcium-binding protein S100β [27, 28], an astrocyte activation marker particularly associated with the proliferation of astrocytes [29]. In streptozotocin- (STZ-) induced diabetes the phenotypic change in astrocytes and microglia glial cells is evidenced by increased expression of S100β and GFAP (astrocytic markers) and Iba-1 and MHC II receptors (reactive microglia markers) [30]. In addition, the death of neurons and such glial cells (the caspase-3 pathway) are also observed in the hippocampus, which is directly related to cognitive impairment [30, 31].

Reactive astrogliosis is a hallmark of PD and AD, supporting the hypothesis that astrocytes, together with microglial cells, play a central role in neurodegenerative diseases [32, 33]. Interestingly, attenuation of reactive gliosis by genetic ablation of the astrocyte intermediate filaments leads to more severe pathologies of AD, suggesting that reactive gliosis could have a protective role in the postacute and early chronic stage of neurodegenerative diseases (review in Pekny et al., 2014) [34].

Furthermore, astrocytes play an important role in central immunity. These cells either respond quickly to the presence of pathogens or other types of damage to the tissue (endogenous aggregated and oxidized proteins), assuming the role of immune cells by releasing cytokines and chemokines, influencing other cells, and modulating the BBB [26]. Astrocytes express toll-like receptors (TLRs) [35]. The in vitro activation of the astrocytic receptors TLRs 2, 4, 5, and 6 induces ROS production, IL-1β, IL-6, TNF-α, and glutamate, favoring neuronal damage [36]. Additionally, astrocytes from the brain and spinal cord of patients with MS display increased expression of TLR3 and TLR4 in inflammation regions [37]. Most TLRs initiate a signal via the myeloid differentiation primary response protein 88 (Myd88) and TRIFF, with consequent activation of MAP kinases and IκB. The phosphorylation of IκB results in translocation of NF-κB to the nucleus, whereas the activation of MAP kinases results in phosphorylation of the AP-1 transcription factor. Activation of both signaling pathways culminates in the secretion of proinflammatory cytokine (IL-1β, IL-6, TNF-α, IL-12, and type I IFNs). Activated astrocytes can also produce chemokines (CCL and CXCL families) that recruit microglial cells, lymphocytes, and dendritic cells to the local of injuries (review in González et al., 2014) [38].

In the last decade, new evidence has been discovered about the importance of glial cells in acute and chronic inflammation of the brain. Hepatic encephalopathy (HE) is a major neurological complication of severe liver disease, characterized by impaired neurological function, including changes in personality, altered mood, diminished intellectual capacity, and abnormal muscle tone and tremor. The principal pathological change in chronic HE is characterized by Alzheimer type II astrocytosis [39]. Astrocytes are the main target of pathology in HE as they contain glutamine

synthetase, the only enzyme in the brain that can remove ammonia [40]. Ammonia triggers inflammatory responses in microglia and the brain endothelial cells (ECs), most likely through the activation of the toll-like receptor-4 and consequent production of cytokines, and leads to severe astrocyte swelling/brain edema in acute hepatic encephalopathy [41]. Astrocyte swelling activates Erk-1/Erk-2- and p38-type MAP kinases, which may represent a signal for the proliferation and development of gliosis [42]. Proinflammatory cytokines (TNF-α, IL-1β, and IL-6), synthesized by astrocytes and microglia, induce disruption of the permeability barrier of the brain and can alter the normal balance and physiological function of cytokines in synaptic plasticity, learning, and memory functions [43–45]. Moreover, in a rat model of minimal hepatic encephalopathy (MHE), elevated levels of serum dopamine, released from cirrhotic livers, crossed the BBB and inactivated the Glutamate-NO-cGMP pathway in astrocytes, triggering memory impairment [46].

Evidence indicates that the cGMP-PKG pathway is involved in the regulation of astrocyte activity. NO/cGMP/PKG inhibits the expression of MMP-9 expression in LPS-stimulated rat primary astrocytes, suggesting that NO can downregulate MMP-9 in brain injury [47, 48]. According to these authors, MMP-9 expression is dependent on ERK 1/2 activation via NF-κB. This data supports the hypothesis that the NO/cGMP/PKG pathway plays a role in astrocytic cells that contributes to the resolution of neuroinflammation.

2.3. Microglia. Microglia play a critical role in the innate and adaptive immune responses of the CNS. Microglia are derived from mesodermal/mesenchymal cells, which enter the brain parenchyma and acquire a specific ramified morphological phenotype described as "resting" microglia [49, 50]. Circulating monocytes provide another important source of microglia in the brain [51]. The physiological functions of microglia are important for maintaining homeostasis. They remodel synapses by phagocytosis [52], secret neurotrophic factors such as brain-derived neurotrophic factor (BNDF) [49], and remove accumulating debris and aggregated proteins [53]. However, when exposed to infections, injuries, or nervous system dysfunction, microglial cells undergo a complex activation process that converts them into activated microglial cells. The phenotypic changes after activation of the microglia are functionally identical to those observed in macrophages. In the absence of pathology, the "resting" microglia are cells with small bodies and long, thin processes ("ramified" phenotype). When activated, microglia lose the long extensions typical of inactive microglia and reveal stubby processes ("amoeboid" phenotype), finally exerting a phagocytic form [54, 55]. This physiological transformation is associated with changes in surface antigen expression and cytokine release, the suppressive effects of which may contribute to the impaired synaptic plasticity of neurodegenerative diseases [56].

Several neurodegenerative-derived molecules (aggregated amyloid-β, Aβ, α-synuclein of Lewy bodies in Parkinson's disease, mutant huntingtin, superoxide dismutase-1, and chromogranin A) can activate pattern recognition receptors (PRRs) expressed on the microglial surface, such as toll-like receptors (TLR2, TLR4, and TLR6), CD36, and triggering receptor expressed by myeloid cells 2 (TREM2). CD36, CD14, TLR2, TLR4, and TLR6 ligation activates the proinflammatory MyD88-NF-κB signaling pathways, while TREM2 ligation increases the clearance of some aggregated molecules such as Aβ. Both pathways lead to formation of the NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) inflammasome, which culminates with the secretion of active IL1-β or IL-18. NLRP3 inflammasome activation has been described in several neurodegenerative diseases such as AD and amyotrophic lateral sclerosis (for review, see Heneka et al., 2014) [56]. Activated microglial cells become a source of TNF-α, IL1-β, IL1-α, superoxide, nitric oxide (NO), chemokines, and glutamate, which can promote neuronal death. TNF-α, secreted by both microglia and astrocytes, can directly promote neuronal death by ligation to their correspondent receptors (TNFRs). Evidence indicates that TNF-α induces apoptosis in oligodendrocytes in demyelinating inflammatory diseases [57] and plays a fundamental role in neurodegeneration in PD and AD [38].

Another microglial neurotoxic mechanism is the excessive production of glutamate, which triggers synaptic and extrasynaptic NMDA receptors in neurons, causing neuronal death through increased calcium influx, a process denominated excitotoxicity involved in several neurodegenerative diseases [58]. As well as neuronal damage, glutamate also induces apoptosis of precursor, immature, and mature oligodendrocytes contributing to myelin damage in hypoxic-ischaemic pathologies [59]. Another mechanism of injury of high levels of glutamate in the brain is the release of TNF-α and IL1-β by microglia, which further aggravates the demyelinating process [60]. Microglial-derived interleukin 1 (IL-1) is also associated with phosphorylation and the aggregation of microtubule-associated protein tau (MAPT), a neuropathological feature of tau diseases [61].

Activated microglia contributes to neurodegenerative processes by secreting ROS and nitric oxide (NO) through NADPH [62], myeloperoxidase (MPO) [63], and inducible NO synthase (iNOS) activation [64]. Under conditions of oxidative stress reactive oxygen species (ROS) act as critical signal molecules that trigger an inflammatory response in the CNS through the activation of transcription factors, such NF-κB and activator protein 1 (AP-1), promoting BBB damage and enabling immune cells to penetrate into the CNS [65, 66]. Furthermore, the upregulation of these enzymes increases the production of superoxide and NO, generating peroxynitrite, a potent oxidant that is thought to cause protein nitration. Increased levels of nitrated proteins represent a pathological event associated with several neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (AD), demonstrating the potential involvement of reactive nitrogen species (RNS) in neurodegeneration [67, 68]. Another study showed that the ROS/ERK/JNK/MAPK signaling pathway contributes to microglial NO production [69]. The oxidative stress response of microglial cells is mostly due to the activity of NADPH oxidase, which appears to have a central role in the pathology of dopaminergic neuron death and the progression of PD [70]. Interestingly, Park et al. (2015) [71] proposed that

although ROS involved in microglia activation are thought to be generated primarily by NADPH oxidase, mitochondria constitute a major source of ROS generation in LPS-mediated activated microglia cells.

A group of studies has shown evidence of bidirectional crosstalk between microglial cells and astrocytes involved in the physiopathology of neurodegenerative diseases. High levels of TNF-α produced by microglial cells can induce a dramatic glutamate release by astrocytes, leading to neuronal excitotoxicity [72]. Another important cellular interaction is microglia-neuron crosstalk. Intracellular chaperone heat shock protein 60 (HSP60) released from dying neurons activates microglial cells through the TLR4 and MyD88 pathway, leading to the synthesis of neurotoxic NO from microglia [73]. Similarly, the release of high mobility group box (HMGB1) by damaged neurons binds to the microglial Mac1 (macrophage antigen complex 1) and activates the nuclear factor-κB pathway and NADPH oxidase, stimulating the production of multiple inflammatory and neurotoxic factors, in a vicious cycle that mediates chronic and progressive neurodegeneration [74].

