KENJI WILLIAM RUIZ MIYAZAWA

O EFEITO ANALGÉSICO DA VIMPOCETINA DEPENDE DA INIBIÇÃO DA PRODUÇÃO DE CITOCINAS E ESTRESSE OXIDATIVO PERIFÉRICO E ESPINAL

Londrina
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Orientador: Prof. Dr. Waldiceu A. Verri Jr.

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Londrina, 25 de Abril de 2013.

DEDICO

"Dedico este trabalho primeiramente a Deus, pois sem Ele, nada seria possível e não estaríamos aqui reunidos, desfrutando, juntos, destes momentos que nos são tão importantes".

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“Todos os homens sonham, mas não da mesma maneira. Aqueles que sonham de noite nos recantos poeirentos das suas mentes acordam para descobrir que foi tudo em vão; Mas os que sonham de dia são homens perigosos, pois podem realizar os seus sonhos de olhos abertos, para os tornar possíveis. Foi o que eu fiz.” T. E Lawrence “Lawrence of Arabia”

MIYAZAWA, Kenji William Ruiz**. O efeito analgésico da vimpocetina depende da inibição da produção de citocinas e estresse oxidativo periférico e espinal.** 2013. 84f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina. Londrina-Pr, 2013.

RESUMO

A vimpocetina é um agente nootrópico utilizado para o tratamento de disfunção cognitiva, como acidente vascular encefálico e distúrbios de memória. Estudos recentes em diferentes modelos demonstraram que além dos efeitos nootrópicos, já conhecidos, este fármaco apresenta atividade anti-inflamatória e analgésica. Baseados nestes dados, propomos investigar o efeito analgésico da vimpocetina em diferentes modelos de estímulos inflamatórios para determinar seus efeitos anti-inflamatórios e antinociceptivos, assim como determinar seus possíveis mecanismos de ação. O comportamento da dor manifesta foi avaliado por ácido acético e fenil-p-benzoquinona (PBQ) responsáveis por induzir contorções abdominais e formalina e adjuvante completo de Freund (CFA) responsáveis por induzir os movimentos de agitação da pata. Hiperalgesia mecânica foi avaliada por uma versão eletrônica do método de von Frey, hiperalgesia térmica pela placa quente, o recrutamento de neutrófilos pela atividade de mieloperoxidade (MPO), a produção de citocinas foram avaliadas nas amostras da medula (L4-L6) e pele da pata por ELISA, já a atividade antioxidante foi analisada pela diminuição dos níveis de glutationa (GSH). Os resultados obtidos apontam que a vimpocetina quando administrada via oral inibe a dor manifesta induzidas pelo ácido acético (até 56%), fenil-p-benzoquinona (81%), formalina (0-5 min-36% e 15-30 min-54%) e CFA (36%). A vimpocetina também foi capaz de inibir a atividade de MPO induzida por carragenina (até 69%), hiperalgesia mecânica (até 78%) e térmica (até 100%), produção de IL-1β e TNFα na medula (65 e 64%, respectivamente) e na pele da pata (49 e 73%, respectivamente), e a depleção dos níveis de GSH na pele da pata e na medula (99 e 50%, respectivamente). No modelo do CFA, a vimpocetina inibiu a atividade de MPO (68%); hiperalgesia mecânica (até 30%) e térmica (até 100%), a produção de IL-1β e TNFα na pele da pata (41 e 53%, respectivamente) e na medula (84 e 79%, respectivamente), e a depleção dos níveis de GSH na pele da pata e na medula (60 e 61%, respectivamente). Em conclusão, a vimpocetina foi capaz de reduzir a dor inflamatória por mecanismos relacionados à redução do recrutamento de neutrófilos, produção de citocinas e estresse oxidativo.

Palavras chaves: Vimpocetina. Inflamação. Citocinas. Estresse oxidativo. Dor. Hiperalgesia.

Miyazawa, kenji William Ruiz. **Analgesic effect of vinpocetine: spinal and peripheral inhibition of cytokines production and oxidative stress.** 2013. 84f. Dissertation (Master's degree in Experimental Pathology) – Universidade Estadual de Londrina. Londrina-Pr, 2013.