2.4. Oligodendrocytes. Oligodendrocytes are specialized cells responsible for myelin synthesis and maintenance and provide a supporting role for neurons [75, 76]. There is intense crosstalk between oligodendrocytes and the other glial cells. Activated microglial cells are considered key initiators of the demyelination process mediated by ROS, which is the most important damage factor of oligodendrocytes. Dysfunction of oligodendrocytes and myelin abnormalities are found in a wide variety of neurological disorders and may be involved in the pathophysiology of several diseases, including genetic leukodystrophies [77], schizophrenia and bipolar disorder [78, 79], brain injury [80], and endocrine and metabolic abnormalities [81, 82] and in neurodegenerative conditions such as strokes [83, 84], Parkinson's disease [85], Alzheimer's disease [86–88], and multiple sclerosis [89] and in diabetic encephalopathy [90].

Oligodendrocytes are very vulnerable to ROS and RNS because of their low concentration or the activity of antioxidant enzymes (superoxide dismutase, glutathione reductase/peroxidase) [66], resulting in neuronal death [91]. During pathological processes, the insult to white matter, originating from oxidative stress, inflammation, and mechanical injury, leads to degenerative loss (demyelination) or inadequate or abnormal formation of myelin (hypomyelination) [92]. The major consequences include oligodendrocyte death and the destruction of the myelin sheath, recruitment failure, and/or the differentiation of oligodendrocyte precursor cells (OPCs) during remyelination [93–96].

Oxidative stress is widely accepted as playing a key mediatory role in the development and progression of diabetes and its complications [97]. Diabetic encephalopathy can induce CNS disorders such as impairment of learning and memory [30, 98–100], dementia [101], apoptosis of hippocampal neurons [31, 102], increased permeability of the blood-brain barrier [103], edema, disarrangement of the myelin sheath, and oligodendrocyte abnormalities in the cerebral cortex, cerebellum, and hippocampus [102].

Oxidative stress also exerts a critical role in AD pathogenesis [104–106]. Aβ induces oligodendrocyte dysfunction and apoptosis through oxidative stress [107, 108]. During the early stages of AD, toxicity mediated beta-amyloid induces the destruction of myelin in cognition related areas in experimental models [87, 109]. Similarly, damage to the myelin associated with aging is clinically associated with neurological deficits, such as a delay in cognitive processing and memory [110]. Initially, deposits of Aβ plaques appear to enhance the survival, proliferation, and differentiation of OPCs [111]. However, other studies have reported the involvement of Aβ in acute inflammation, oxidative stress, and neuronal apoptosis [65, 112–114].

Oligodendrocyte injury is also evident in other demyelinating diseases. In MS, Th1 and Th17 have been shown to be the main pathogenic T cells, as they promote BBB disruption, demyelination, and neurodegeneration [104, 115]. The process is the initial response of the endothelium of the blood-brain barrier, as it is capable of expressing MHC class II antigens, allowing the migration of CD4+ and CD8+ T cells to the perivascular space [116, 117]. As a result, the activated macrophages and microglia release large amounts of inflammatory cytokines such as IL-1β and TNF-α, thereby amplifying the proinflammatory autoimmune reaction [74]. Both cytokines are involved in oligodendrocyte damage via the MAPK signaling pathway. The binding of TNF-α to its receptor TNF-R1 induces oligodendrocyte apoptosis, whereas the binding of IL-1β to IL-1R1 delays remyelination [118]. In an attempt to repair myelin damage, oligodendrocyte precursor cells (OPCs) transform into mature oligodendrocytes, promoting remyelination. In more advanced stages of the disease, however, OPCs also undergo apoptosis [119].

Oligodendrocytes are not immunological inert cells but secrete a wide range of inflammatory mediators, expressing receptors to such factors. They produce the proinflammatory cytokines IL1-β and IL-6 and the chemokines CCL2 and IL-8, which are involved in the recruitment of immune cells during acute inflammation [120, 121]. In MS experimental models, dying oligodendrocytes also express increased COX-2 levels at the onset of demyelination, which seems to render these cells more susceptible to death by glutamate-mediated excitotoxicity [122].

In summary, glial cells may serve as a potential therapeutic target for neuroinflammatory and neurodegenerative disorders and disturbances in learning processes and memory.

3. Cyclic Nucleotide Signaling and Neuroinflammation/Neurodegeneration

Cyclic nucleotides, cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP) exert many physiological roles such as the regulation of ion channels, relaxation of smooth muscle, immunomodulation, platelet aggregation, phototransduction, neuronal survival, and consolidation of memory [123–125].

cAMP is synthesized from adenosine 5′-triphosphate (ATP) by membrane-bound adenylyl cyclase (AC), which is mainly regulated in neurons by G-proteins and additionally stimulated by and calmodulin. The main transducer of cAMP signals is the cAMP-dependent protein kinase A (PKA) [126].

Synthesis of cGMP is regulated by membrane-bound/particulate (pGC) and cytosolic/soluble (sGC) guanylate cyclases, which convert guanosine 5′-triphosphate (GTP) into cGMP. The physiological effects of cGMP activities are determined by three types of intracellular receptors: cGMP dependent kinases (PKG), ion channels regulated by cGMP, and PDEs regulated by cGMP. cGMP can also activate the cAMP pathway by activating the cAMP intracellular receptors, such as its main target protein kinase, PKA [127].

Both cAMP and cGMP can alter cell function by activating or inactivating proteins by phosphorylation. The most important regulation of cyclic nucleotides is achieved by the breakdown of cAMP and cGMP in their inactive forms, 5′AMP and 5′GMP, respectively, by phosphodiesterases (PDEs) [4]. In several cell types, cGMP modulates the concentration of cAMP by activating or inhibiting cAMP-specific phosphodiesterases (PDEs) [127].

In the central nervous system (CNS), cGMP plays an important role as a mediator of the action of nitric oxide (NO) and natriuretic peptides. NO is a gaseous free radical that acts as an important sign of intra- and extracellular processes [128] and is synthesized intracellularly by three isoforms of the nitric oxide synthase enzyme (NOS): (1) dependent Ca^{+2} constitutive forms, consisting of the endothelial form or type III (eNOS or NOS-III) and the neuronal form or type I (n-NOS or NOS-I), present in endothelial cells, neurons, and glial cells, which produce NO under physiological conditions; (2) Ca^{+2} independent inducible form (i-NOS) or type II, present in macrophages, hepatocytes, smooth muscle, endothelium, and glial cells, which produce NO after immunological stimulation (i.e., IFN-γ, TNF-α, and LPS) [5].

The role of NO in inflammation is complex. At micromolar range, NO produced by iNOS exerts cytotoxic and proinflammatory effects that are opposite to those induced by low nanomolar concentrations of NO produced by the eNOS isoform, which exhibits anti-inflammatory effects via the cGMP-PKG pathway [129].

Some studies indicate that NO derived from both nNOS and eNOS, but not the iNOS isoform, is critical in the regulation of leukocyte-endothelial cell interactions [129–131]. Nitric oxide (NO) modulates leukocyte adherence and recruitment on the vascular endothelium, exerting a cytoprotective and antithrombotic role. The anti-inflammatory effects of NO are mediated predominantly via the activation of sGC/cGMP. The production of cGMP causes specific downregulation of the expression of P-selectin on endothelial cells and platelets to prevent leukocyte rolling [132].

Moreover, it has been reported that the intracellular accumulation of cGMP in different models of inflammation reduces the production of proinflammatory cytokines such as IFN-γ, TNF-α, and interleukins (ILs), lowers oxidative stress, and diminishes the production of chemokines and chemokine receptors such as monocyte chemoattractant protein (MCP-1) and its receptor CCR2. Therefore, intracellular levels of cGMP exert a role in modulating inflammatory response [133, 134].

Inflammatory response is a tightly regulated physiological process, involving the orchestrated expression of inflammatory mediators. cAMP interferes with the function of the proinflammatory transcription factor Nuclear Factor-kappaB (NF-κB), as a result of the inhibition of IκB degradation due to blocking of IKK activity by cAMP/PKA [135, 136] or enhanced levels of resynthesized IκB [137]. NF-κB plays a crucial role in switching on the gene expression of a plethora of inflammatory and immune mediators. Cyclic AMP modulates NF-κB when activated by typical stimuli, such as proinflammatory cytokines, B- and T-cell activators, pathogen-associated molecular patterns (PAMPs), and oxidative stress, but also has an effect on the triggering of NF-κB by less common activators such as amyloidogenic peptides, thrombin, and high levels of glucose. However, other observations suggest that cAMP/PKA can induce NF-κB transactivation. A possible explanation for the seemingly conflicting effects of cAMP/PKA on NF-κB activation may lie in the existence of different PKA pools, with distinct subcellular localization and different functions (review in Gerlo et al., 2011) [126].

The NO-cGMP pathway can directly inhibit vascular NF-κB inflammatory activity by increasing the cytoplasmic and nuclear levels of IκBα expression [138] and inhibition of NF-κB binding [139] or indirectly by activating the kinase-A protein in the cGMP-dependent pathway [133]. Moreover, eNOS regulates NF-κB expression in a negative feedback mechanism, limiting local inflammation [140].