ABSTRACT

Vinpocetine is a nootropic agent used for memory improvement. Recent studies demonstrated some of its anti-inflammatory and analgesic effects. Herein, the analgesic effect and mechanisms of vinpocetine were further addressed. Overt pain-like behavior was assessed by acetic acid and phenyl-p-benzoquinone-induced writhing response and formalin and complete Freund's adjuvant (CFA)-induced paw flinches. Mechanical hyperalgesia was investigated using an electronic anesthesiometer, thermal hyperalgesia using the hot-plate, the recruitment of neutrophils by myeloperoxidase (MPO) activity, cytokines production in spinal cord (L4-L6) and paw skin samples by ELISA, antioxidant activity by the levels of reduced glutathione (GSH). Vinpocetine inhibited the overt pain-like behavior induced by acetic acid (up to 56%), phenyl-p-benzoquinone (81%), formalin (0-5 min-36% and 15-30 min-54%) and CFA (36%). Vinpocetine also inhibited carrageenin-induced MPO activity (up to 69%), mechanical (up to 78%) and thermal (up to 100%) hyperalgesia, IL-1β and TNF-α production in the spinal cord and paw skin (65 and 64%, and 49 and 73%, respectively), and GSH depletion in paw skin and spinal cord (99 and 50%, respectively). In the model of CFA model, vinpocetine inhibited the MPO activity (68%); mechanical (up to 30%) and thermal (up to 100%) hyperalgesia, IL-1β and TNF-α production in paw skin and spinal cord (41 and 53%, and 84 and 79%, respectively), and GSH depletion in paw skin and spinal cord (60 and 61%, respectively). Vinpocetine reduces inflammatory pain by mechanisms related to reduction of cytokine production and oxidative stress.

Key words: Vinpocetine. Inflammation. Cytokine. Oxidative stress. Pain. Hyperalgesia.

Abbreviations: AST, Aspartate aminotransferase); ALT, Alanine aminotransferase; cAMP, cyclic adenosine monophosphate; CFA, Complete Freund's adjuvant; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); ELISA, Enzyme-linked immunosorbent assay; GSH, Reduced glutathione; HTAB, Hexadecyltrimethylammonium bromide; IL-1β, Interleukin-1 beta; MPO, Myeloperoxidase; PBQ, Pphenyl-p-benzoquinone; TNFα, Tumor necrosis factor alpha.

LISTA DE ABREVIATURAS E SIGLAS

ALT –	Alanina Aminotransferase
AST –	Aspartato Aminotransferase
ATP-	Trifosfato de adenosina
Aβ-	Fibra nervosa do tipo A-beta
Aδ-	Fibra nervosa do tipo A-delta
C-	Fibra nervosa do tipo C
Ca+2-	Cálcio
CINC-1-	Quimiocina de ratos análoga a IL-8 em humanos
CGRP-	Peptídeo relacionado ao gene da calcitonina
Cg –	Carragenina
cAMP -	Monofosfato cíclico de adenosina
cGMP -	Monofosfato cíclico de guanosina
CFA -	Complete Freund's adjuvant
DMSO –	Dimetilsulfóxido
EDTA -	ácido etilenodiamino tetra-acético
ELISA –	Ensaio Imunoenzimático
GRD -	Gânglio da raiz dorsal
GSH –	Glutationa
HTAB -	Hexadecyltrimethylammonium bromide
IL-1β -	Interleucina-1 beta
h-	Hora
IASP-	Associação Internacional para o Estudo da Dor
IL-	Interleucina
IL-1-	Interleucina 1
IL-1β-	Interleucina 1 Beta
IL-8-	Interleucina 8
IL-10-	Interleucina 10
i.p -	Intraperitoneal
i.pl –	Intraplantar
K^+-	Potássio
KC -	Quimiocina derivada de queratinócitos corresponde à IL-8 em humanos

Mg-	Miligrama
mg/kg-	Miligrama por quilo
min-	Minuto(s)
mL-	Mililitro
mL/kg-	Mililitro por quilograma
m/s-	Metro por segundo
µg/kg-	Micrograma por quilograma
µL-	Microlitro
µL/grama-	Microlitro por grama
Na^+-	Sódio
MPO -	Mieloperoxidase
PBQ -	phenyl-p-benzoquinone
PGE_2-	Prostaglandina E_2
PKA-	Proteinocinases A
PKC-	Proteinocinases C
pg -	picogramas
p.o –	per oral
TNF-	Fator de Necrose Tumoral
TNFα-	Fator de Necrose Tumoral Alfa
TNFR1-	Receptor de TNF Tipo 1TRIS - tris(hidroximetil)aminometano
v.o –	via oral

SUMÁRIO

1 INTRODUÇÃO

1.1 Processos Inflamatórios e Dor

A primeira defesa do organismo frente a um dano tecidual é a resposta inflamatória. A inflamação é um processo biológico complexo desencadeado pelas células imune no local da lesão a fim de promover eventos vasculares culminando na passagem de células e moléculas do sangue para os tecidos, para que ocorra a eliminação do agente agressor (MESQUITA et al., 2010; BALESTIERI, 2006).

Os eventos inflamatórios podem ser desencadeados após dano celular causado por microrganismos, agentes físicos (radiação, trauma, queimaduras), químicos (toxinas, substâncias cáusticas), necrose tecidual e/ou reações imunológicas (ABBAS; JANEWA, 2000; GOMES-LEAL, 2002).

Os mediadores inflamatórios produzidos pelas células no local da lesão agem em conjunto, induzindo modificações morfológicas e funcionais peculiares ao processo tais como rubor, calor, dor, edema e perda da função (ABBAS; JANEWA, 2000; CONTRAN, 2000; GOMES-LEAL, 2002). Vários são os mediadores pró-inflamatórios envolvidos nesse processo, como por exemplo, os eicosanoides (prostaglandinas, tromboxanos e leucotrienos), fator de ativação plaquetária (PAF), aminas vasoativas (histamina e serotonina), citocinas pró-inflamatórias (IL-1, IL-8, TNF-α IFN-α), óxido nítrico, ou ainda ser provenientes do plasma, como: componentes do sistema complemento, sistema de coagulação, sistema fibrinolítico e cininas (BOOTHE, 2001; ROCHA, 2001; CARVALHO et al., 2004).

A ativação de células residentes como macrófagos e mastócitos induz a produção de citocinas pró-inflamatórias como fator de necrose tumoral-alfa (TNF-α) e interleucina- 1beta (IL-1β), às quais ativam as células endoteliais que passam a expressar moléculas de adesão da família das selectinas e integrinas, importantes no processo de transmigração dos leucócitos do sangue para o tecido (COTRAN, 2000). Esta emigração dos leucócitos ativados para o foco inflamatório ocorre por meio de substâncias quimioatraentes. Dentre elas podemos citar como moléculas endógenas os componentes do sistema complemento C3a e C5a, leucotrienos, citocinas e quimiocinas como IL-8 e como moléculas exógenas, os produtos bacterianos (lipopolisacarídeos de bactérias gram negativas- LPS). As principais células a chegarem ao local do foco inflamatório são neutrófilos seguidos de macrófagos e linfócitos

que agem principalmente na fagocitose e apresentação ou reconhecimento de peptídeos do agente agressor (BALESTIERE, 2006).

Diversos mediadores envolvidos no processo inflamatório são responsáveis pelos eventos vasculares da inflamação, podendo, muitos deles estimular os neurônios sensoriais locais, contribuindo para o desenvolvimento da dor (GILMAN et al., 2006; SPINOSA et al., 2006; RANG et al., 2007; ROTH et al., 2009).

A dor é uma sensação extremante importante e essencial para o sistema de defesa do corpo, pois nos adverte de que a integridade do organismo está sendo ameaçada ou que ocorre alguma disfunção (FERREIRA et al., 2013), porém, por outro lado, pode trazer consequências desagradáveis tais como estresse, sofrimento, distúrbios nas relações sociais e econômicas (LOESER; MELZACK, 1999; BRENNAN et al., 2007).

A Associação Internacional para o Estudo da Dor (*International Associaton for the Study of Pain,* IASP) definiu dor como "uma experiência sensorial e/ou emocional desagradável, associada ou não ao dano potencial dos tecidos". Deste mesmo modo, a IASP redefiniu a sensibilização dos nociceptores em alodinia, que é caracterizada como uma resposta dolorosa a estímulos inócuos que envolvem uma mudança no sentido da dor, da qualidade de uma sensação sendo um estímulo sensorial que em situações normais não provocam dor, já a hiperalgesia seria a resposta dolorosa aumentada a um estímulo previamente doloroso (IASP, 2012).