The NO/cGMP/PKG pathway appears to play an essential role in preventing the activation of a proapoptotic pathway, thus promoting neural cell survival. This neuroprotective mechanism may be especially important during brain ischemia, inflammation, or trauma [141]. In retinal neuroglial progenitor cells, NO/cGMP/PKG antiapoptotic cascade is activated through the cAMP-responsive element binding protein (CREB) [142], the transcription factor involved with neurotransmitters, growth factors, and other signaling molecules with essential functions for long-lasting changes in synaptic plasticity, which mediates the conversion of short-term memory to long-term memory and neuronal survival [143, 144].

The brain-derived neurotrophic factor (BDNF) is one of the major gene products of CREB-mediated transcription that is upregulated on cyclic nucleotide level elevation. The neurotrophin BDNF and its major receptor TrκB have the most abundant and widespread expression in the developing and adult mammalian brain and have a critical role in the differentiation and survival of neurons of the CNS and in long-term potentiation (LTP), a form of synaptic plasticity. The activation of the BDNF/ TrκB pathway is directly implicated in the rise of intracellular Ca^{+2} via its release from intracellular stores, and in the activation of the Ca^{+2}-calmodulin dependent kinase, CaMKII. The elevation of intracellular Ca^{+2} is one of the most important biochemical outcomes of BDNF signaling in the postsynaptic cell (review in Cunha

et al., 2010) [145]. Moreover, BDNF/ TrκB signaling activates phosphatidylinositol-3-kinase/Akt cascades, responsible for neuronal survival via bcl-2 activation and Bad inactivation [146]. The inhibition of CREB phosphorylation may impair synaptic plasticity and apoptosis due a reduction of BDNF levels.

4. Effect of Phosphodiesterase-5 Inhibitors on Neuroinflammation and Neurodegeneration

4.1. Neurogenesis and Antioxidant Activity. Neurogenesis is the biological process of generating new neurons from progenitor or neural stem cells (NSCs). NSCs proliferate in two main regions of the adult mammalian brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus [147]. Neurogenesis can be influenced by several factors, including the release of growth factors, serotonin, estrogen, and glucocorticoids, among others [148, 149]. PDE-5 inhibitors have been reported to promote neurogenesis [113, 150].

Santos et al. (2014) [147] reported that inhibitors with different selectivity for PDE5, such as T0156, sildenafil, and zaprinast, enhanced the proliferation of neural stem cells (NSCs), the first step of neurogenesis, through the sGC/PKG/ERK/MAPK pathway, with the exception of sildenafil, which did not alter ERK1/2 phosphorylation. A similar lack of an effect of ERK1/2 on phosphorylation by sildenafil was previously described by Zhang et al. (2005) [151], who suggested that the increase in cGMP levels via the inhibition of PDE5 activity enhances neurogenesis through the PI3K/Akt pathway. As already mentioned, the NO/cGMP/PKG/CREB/BDNF pathway has a fundamental role in neurogenesis and synaptic plasticity by activating PI3K/Akt.

Several studies have reported that PDE5Is inhibitors have a neuroprotective effect though elevating cGMP brain levels. Sildenafil has been found to reduce the neurologic deficit, improve neurogenesis and memory, and promote functional recovery after a stroke and focal cerebral ischemia in young and aged rats [152, 153]. Tadalafil also improved neurogenesis in an embolic stroke model in rats [153]. Another study demonstrated that pretreatment with tadalafil attenuated the deleterious effect of cerebral ischemia-reperfusion on infarct size, nitrosative and oxidative stress, memory, and motor coordination. These effects were attenuated by administration of L-NAME, a nonselective nitric oxide synthase inhibitor [154].

Antioxidant activity has also been described with the use of vardenafil on cerebral vasospasm in an experimental rat subarachnoid hemorrhage model, which induced dose dependent vasodilation of the basilar artery and also had an antioxidant effect by reducing lipid peroxidation [155].

Sildenafil also has an antioxidant and anti-inflammatory effect. An in vitro study using N9 microglial cells demonstrated that sildenafil suppressed NO, interleukin 1β (IL-1β), and tumor necrosis factor α (TNF-α) production induced by LPS. Sildenafil also blocked IκBα phosphorylation and degradation, inhibited the phosphorylation of mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, and c- Jun N-terminal kinase (JNK). Moreover, sildenafil downregulated gp91phox, a critical and catalytic subunit of NADPH oxidase, and levels of intracellular ROS. A possible mechanism for the anti-inflammatory and antioxidant effects of sildenafil may be at least in part due to suppression of the MAPKs/NF-κB pathways through the inhibition of NADPH oxidase-mediated ROS generation [156].

Chronic administration of sildenafil in diabetic type II (T2DM) patients reduces levels of endothelin, C-reactive protein, interleukin-6, intercellular adhesion molecules (ICAM), and vascular adhesion molecules (VCAM), as well as reducing nitrate/nitrite levels [157]. Recent clinical research showed that treatment of T2DM patients with sildenafil for three months reduced the endothelial function marker P-selectin and exerted a beneficial effect on glycometabolic control [158].

Similarly, daily administration of tadalafil also reduced circulating levels of the proinflammatory cytokines TNF-α and IL-1β, improved fasting glucose levels, and reduced infarct size following I/R injury in the heart of diabetic type II mice [159]. The diabetic myocardium is exposed to intense oxidative stress that can eventually lead to cardiac tissue injury and dysfunction [160]. Chronic treatment with tadalafil reduced ROS production, cardiac NADPH oxidase activity, lipid peroxidation, and oxidized glutathione [161]. Clinical studies also confirmed that the beneficial effects of tadalafil treatment on vascular function occur via improvement of endothelial function markers such as C-reactive protein, endothelin-1, and ICAM-1 [162].

4.2. Neuropathy and Motor Neuron Diseases. The early impairment of endothelial NO in diabetic patients may contribute to increased susceptibility to damage to neurons that are normally protected by the NO/cGMP/PKG signaling pathway, which may be responsible, in part, for diabetic polyneuropathies (review in Fiscus, 2002) [141]. Diabetic peripheral neuropathy is characterized by the loss and/or degeneration of neurons, Schwann cells, and neuronal fibers and by the slowing of nerve conduction velocities [163]. Diabetic patients treated with sildenafil reported an improvement in peripheral neuropathy symptoms [164]. In rodents with diabetic peripheral neuropathy, treatment with sildenafil improved blood supply to the vasa nervorum and functional outcome through the nitric oxide- (NO-) cGMP pathway [165].

According to the elegant studies performed by Wang et al. (2011) [166], diabetic type II mice present upregulated PDE5 expression in the sciatic nerve, whereas myelin sheath thickness, myelin basic protein (MBP), and the subcutaneous nerve fibers are significantly reduced. Treatment with sildenafil significantly counteracted these effects and concomitantly improved neurological function, assayed by motor and sensory conducting velocities and thermal and mechanical noxious stimuli. In vivo and in vitro analysis demonstrated that PDE5/cGMP regulates BDNF expression

in the Schwann cells of diabetic mice, enhancing myelin formation in the sciatic nerve. The same research group also studied the therapeutic effect of sildenafil in middle aged diabetic mice with long-term peripheral neuropathy, concluding that sildenafil is likely to contribute to the amelioration of nerve function through angiopoietin-1 (Ang1) and its receptor Tie-2 signaling, promoting the beneficial effects of sildenafil on neurovascular function in diabetic mice [69].

It is apparent that increases in cGMP levels favor the proliferation of motor neurons. Amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder characterized by the rapid degeneration of motor neurons, has also been the subject of many studies. While the pathogenesis of ALS is clear, the results of such studies suggest the involvement of excitotoxicity [167], peroxynitrite toxicity [168], or other oxidative damage [169]. An in vitro study using motor neuron culture showed that both PDE-5 inhibitors such as sildenafil and 8q-cGMP, an analogue of cGMP, provided neuroprotection against neurotoxicity induced by reactive oxygen species (ROS) and could therefore be a possible therapeutic tool for the treatment of ALS [170].

Another disease that causes impairment of movement is Huntington's disease (HD), an autosomal dominant neurodegenerative disorder caused by an expanded CAG repeat in the coding region of the huntingtin gene. Drugs such as (PDE) inhibitors targeted at counteracting loss of CREB function and decreased BDNF have been considered as powerful tools for the treatment of HD [171]. Some studies showed that rolipram PDE-4 inhibitors are able to exert a neuroprotective effect and to significantly increase levels of activated CREB in the striatal spiny neurons, in a surgical model of HD [172, 173]. There are also reports that treatment of HD with PDE-10 inhibitors reduces the death of cortical neurons and increased phosphorylation of CREB and BDNF levels (review in Fusco and Giampà, 2015) [174]. Similarly, Puerta et al. (2010) [175], showed that sildenafil and vardenafil can improve neurological symptoms, reduce neuronal death, and increase levels of phosphorylated CREB in a HD model, indicating a possible neuroprotective effect.

4.3. Demyelinating Diseases. In demyelinating diseases, important functions such as electrical conduction, connectivity, and axolemmal organization are compromised. Consequently, the injured axons are unable to function efficiently, leading to severe psychomotor deficits [176]. The demyelination process is usually accompanied by an inflammatory condition caused by the release of cytokines and activation of glia cells (astrocytes and microglia), leading to the death of oligodendrocytes (review in Peferoen et al., 2014) [118].

Multiple sclerosis (MS) is a chronic immune-inflammatory disease of the central nervous system (CNS) characterized by demyelination of white matter and axonal injury. The action of sildenafil in improving the clinical symptoms of multiple sclerosis (MS) patients initially was assigned to neurogenesis induction, but recent information also points to the role of the drug as a modulator of inflammation and protection of the myelin sheath [6, 177–179].

Sildenafil improved clinical signs and neuropathology in a murine model of multiple sclerosis (EAE), promoting remyelination and reducing infiltration of CD3+ leukocytes and microglia/macrophages activation [177]. Recently, Pifarré et al. (2014) [180] showed that daily treatment with sildenafil from the onset of symptoms of EAE prevented further clinical deterioration by stimulating immunomodulatory and neuroprotective mechanisms. According to these authors, early administration of sildenafil downregulated adaptative immune responses switching from the M1 to the M2 phenotype in microglia/macrophages. Furthermore, in vitro analyses of splenocytes found that sildenafil downregulated Th1/Th2/Th17 responses, while upregulating Tregs.

Additionally, Nunes et al. (2012) [178] demonstrated that sildenafil inhibited the demyelination process and reduced micro- and astrogliosis and expression of proinflammatory cytokines (TNF-α, IFN-γ, and IL-2 IL1β) in a cuprizone-induced demyelination model. In another study by the same group, sildenafil increased levels of protein expressed by oligodendrocytes and MBP (myelin basic protein) and restored the morphology of the myelin sheath, indicating remyelination. In addition, sildenafil induced OPC differentiation into mature oligodendrocytes, demonstrated by increased GST-pi (marker of mature oligodendrocytes) [6]. Sildenafil also induced myelin repair and myelin debris clearance, possibly associated with the release by the microglia of MCP-1 chemokine and metalloproteinase MMP-9 (unpublished data).

5. Cyclic Nucleotides Signaling Pathways and Cognition

Memory consolidation is the process by which newly acquired information is stabilized and stored [181]. Consolidation is a very complex brain function which requires specific molecular mechanisms and evolves two stages over time: a short-term and a long-term phase, which differ in their dependence on new protein synthesis [182]. Long-term potentiation (LTP), an electrophysiological measure involving the sustained increase in synaptic efficacy of the hippocampal synapses, is the neurophysiological correlate of memory [183]. LTP and memory share the same molecular mechanism, and, therefore, changes in synaptic strength can also be divided into two temporally and mechanistically distinct phases. A single train of high frequency stimulation (e.g., 100 Hz, 1 s), which mimics the physiological bursts of neuronal activity in the hippocampus, induces a transient increase in synaptic efficacy called early phase LTP (E-LTP). This early phase involves the short-term modification of preexisting synapses and posttranscriptional modification events [183, 184]. In contrast, repeated high-frequency stimulation (e.g., four trains of 100 Hz every 10 min) induces long-lasting, late-phase LTP (L-LTP) [185]. L-LTP, as well as long-term memory, induces the synthesis of new proteins which are responsible for the stable structural changes required for memory trace stabilization [186].

Both the cAMP/PKA and cGMP/PKG signal transduction pathways regulate the molecular mechanism that

underlies LTP. It had been suggested that the two pathways were differentially involved in the distinct phases of the memory consolidation process. Whereas cAMP was described as being involved in the formation of late-LTP (long-lasting synaptic changes) [187], cGMP was related to the transient early-LTP phase (labile synaptic changes) [188, 189]. Bacskai et al. (1993) [190] demonstrated how cAMP and PKA participate in the RNA and long-term protein synthesis-dependent process. An increase in cAMP concentration can induce gene transcription through the phosphorylation of CREB (Ser133) [191]. Interestingly, the activation of CREB can also be triggered by cGMP, by increasing intracellular Ca^{+2} levels [192]. The cGMP and PKG pathways cause the release of Ca^{+2} from ryanodine-sensitive stores, and when the Ca^{+2} signal is sufficiently large, it causes phosphorylation of CREB and can induce LTP in parallel with PKA [193]. cGMP-induced potentiation was blocked by protein inhibitors, RNA synthesis, and a PKG inhibitor, but not by a PKA inhibitor, suggesting that it is PKA-independent [192]. Nonetheless, it has been shown that these merge at some stage as both PKG- and PKA-induced late-phase potentiation are blocked by protein inhibitors and RNA synthesis [185].

In the same way, whereas cGMP is implicated in early memory consolidation processes, a role has been attributed to cAMP in late memory consolidation [194, 195]. However, behavioral studies have shown that NO was also involved in long-term memory [196], raising the question of whether cGMP signaling might also be involved. In fact it has recently been demonstrated that NO contributes to long-term memory via the activation of soluble guanylyl cyclase (sGC), cGMP-dependent protein kinase, and CRE-binding protein (CREB) phosphorylation [197]. In a recent study, Bollen and Prickaerts, 2012 [4], showed that cAMP and cGMP signaling act independently to improve memory formation and that cGMP/PKG signaling mediates both early and late memory consolidation, whereas cAMP/PKA signaling mediates late consolidation. Importantly, the cGMP/PKG pathway requires cAMP signaling to enhance consolidation, implying that this is the common pathway in long-term memory formation.

Thus, the late protein synthesis-dependent phase of LTP involves the induction of immediate early genes via CREB phosphorylation, which is mediated in part via PKA [198, 199] and also PKG [200, 201]. New synthesized proteins are required for the growth of new synapses, which may underlie the nootropic effects of PDE inhibitors.

Other downstream targets of PKG have been described in addition to the canonical cGMP/PKG/CREB signaling pathway. While most previous studies were carried out in the hippocampus, in the amygdala the NO-cGMP-PKG signaling pathway regulates LTP and promotes fear memory consolidation via activation of ERK/MAPK signaling, which promotes ERK-driven immediate early gene expression [202, 203]. The cGMP pathway also plays an important role in the specific signaling mechanism that promotes branch formation in neurons [204]. In this case, a direct link has been found between cGMP signaling and GSK-3β, a kinase known to phosphorylate cytoskeletal proteins in neurons [205]. GSK-3β is an important kinase involved in tau pathology associated with AD and therapeutic approaches aimed at the inhibition of these kinases present a novel perspective for the management of AD [206]. It has been hypothesized that the inactivation of GSK3β and the consequent decrease in tau phosphorylation also contribute to the restoration of cognitive function caused by PDE5 inhibitors in AD mice (review in García-Osta et al., 2012) [207].

6. Phosphodiesterase Inhibitors 5 on Cognition

PDEs inhibitors can play a major role in memory function, regulating cell signaling by increasing the concentration of cGMP or cAMP throughout the brain [207]. One of the first studies investigating the effects of PDE inhibitors as memory enhancers in AD patients demonstrated that vinpocetine, a PDE1 inhibitor, failed to improve cognition or slow the rate of memory decline [208]. However, more recent studies have demonstrated the effectiveness of PDE3 (cilostazol), PDE4 (rolipram), or PDE5 (sildenafil or tadalafil) inhibitors in reversing memory impairments in several mouse models of AD [10, 209–212]. The prominent expression of PDE5 in the smooth muscle of the meningeal arteries and in blood vessels suggests that a peripheral effect of PDE5 inhibitors, which may lead to an improvement in cerebral blood flow, may also contribute to the procognitive action of these drugs [211]. However, some authors demonstrated that systemic administration of vardenafil does not affect cerebral blood flow in the hippocampus and even decreases it in some of the brain areas studied [213]. Moreover, a recent report by Akkerman et al., 2015, demonstrated the effectiveness of vardenafil after intracerebrovascular administration, providing evidence that PDE5 inhibitors enhance memory via a central mechanism [9]. Nevertheless, the underlying mechanism has yet to be fully elucidated.

In physiological conditions, PDE5 inhibitors have a more pronounced effect as memory enhancers in aged mice than in young mice. Recently, a report has demonstrated that chronic treatment with sildenafil improved memory in the object recognition and Morris Water Maze (MWM) task in aged mice but not in young mice [214]. The effect on memory is paralleled with an increase in synaptic plasticity and a restoration of pCREB levels in the hippocampus [214]. Interestingly, in the senescence-accelerated prone mouse (SAMP8), a model of age-related cognitive decline, chronic treatment with sildenafil attenuated learning and spatial memory impairments in the MWM [215]. The authors argued that this nootropic effect in SAMP8 mice is mediated by a decrease in tau hyperphosphorylation, an effect that could be mediated by the modulation of the Cdk5/p25 and Akt/GSK-3β pathways by sildenafil [215]. Similar results were obtained with chronic tadalafil treatment in aged mice, where tadalafil enhanced memory by increasing BDNF levels and dendritic spine density in apical dendrites on CA1 hippocampal pyramidal neurons, which may contribute to enhancing learning and memory process [11].

In relation to human reports, udenafil has been used to study the effect of repeated dosing of PDE5-I on cognition

[216, 217]. In a pilot study involving 60 men with erectile dysfunction, udenafil improved cognitive function and depression after two months of daily dosing treatment. The authors argued that the effect was mediated by an increase in the NO-cGMP signaling pathway, increased glucose and oxygen delivery to the brain, and increase in self-esteem in patients [216].