A dor, quando desencadeada pelo processo inflamatório, é resultante, basicamente, da participação de mediadores inflamatórios específicos, provenientes do tecido lesionado, com os neurônios sensoriais nociceptivos periféricos. Estes mediadores podem não somente sensibilizar estes nociceptores, como também pode desencadear resposta dolorosa manifesta (HARDY et al., 1950; BJÖRKMAN, 1995).

Esta resposta dolorosa, de maneira geral, é devida a transformação dos estímulos em potenciais de ação que, por meio das fibras nervosas periféricas, são transferidos para o sistema nervoso central de forma organizada (RANG et al., 1991; FERREIRA, 2008).

A percepção do estímulo nocivo ou nociceptivos seja ele mecânico, químico, térmico ou de um mediador inflamatório, como a bradicinina, que ativa os neurônios periféricos sensibilizados (HARDY et al., 1950), são capazes de provocar injúria tecidual, sendo estes detectados pelos nociceptores (neurônios nociceptivos). Os nociceptores são terminações nervosas livres de fibras aferentes primárias, que quando ativadas, conduzem os estímulos nocivos (BJÖRKMAN, 1995). Estes estímulos são recebidos pelos nociceptores localizados nas extremidades periféricas das fibras nervosas aferentes, que sofrem modificações

funcionais de sua excitabilidade neuronal após o contato com mediadores inflamatórios. Estes mediadores podem ser liberados por meio das células danificadas no trauma ou após o reconhecimento de um antígeno no organismo por células residentes, como os macrófagos (FERREIRA, 1993; RIBEIRO etal., 2000).

Existem três tipos principais de fibras aferentes primárias (A- beta (Aβ), A- delta (Aδ) e as do tipo C), que transmitem os estímulos periféricos ao sistema nervoso central. Com isto, forma-se o sistema nociceptivo, que é um segmento do sistema nervoso que possui fibras nervosas caracteristicamente distribuídas nos tecidos, sendo responsáveis pela informação dolorosa (RANG et al., 1991). As fibras Aβ e Aδ são mielinizadas e as do tipo C são amielinizadas. As fibras A-β, são importantes para a manutenção da integridade do animal e desencadeiam respostas rápidas. A informação sensorial que chega a essas camadas é transmitida para os centros superiores de processamento por neurônios secundários, conhecidos como neurônios de ampla faixa dinâmica (WDR – *wide dynamic range neurons*), os quais conduzem as informações geradas por estímulos de diferentes naturezas (DUBNER et al., 1989; MILLAN, 1999; BONICA, 1990). A fibra Aδ conduzem os estímulos mais rapidamente, em torno de 12-30 m/s e respondem a estímulos térmicos e mecânicos, enquanto as fibras do tipo C conduzem os estímulos de baixa velocidade (0.5 – 2 m/s), porém, com maior intensidade e respondem a estímulos de origem térmica, mecânica e química (RANG et al., 1991; JULIUS, BASBAUM, 2001). Existe também "outra classe de nociceptores chamados que "silenciosos" ou "adormecidos" (*silent ou sleeping noceceptors*). Estes nociceptores não respondem a estímulos térmicos e mecânicos em tecidos não inflamados, mas são ativados durante o processo inflamatório (SCHAIBLE; SCHIMIDT, 1988).

A interpretação ou compreendimento da experiência da sensação da dor é entendida como função cortical, que analisa a informação recebida em locais especializados em detectar, localizar e determinar a intensidade do estímulo em áreas corticais responsáveis pela interpretação do processo doloroso (FERREIRA et al., 2010).

1.2 Participação das citocinas no processo doloroso

As citocinas possuem papel essencial na formação dos sinais locais ou sistêmicos de inflamação. São polipeptídeos produzidos por diferentes tipos celulares, principalmente linfócitos e macrófagos ativados, bem como células do endotélio, epitélio e tecido conjuntivo, em resposta a uma variedade de estímulos desencadeados por vírus, parasitas, bactérias e seus

produtos ou em resposta a outras citocinas (VERRI, 2007). Atualmente, já foram descritas mais de 200 citocinas diferentes (BILATE, 2007), que são responsáveis por modular funções celulares e são produzidas durante respostas imunes e inflamatórias, sendo sua secreção transitória e estreitamente regulada (COTRAN et al., 2006; LIN et al., 2000; SOMMER et al., 2010).