Aging and accumulation of amyloid β (Aβ) peptides are important risk factors for the development of dementia. It has been reported that a decrease in the basal level of cGMP occurs in the brain with aging [218]. cGMP synthesis in the central nervous system occurs mainly through the activation of NO-dependent soluble guanylate cyclase (sGC). At the same time, NO formation is typically coupled to the activation of NMDA receptors meaning, therefore, that the activation of the NMDA receptor significantly stimulates NO/cGMP production in the hippocampus. On the other hand, cGMP hydrolysis is regulated by PDEs, specifically PDE2, PDE5, and PDE9, which are the most prevalently expressed in the brain. Thus, a decrease in cGMP could be a consequence of a more active degradation of cGMP by PDEs and/or a decrease in NMDA receptor-mediated cGMP formation in the aged brain when compared with the adult brain. The accumulation of Aβ peptides in AD can also decrease the NO/cGMP dependent signal transduction mediated by NMDA receptors [219]. Furthermore, changes in the expression and activity of both constitutive NOS isoforms, eNOS and nNOS, have been implicated in the decline of cognitive functions in the senescent brain by decreasing cGMP synthesis [219, 220].

Due to the possible regulation of the cGMP/PKG/CREB and cGMP/PKG/pGSK3 β pathways through the increase in levels of cGMP in the brain, PDE5 inhibitors appear to be good candidates for AD treatment. The expression of PDE5 in brain areas involved in cognition (e.g., the hippocampus and cortex) supports this hypothesis [11]. Nevertheless, the lack of availability of good brain-penetrant PDE5 inhibitors has been crucial to the carrying out of studies in AD animal models. At the same time, although currently available selective PDE5 inhibitors have low blood-brain barrier (BBB) permeability, tadalafil and sildenafil have been used in chronic treatments and in a range of AD models, confirming their efficacy in reversing cognitive impairment [10, 207, 211]. Increased cGMP levels have been detected in the brain after the administration of tadalafil and sildenafil, confirming that both drugs cross the BBB and reach the brain at a sufficient concentration to inhibit PDE5 [3, 11], the stimulation of the cGMP/PKG pathway and the restoration of CREB signaling may underlie the therapeutic effect of PDE5 inhibitors in AD. The activation of CREB promotes the transcription of genes, such as BDNF, which, as has been proposed, contributes to the neuroprotective effects of sildenafil [175]. Indeed, the upregulation of BDNF has also been observed in the hippocampus after chronic sildenafil treatment in a mouse model of AD. The full mechanism of action, however, has yet to be elucidated [211].

The downstream activation of CREB by the NO/cGMP pathway provides an interesting link to cognitive dysfunction and decreased synaptic plasticity in AD. The NO-cGMP-PKG signaling pathway contributes to CREB phosphorylation and LTP in the hippocampus, evidently acting in parallel with PKA and MAP kinase [192]. In fact, dysfunction in CREB signaling contributes to the pathology of AD, leading to synaptic dysfunction and cognitive impairment in both AD patients and AD animal models [209, 221]. It has been suggested that in AD, Aβ impaired synaptic plasticity by downregulating the NO/cGMP/PKG/CREB pathway in hippocampal slices [222]. It has been found that Aβ decreases the activity of sGC, and therefore the synthesis of cGMP in brain astroglial cells and in the temporal cortex of AD patients [223, 224]. It has recently been found that cGMP levels are significantly lower in the Cerebral Spinal Fluid (CSF) of AD patients compared with nondemented controls. Importantly, a significant association was found between cGMP levels and cognitive decline and Aβ42 levels in AD patients. The authors also identified an association between a decrease in cGMP and an increase in PDE5 expression in the temporal cortex of AD patients [225]. Taken together these findings suggest that the cGMP/PKG/CREB pathway is downregulated in AD and therapeutic strategies aimed at increasing cGMP seem to be a good option in dementia associated with aging and AD. Moreover, downregulation of the AC/cAMP/PKA pathway can also account for a loss of synaptic plasticity and memory in AD patients [226].

The beneficial effect of PDE-5 inhibitors in reducing Aβ levels is controversial. While some studies have demonstrated the beneficial effects of PDE5 inhibitors in reducing Aβ levels in different mouse models [10, 218, 227, 228], other authors have argued that PDE5 inhibitors did not affect the Aβ burden [11, 211]. This discrepancy may be due to differences in animal models and the severity of the amyloid pathology. Some authors argued that a decrease in amyloidogenic APP processing, and consequent Aβ formation, is induced by the inhibition of PDE5 [227, 229]. Nevertheless, the mechanisms by which PDE5 inhibitors decrease Aβ levels are not yet clear. In terms of tau pathology, another major pathological hallmark of AD, most authors agree that PDE5 inhibitors reduce levels of phosphorylated tau through the inhibition of GSK3beta [11, 211, 215].

In addition to sildenafil, tadalafil, and vardenafil, the most commonly used PDE5 inhibitors, other novel selective PDE5-Is have recently been identified. A selective quinoline-based compound with an IC50 of 0.27 nM and favorable permeability has also been shown to recover memory defects in a mouse model of AD [230]. Icariin, a flavonoid extracted from a Chinese herb (Berberidaceae *Epimedium* L.), has been described as an effective PDE5 inhibitor that is also an effective AD reversion phenotype in mice, through the stimulation of the NO/cGMP signaling pathway in the brain [218]. Finally, Yonkenafil, a novel PDE5 inhibitor [231] which has a strong inhibiting effect against PDE-5 (IC50 of 2.01 nM), improved cognitive function and ameliorated the amyloid burden in an APP/PS1 transgenic mice model, by inhibiting the activation of glial cells and restoring neurogenesis [231].

7. Concluding Remarks

In recent years, knowledge about NO-cGMP signaling has emerged as a promising target for neuroinflammation and cognitive disorders. Special attention, however, must be paid to the effect of NO-cGMP on glial cells, particularly oligodendrocytes, as little is known about their immunological function or their possible crosstalk with microglia and astrocytes in the progression of neuroinflammatory and demyelinating diseases. Moreover, little is known about the relationship between NO-cGMP and glial cells in the development of some important neurodiseases such as PD, HD, and ALS. Given the crucial role of NO-cGMP in neuroinflammation and cognition, there is a wide range of possible therapeutic applications of PDE5-Is. Furthermore, as the majority of studies to date have concentrated on sildenafil, the potential of other inhibitors, such as tadalafil, which has a long-lasting half-life and crosses the BBB, remains underexplored. Increased knowledge of this subject will provide a conceptual framework for the use of PDE5 inhibitors and the design and delivery of novel selective agents.

Conflict of Interests

The authors declare that no conflict of interests exists regarding the publication of this paper.

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CAPÍTULO 6

ARTIGO EM ELABORAÇÃO

CAPÍTULO 6- SILDENAFIL (VIAGRA®) PREVINE E RESTAURA INFLAMAÇÃO INDUZIDA POR LPS EM ASTRÓCITOS

METODOLOGIA

Grupo 1: Controle

Grupo 2: LPS 10 ng/ml (24 horas).

Grupo 3 (Sildenafil 1 µM preventivo): Sildenafil 1 µM por 4 horas, seguido por LPS 10 ng/ml + Sildenafil 1 µM por 24 horas.

Grupo 4 (Sildenafil 10 µM preventivo): Sildenafil 10 µM por 4 horas, seguido por LPS 10 ng/ml + Sildenafil 10 µM por 24 horas.

Grupo 5 (Sidenafil 1 µM terapêutico): LPS 10 ng/ml por 24 horas, seguido por Sildenafil 1 µM por 4 horas.

Grupo 6 (Sidenafil 10 µM terapêutico): LPS 10 ng/ml por 24 horas, seguido por Sildenafil 10 µM por 4 horas.

Procedimentos *in vitro* – cultura de astrócitos

Os astrócitos corticais primários de rato foram adquiridos daInvitrogen (Thermo Fisher Scientific, Waltham, MA, EUA) e preparados de acordo com as instruções do fabricante com algumas modificações. Resumidamente, um frasco com 1 x 10^6 células viáveis foi rapidamente descongelado e lavado suavemente com meio de crescimento constituído por meio Eagle Dulbecco modificado (DMEM) (elevado teor de glucose) suplementado com 15% de soro bovino fetal (ambos da Invitrogen). Após centrifugação, o sobrenadante foi removido e as células ressuspensas em meio de crescimento astrocitário. As células foram plaqueadas a uma densidade de 1 x 10^4 células por cm^2, sobre uma

lamínula de vidro não revestido (nr 1, 20 mm de diâmetro) (Bergman Labora, Estocolmo, Suécia) colocada numa placa de 12 poços. As células foram incubadas a 37$_o$C, 5% de CO2 em 90% de humidade e o meio foi substituído duas vezes por semana. Os astrócitos foram usadas após 16 - 17 dias de cultivo.

Imagem de cálcio latente

Os astrócitos foram incubadas à temperatura ambiente com a sonda sensível ao Ca2 , Fura-2 / AM (Invitrogen Molecular Probes, Eugene, OR, EUA) durante 30 min (8 uL em 990 ul de HEPES de Hank tamponada com solução salina [HHBSS], contendo 137 mM de NaCl, 5,4 mM de KCl, MgSO4 0,4 mM, MgCl2 0,4 mM, CaCl2 1,26 mM, KH2PO4 0,64 mM, NaHCO3 3,0 mM, glicose 5,5 mM e HEPES 20 mM, dissolvido em água destilada, pH 7,4). Todas as substâncias usadas durante o experimento foram diluídas com a mesma solução. A sonda fluorescentefoi dissolvida com 40 ul de dimetilsulfóxido (DMSO) e 10 ul de ácido plurónico (Molecular Probes, Leiden, The Netherlands). Após a incubação, as células foram lavadas três vezes com HHBSS. Depois de um minuto a partir do início do experimento, o glutamato (10-3 M) foi utilizado como o estimulador. Os experimentos foram realizados em temperatura ambiente usando um sistema de imagem de Ca^{2+} e software simples PCI (Compix Inc., Imaging Systems, Hamamatsu Photonics Management Corporation, Cranberry Twp, PA, EUA) e um microscópio de epifluorescência invertido (Nikon ECLIPSE TE2000-E), com uma lente seca x20 (NA 0,45) de fluorescência e um, sistema de iluminação monocromático policromia V (ATE Photonics GmbH, Pleasanton, CA, EUA). As diversas substâncias foram fornecidos por uma bomba peristáltica (Instech Laboratories, Plymouth Meeting, PA, EUA) a um fluxo aproximado de 600 ul / min. As substâncias levaram aproximadamente 60 segundos para atingir as células através dos tubos. As imagens foram capturadas com uma câmera ORCA-12AG (C4742-80-12AG), High Res Digital Refrigerado CCD (Hamamatsu Photonics Corporation, Hamamatsu, Japão). As áreas totais sob os transientes (AUC), refletindo quantidades de Ca^{2+} liberados (Berridge 2007), foram analisadas para

fornecer medidas do vigor das respostas de Ca^{2+} . O número de picos foi calculado manualmente para cada célula. A área sob os picos de Ca^{2+} foi calculado utilizando o programa Origin (Microcal Software Inc., Northampton, MA, EUA).

Imunocitoquímica

Após o período de incubação, as células foram fixadas com paraformaldeído a 4% (Bie & Berntsen, Herlev, Dinamarca) durante 10 min e lavadas duas vezes com solução salina tamponada com fosfato (Invitrogen) contendo 1% de albumina de soro bovino (PBS-BSA). As células foram permeabilizadas com PBS-BSA contendo 0,05% de saponina (PBS-BSA-sap) durante 20 min. Em seguida, as células foram incubadas durante 1 h com anticorpo policlonal de coelho contra GFAP (Dako, Glostrup, Dinamarca). O anticorpo foi diluído 1: 100 em PBS-BSA-sap. As células foram lavadas com PBS-BSA-sap 3 x 5 min e em seguida incubadas com anticorpo secundário anti-rabbit F(ab')2 conjugado com 594 DyLight (Jackson Immuno Research, Westgrove, PA, USA), diluídas 1: 150 . As células foram lavadas com PBS-BSA-sap 3 x 5 min e, finalmente, lavadas com PBS. As lamínulas foram montadas em lâminas de microscópio com um meio de montagem fluorescente (Dako) e visualizadas em um microscópio Nikon Eclipse 80i. As fotos foram tiradas com uma câmera Hamamatsu C5810 3 CCDs.

Visualização das fibras de estresse (*stress fibers*)

Após a incubação com os tratamentos descritos acima, as células foram fixadas com 4% de paraformaldeído e permeabilizadas com PBS (Invitrogen) contendo 1% de BSA e 0,05% de saponina, seguida por uma sonda de faloidina conjugada com Alexa488 (Invitrogen) diluída 1:40 em PBS suplementado com BSA a 1% para marcação de citoesqueleto dos astrócitos. As lamínulas foram lavadas três vezes em PBS e montadas em lâminas de microscópio usando meio de montagem para

fuorescência da Dako (Dako), antes de serem observadas com lente objetivo seca, em um microscópio invertido de fluorescência Nikon Optiphot-2.

Análise estatística

Os valores foram comparados pelo teste de variância de uma via (ANOVA-one way), seguido por pós-teste de Student T, para comparações múltiplas, utilizando o programa GraphPad Prism (San Diego, CA, USA). Foram consideradas significativas as diferenças com $p < 0,05$. Os dados foram apresentados como a média ± desvio padrão.

RESULTADOS

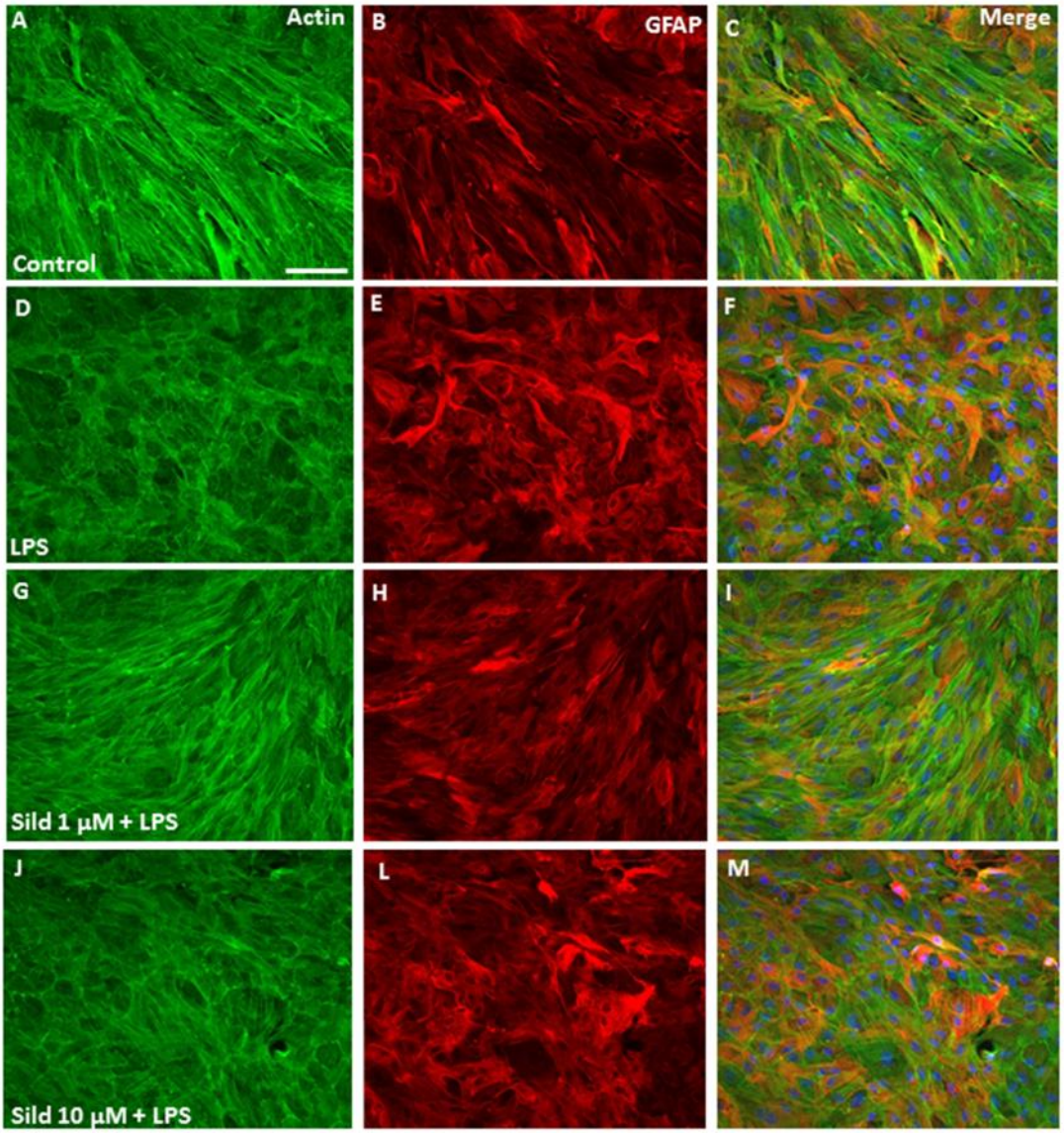

Figura 1 : Tratamento preventivo com sildenafil 1 µM preservou filamentos de actina e morfologia astrocitária. Os astrócitos foram corados com uma sonda faloidina conjugada com Alexa488 . A imunomarcação para GFAP foi feita para confirmar o fenótipo de astrócitos. As imagens sobrepostas são também demonstradas. A-C mostram as células não tratadas, com filamentos de actina organizados em fibras de stress e morfologia tipicamente normal. D-F mostram que o LPS induz desorganização dos filamentos de actina e alterações da morfologia celular. G-I Os astrócitos que foram incubados peviamente com Sildenafil 1 µM mostraram filamentos de actina, organizados em fibras de estresse e morfologia similar ao grupo controle. J-M tratamento com sildenafil 10 µM mostrou melhora discreta na preservação de filamentos de actina e com morfologia dos astrócitos típica.