Um dos papeis desenvolvidas pela citocinas está na indução de mediadores necessários para conduzir a resposta inflamatória aos locais de infecção e lesão. De modo geral, esses mediadores são fundamentais para desenvolvimento da dor e da inflamação. As primeiras citocinas descritas na fisiopatologia do processo inflamatório foram: IL-1β, TNF-α, IL-6 e as quimiocinas, IL-8, quimiocina quimioatraentes de neutrófilos 1 (CINC-1) e quimiocina derivada dos queratinócitos (KC) (VERRI et al., 2006). A IL-1β e o TNF-α participam das reações pró-inflamatórias agudas e crônicas, assim como dos processos de reparo e de resolução. São principalmente produzidas por macrófagos ativados e podem estimular, secundariamente, a liberação de mais citocinas (COTRAN et al., 2006; RANG et al., 2007).

As citocinas IL-1β e TNFα, são responsáveis por atuar diretamente sobre receptores específicos dos neurônios sensitivos e levam à síntese de outros efetores, como por exemplos outras citocinas, quimiocinas, prostanoides, neurotrofinas, óxido nítrico, cininas, lipídeos, trifosfato de adenosina (ATP) e membros da via do complemento. Esses elementos, por sua vez, causam proliferação e hipertrofia de células glias no sistema nervoso central, com a liberação de citocinas pró-inflamatórias relevantes, como TNFα, IL-1β e IL-6, formando uma rede complexa de ativação interdependente (ZHANG; AN, 2007; SHAVIT et al., 2006, OBATA et al., 2006; MILLER et al., 2009).

O TNFα reduz o limiar para a ativação de fibras nervosas periféricas do tipo C relativas a estímulos mecânicos, através de extravasamento de plasma, gerando alodínia mecânica. Este aumenta as correntes iônicas nos canais de sódio resistentes à tetrodotoxina nos neurônios do gânglio da raiz dorsal (GRD) através da ativação de receptores TNFR1 e da proteína quinase ativada por mitógeno p38 (p38 MAPK). Este, em geral, é encontrado junto aos canais de sódio Nav 1.8 no GRD, e sua fosforilação direta provoca aumento na intensidade de corrente, o que contribui para dor inflamatória e neuropática. O TNF também atua na condutância dos canais de potássio por meio da ativação da PKC, o que afeta a capacidade de as células gliais permitirem a saída de potássio intracelular e removerem o glutamato liberado após um estímulo, resultando em maior vulnerabilidade neuronal (SOMMER et al., 2010; HUDMON et al., 2008).

O TNFα e a IL-1β ativam os receptores B2, causando hiperalgesia inflamatória. A própria bradicinina pode induzir a secreção dessas citocinas a partir de macrófagos, formando um ciclo vicioso de nocicepção. É importante notar que a IL-1β isolada é incapaz de estimular os neurônios do GRD, porém, com IL-6 e TNFα, produz aumento rápido da sensibilidade de TRPV1 e da liberação de CGRP, o que leva à sensibilização térmica. O TNF, IL-1β e a IL-6 são potentes indutores da cicloxigenase-2 e, em consequência, da PGE_2, tanto no local da lesão quanto na medula espinal, aumentando a sensibilidade dos neurônios a estímulos dolorosos químicos, térmicos e mecânicos (SOMMER et al., 2010; SHAVIT et al., 2006; MILLER et al., 2009; MCMAHON et al., 2006; BUVANENDRAN et al., 2006; CUNHA et al., 2007).