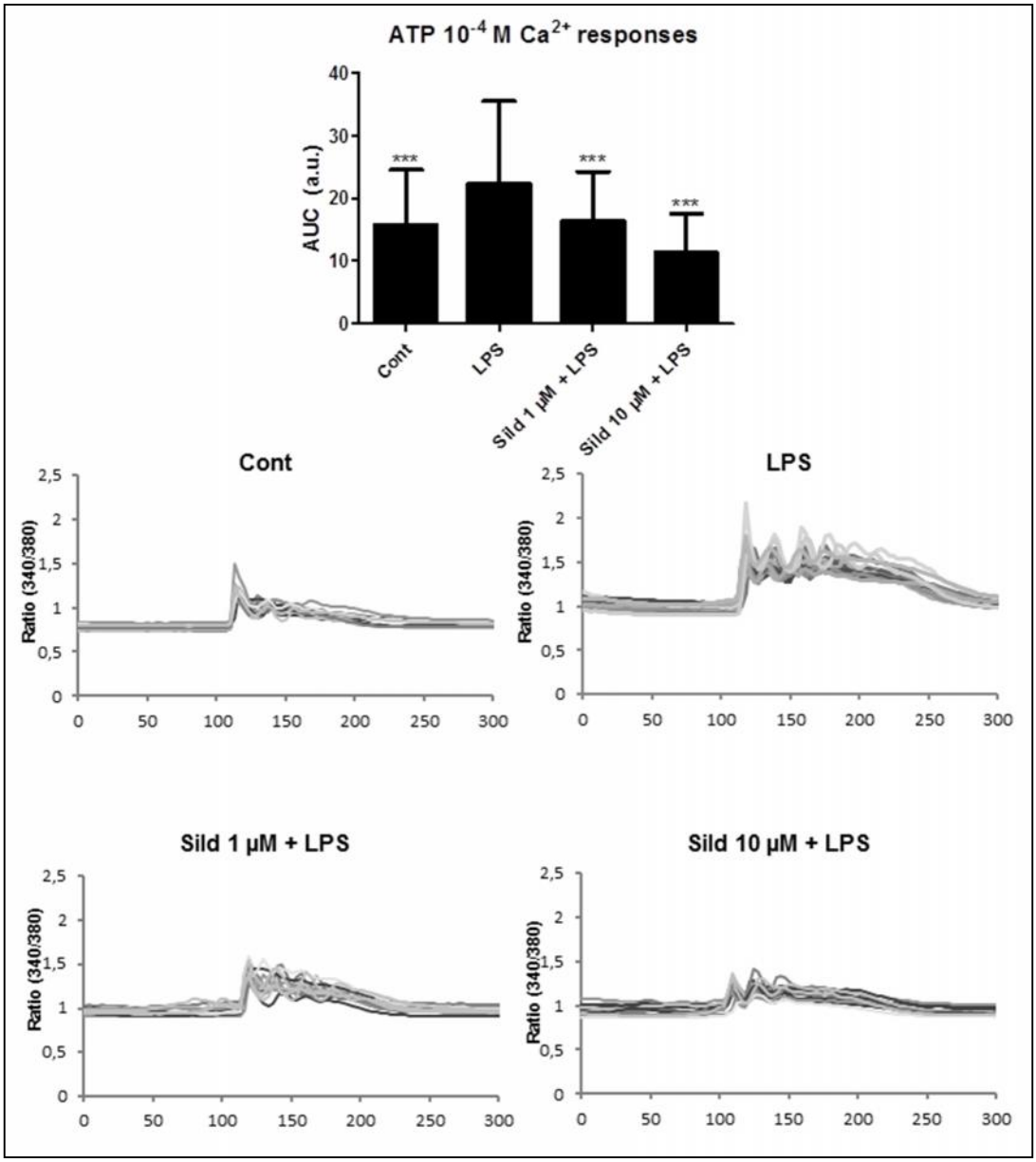

Figura 2: Tratamento preventivo com Sildenafil diminui a resposta de Ca^{2+} e atividade oscilante evocadas pelo ATP. Dados estatísticos mostram que o células do grupo controle apresentaram diminuição significativa na de resposta de Ca^{2+} em relação ao grupo LPS , indicando fisiologia normal dessas células. O tratamento preventivo com Sildenafil 1 e 10 µM promoveu redução nas respostas de Ca2+, comparado ao grupo LPS. . Ambos os tratamentos apresentarm diminuição significativa (*** $p<0.05$) em relação ao grupo LPS.. O grupo LPS apresentou resposta típica de estados inflamatório das células. O ATP induziu respostas de Ca^{2+} em todos os astrócitos em um sistema de imagem de Ca^{2+}. As células foram carregadas com a sonda de Ca^{2+}, Fura-2 / AM, durante 20 min. As áreas sob as curvas (AUC) do Ca^{2+} transientes foram calculados.

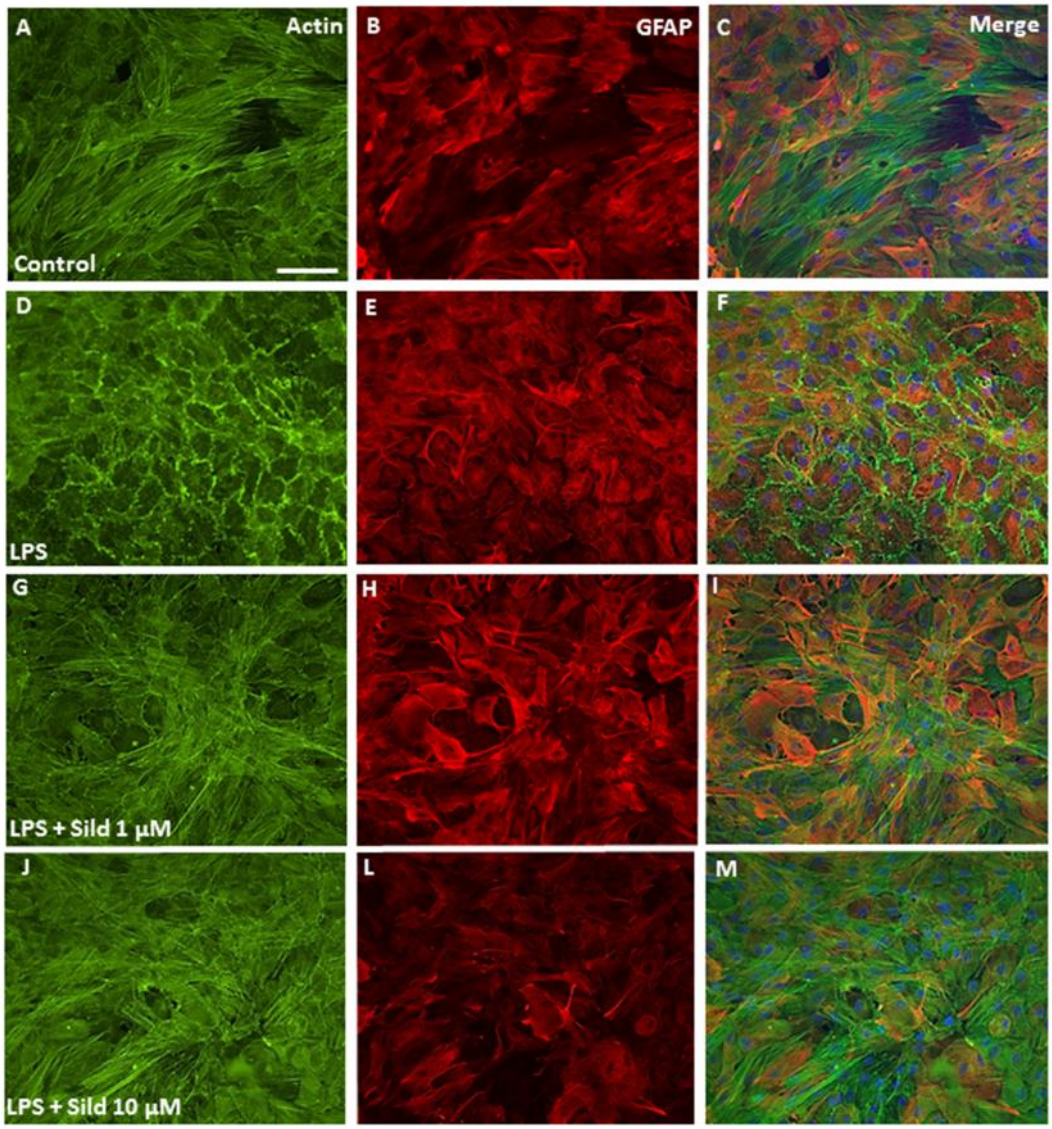

Figura 3: Tratamento com sildenafil 10 µM restaurou filamentos de actina e morfologia astrocitária. Os astrócitos foram corados com uma sonda faloidina conjugada com Alexa488 . A imunomarcação para GFAP foi feita para confirmar o fenótipo de astrócitos. As imagens sobrepostas são também demonstradas. A-C Mostram as células não tratadas, com filamentos de actina organizados em fibras de stress e morfologia tipicamente normal. D-F LPS induz a desorganização dos filamentos de actina e alterações da morfologia celular. G-I Tratamento com sildenafil 1 µM mostrou melhora discreta na preservação de filamentos de actina e com morfologia dos astrócitos típica em gliose reativa. J-M As células incubadas com Sildenafil 10 µM mostraram filamentos de actina organizado em fibras de stress e morfologia similar ao grupo controle.