Existem diferentes modelos experimentais no qual se pode avaliar a eficácia de um fármaco com prováveis propriedades analgésica e anti-inflamatória. Dentre eles podemos citar os modelos de dor manifesta como as contorções abdominais induzidas por ácido acético e fenil-p-benzoquinona, adjuvante completo de Freund (CFA) e teste da formalina. Nesses modelos, os estímulos inflamatórios induz a liberação de mediadores que ativam os nociceptores induzindo uma resposta comportamental considerada nociceptiva por ser inibida por um fármaco analgésico. No teste da formalina, apresenta-se em duas fases, sendo a primeira fase neurogênica 0-5 minutos mediados pela liberação de mediadores como histamina e serotonina, e receptores acoplados a canal iônico. Já a segunda fase conhecida como fase inflamatória, pois depende da produção de citocinas e outros mediadores (VERRI et al., 2006). Em outro modelo, como da carragenina, a hiperalgesia inflamatória é mediada pela secreção de TNF-α e quimiocina derivada de queratinócitos (KC) (corresponde à IL-8 em humanos). O TNF-α e o KC estimulam a subsequente produção de IL-1β que, por sua vez, induz a expressão de cicloxigenase 2 (COX-2), a qual participa da transformação de ácido araquidônico em prostanóides. Dentre os prostanóides produzidos está a PGE_2, que promove a sensibilização dos nociceptores e a hiperalgesia (CUNHA et al., 2005; LORAM et al., 2007). Além da IL-1β propiciar a indução da expressão de COX-2, o próprio TNF-α e a PGE2 podem induzi-la (BERTOLINI; OTTANI; SANDRINI, 2001). O KC também pode contribuir com a sensibilização do nociceptor por mediar à produção de aminas simpatomiméticas, como noradrenalina e dopamina, as quais atuam diretamente sobre o nociceptor aumentando a sua excitabilidade (CUNHA et al., 2005; KHASAR; MCCARTER; LEVINE, 1999; NAKAMURA; FERREIRA, 1987).

Logo existem diversos modelos que são extremamente úteis para avaliação do efeito e mecanismo de ação de drogas analgésicas e anti-inflamatórias.

1.3 Vimpocetina

A vimpocetina ((3α,16α)-Eburnamenine-14-carboxylic acid ethyl ester) um derivado sintético do alcalóide vincamina extraído da planta pervinca, *Vinca minor* , que foi originalmente descrito como sendo um vasodilatador cerebral (KÁRPÁTI; SZPORNY, 1976). Este fármaco possui um efeito neuroprotetor em vários modelos experimentais, reduzindo a lesão neuronal que ocorre em condições de hipóxia ou isquemia (KÁRPÁTI; SZPORNY, 1976; RISCHKE; KRIEGLSTEIN, 1990).

Este fármaco foi comercializado primeiramente sob o nome comercial Cavinton (Hungria) em 1978. Desde então, a vimpocetina tem sido amplamente utilizada em muitos países para o tratamento preventivo de disfunção cognitiva, incluindo demência, acidente vascular encefálico (AVE) senil e distúrbios de memória (BAGOLY et al., 2007).

Vimpocetina, bem como vincamina, são usadas na Europa, Japão e México como agentes farmacêuticos para o tratamento de desordens vasculares cerebrais e cognitivas. Nos países como os Estados Unidos, a vimpocetina é comercializada como um suplemento dietético, e até o presente momento, não há relatos de efeitos colaterais significativos, toxicidade, ou contraindicações nas doses terapêuticas desta. O mecanismo proposto para as ações da vimpocetina é o de vasodilatador cerebral que melhora fluxo sanguíneo cerebral (TAMAKI; MATSUMOTO, 1985; KIM et al., 2001). Este composto diminui a resistência vascular cerebral, a viscosidade do sangue, a agregação plaquetária e aumenta a fluidez dos glóbulos vermelhos sanguíneos (KISS; KÁRPÁTI, 1996).

A vimpocetina atua aumentando o metabolismo cerebral por aumentar a recaptação de oxigênio e glicose do sangue além de estimular a produção de ATP neuronal (SZOBOR; KLEIN, 1976). Além disso, estudos recentes demonstraram que a vimpocetina age via modulação dos níveis de AMP ciclico (AMPc) e GMPc por ser um inibidor de fosfodiesterase (PDE-1) (BEAVO, 1995), devido a esses fatores com o aumento dos níveis de GMPc e consequentemente de proteína quinase G (PKG), observa-se que há abertura (fosforilação) de canais de potássio (K+ATP), o que contrabalança o aumento do potencial de membrana, evitando a despolarização e o disparo de potenciais de ação, causando a analgesia (SACHS; CUNHA; FERREIRA, 2004; DASTIDAR et al., 2007; FAN CHUNG , 2006). Além disso, estudos realizados com a vimpocetina demonstraram que IkB quinase (IKK), é um outro alvo celular da vimpocetina, e que esse fármaco é capaz de atenuar a ativação do NF-kB induzida por TNF-α e subsequente produção mediadores pró-inflamatórios em vários tipos de células,