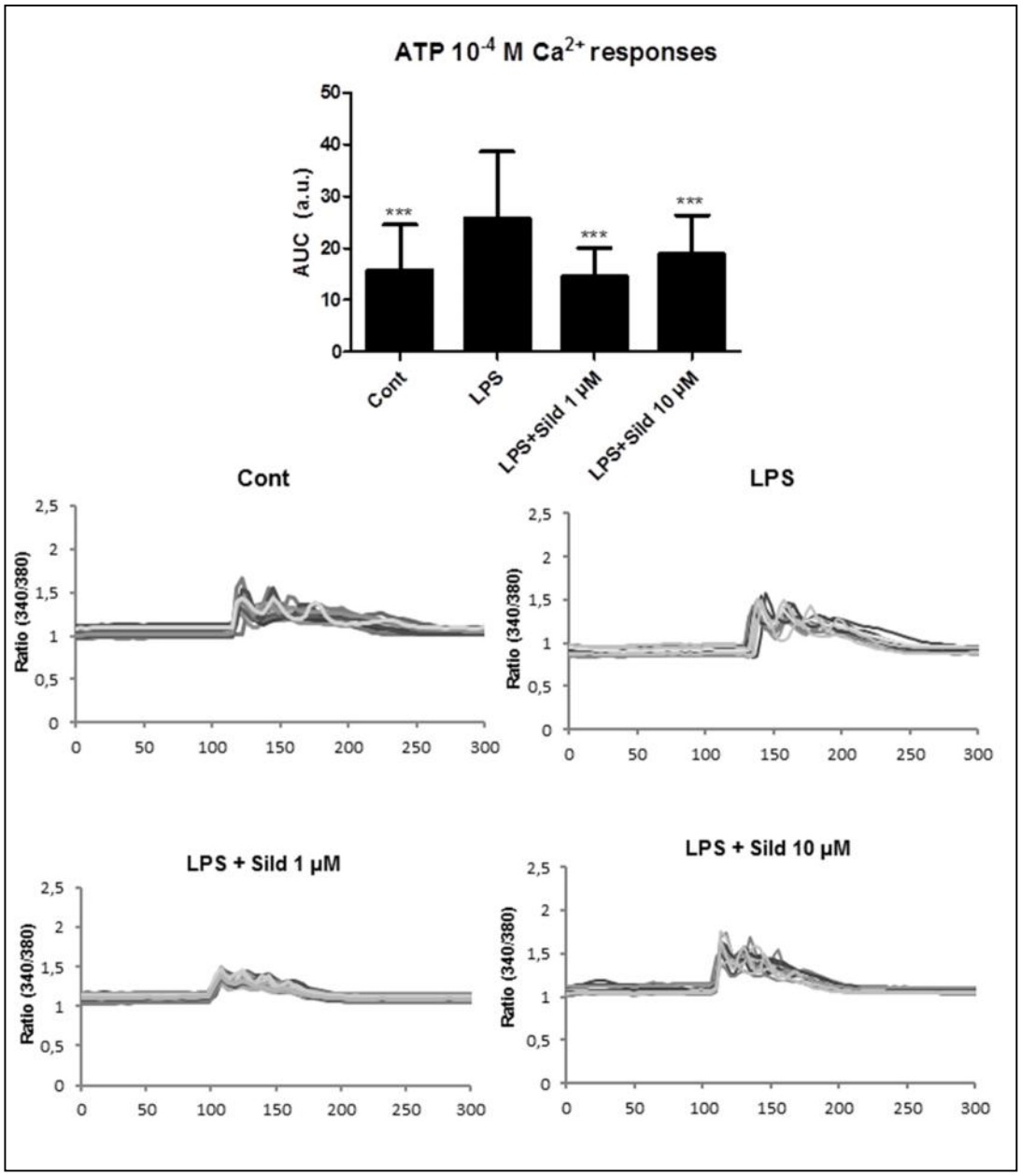

Figura 4: Tratamento terapêutico com Sildenafil diminui a resposta de Ca^{2+} e atividade oscilante evocadas pelo ATP. Dados estatísticos mostram que o células do grupo controle apresentaram resposta de Ca^{2+} significativamente menor em relação ao grupo LPS , indicando fisiologia normal dessas células. O tratamento preventivo com Sildenafil 1 e 10 µM promoveu diminuição da respostas astrocitária, em relação ao grupo LPS Ambos os tratamentos apresentarm diminuição significativa (*** $p<0.05$). O grupo LPS apresentou resposta típica de estado inflamatório das células. O ATP induziu respostas de Ca^{2+} em todos os astrócitos em um sistema de imagem de Ca^{2+}. As células foram marcadas com a sonda de Ca^{2+}, Fura-2 / AM, durante 20 min. As áreas sob as curvas (AUC) dos transientes de Ca^{2+} foram calculados.

5. CONCLUSÃO

Diante desses resultados, concluímos que o Sildenafil pode ser apontado como potente fármaco terapêutico para paciente portadores de doenças desmielinizantes. O presente estudo fornece evidências de que o Sildenafil tem ação neuroprotetora e anti-inflamatória, provavelmente por meio da modulação da AMPK, NFκB e sinalização de eNOS-NO em camundongos C57BL/6. Além disso, a redução de citocinas inflamatórias IL-1β e TNF-α, pode contribuir para o controle da neuroinflamação, uma vez que podem ativar o NFκB, e consequentemente a transcrição de genes pró-inflamatórios. Outros efeitos neuroprotetores do Sildenafil foram a modulação do IL-10 e do NGF. A via AMPK-eNOS-NO-GMPc e GMPc- IKK- NFκB parece estar envolvida, no entanto, outras vias da cascata de sinalização da inflamação/neurodegeneração precisam ser mais investigadas.

O tratamento com sildenafil também contribuiu para recuperação da bainha de mielina, bem como o ambiente neuronal e integridade de zonas danificadas, tais como cerebelo e corpo caloso de animais C57BL/6 com desmielinização induzida por cuprizona. Esse efeito podem estar associado com a interação entre a quimiocina MCP-1/CCR-2 e a metaloproteinase MMP-9, envolvidas no controle da inflamação, processo de reparo de tecido e organização da mielina. A condição para o reparo tecidual é percebido pela mudança fenotípica de microglias que assumem papel fagocitíco.

A investigação em camundongos konckout iNOS$^{-/-}$ evidenciou uma maior susceptibilidade a intoxicação induzida pelo cuprizona. Esse efeito pode esta relacionado ao envolviemnto dos seguintes mecanismos: (1) ausência do mecanismo de feedback negativo iNOS em condições inflamatórias e, conseqüentemente, proteínas pró-inflamatórias, tais como citocinas e COX-2, são excessivamente aumentadas; (2) super-expressão da enzima eNOS como mecanismo compensatório, gerando cronicamente altos níveis de NO, levando ao dano tecidual.

Os resultados do presente estudo sugerem que o sildenafil pode exercer os seus efeitos anti-inflamatórios, principalmente através da inibição da iNOS. Além disso, o sildenafil pode ter um efeito protetor direto em oligodendrócitos, porém outros estudos precisam ser realizados.

O estudo com cultura de astrócitos proporcionou um melhor entendimento quanto ao papel do Sildenafil no papel preventivo e restaurador após estímulos inflamatórios em astrócitos. Estudos complementares devem ser realizados para melhor compreensão do mecanismo de ação deste fármaco.

Portanto, é possível que após ensaios clínicos bem conduzidos, o tratamento preventivo e terapêutico com Sildenafil seja mais uma opção adicional de fármaco aos pacientes acometidos por patologias de caráter neuroinflamatório, proporcionando benefícios adicionais aos tratamentos atuais de pacientes com esclerose múltipla.

ANEXOS

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o Projeto intitulado: **"AVALIAÇÃO DOS EFEITOS DO INIBIDOR DE FOSFODIESTERASE-5 SOBRE A NEUROINFLAMAÇÃO, EM MODELO DE ESCLEROSE MÚLTIPLA (ENCEFALITE AUTOIMUNE EXPERIMENTAL) INDUZIDO EM CAMUNDONGOS C57BL/6 WILD TYPE E KNOCKOUT PARA iNOS."** protocolado sob o Nº 10/2010, coordenado pelo (a) pesquisador(a) **CHRISTINA ALVES PEIXOTO**, está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães/ Fundação Oswaldo Cruz (CEUA-CPqAM) em reunião de 28/04/2011. Na presente versão, este projeto está licenciado e tem validade até o mês de janeiro de 2013.

Quantitativo de Animais Aprovados	
Espécie - linhagem	**Nº de Animais**
Camundongo C57BL/6 SELVAGEM **CAMUNDONGO C57BL/6 B6.129 P2- Nos2 (Knockout iNOS)**	**90** **30**
TOTAL	**120**

We certify that the project entitled "**AVALIAÇÃO DOS EFEITOS DO INIBIDOR DE FOSFODIESTERASE-5 SOBRE A NEUROINFLAMAÇÃO, EM MODELO DE ESCLEROSE MÚLTIPLA (ENCEFALITE AUTOIMUNE EXPERIMENTAL) INDUZIDO EM CAMUNDONGOS C57BL/6 WILD TYPE E KNOCKOUT PARA Inos**." *(CEUA* Protocol Nº 10/2010), coordinated by **CHRISTINA ALVES PEIXOTO** according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães/ Fundação Oswaldo Cruz on April 28, 2011. In the present version this project is licensed and valid until january 2013

Recife (PE, Brazil) may 23 , 2011.

Drª Sheilla Andrade de Oliveira
Vice - Coordenadora da Comissão de Ética no Uso de Animais
Centro de Pesquisas Aggeu Magalhães – FIOCRUZ

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