incluindo células musculares lisas vasculares endoteliais, células e macrófagos mostrando que a vimpocetina é um potente agente anti-inflamatório. A vimpocetina se mostrou capaz de inibir os mediadores pró-inflamatórios (TNF-α e IL-1β) assim como o processo inflamatório intersticial de leucócitos polimorfonucleares em um modelo de inflamação pulmonar em rato induzidos por TNF-α ou LPS, além de demonstras a inibição da quimiotaxia e da adesão de monócitos que são os processos críticos durante a inflamação (JEON et al., 2010). Um efeito adicional da vimpocetina que não tem sido relacionada com a inibição de fosfodiesterase é a redução do stress oxidativo como demonstrado pela prevenção da diminuição de glutationa reduzida (GSH) e de produção de malondialdeído induzidas por injeção intracerebral de brometo etídio (ABDEL-SALAM, 2011). Há evidências de que espécies reativas de oxigênio e nitrogênio produzidas no local da inflamação auxiliam na promoção da hiperalgesia (KEEBLE et al., 2009; NDENGELE et al., 2008). A influência do estresse oxidativo sobre a hiperalgesia se dá pelo fato de que o ânion superóxido e o peroxinitrito ativam fatores de transcrição como a proteína ativadora 1 (AP-1), NFκB, além de MAP quinases, culminando na indução da expressão de COX-2 e aumentando a produção de prostanóides, o que promove a sensibilização dos nociceptores (LITTLE; DOYLE; SALVEMINI, 2012). Estímulos inflamatórios, como a carragenina são capazes de induzirem a formação de produtos do estresse oxidativo e esses produtos medeiam a uma resposta nociceptiva, uma vez que há uma diminuição nos níveis de ânion superóxido e peroxinitrito ocorre uma redução no edema e da hiperalgesia (KHATTAB, 2006; LITTLE; DOYLE; SALVEMINI, 2012; NDENGELE et al., 2008).

Existe também evidência de que a vimpocetina reduz as correntes de sódio tetrodotoxina Nav 1.8 no gânglio da raiz dorsal derivada de células neuronais, o que sugere um efeito analgésico da vimpocetina (ZHOU et al., 2003). Corroborando com esses dados, estudos prévios demonstraram que a vimpocetina apresenta atividade analgésica, que reduz o número de contorções abdominais induzidas por ácido acético por um mecanismo dependente de adenosina (ABDEL-SALAM, 2006), bem como no comportamento de dor induzida por formalina (CSILLIK et al., 2008). Indicando que esta droga nootrópica pode ser útil no controle da dor.

Baseados nestes dados nós propomos investigar o efeito analgésico da vimpocetina em diferentes modelos nociceptivos e determinar seus possíveis mecanismos de ação.

2 OBJETIVOS

2.1 Objetivo geral

Investigar a atividade analgésica da vimpocetina em diferentes modelos de inflamação, bem como seu mecanismo de ação.

2.2 Objetivo específico

- Avaliar o efeito analgésico do tratamento com vimpocetina em modelos experimentais por meio de diferentes estímulos nociceptivos (ácido acético, fenil-p-benzoquinona (PBQ), modelo da carragenina, formalina, CFA);
- Avaliar o efeito analgésico da vimpocetina em modelos de inflamação aguda e prolongada;
- Avaliar se a vimpocetina inibe a hiperalgesia mecânica e térmica;
- Avaliar se a vimpocetina inibe a migração de neutrófilos;
- Avaliar se o efeito analgésico da vimpocetina estaria relacionado à inibição da produção de citocinas pró-hiperalgésicas como IL-1β e TNFα;
- Avaliar o efeito antioxidante da vimpocetina pelos níveis de glutationa reduzida;
- Avaliar se a vimpocetina causa algum dano hepático por meio da dosagem das Alanina transaminase (ALT) e Aspartato transaminase (AST) e/ou danos gástrico por meio da avaliação da atividade de mieloperoxidase (MPO).

3 ARTIGO PARA PUBLICAÇÃO

Este trabalho foi realizado no Laboratório de Dor, Inflamação, Neuropatia e Cancêr, dando origem a o artigo científico Analgesic effect of vinpocetine: spinal and peripheral inhibition of cytokines production and oxidative stress. Kenji W. R. Miyazawa, Ana C. Zarpelon, Larissa Staurengo-Ferrari, Miriam S. N. Hohmann, Gabriela F. Pavão-de-Souza, Sergio H. Ferreira, Jose C. Alves-Filho, Thiago M. Cunha, Fernando Q. Cunha, Rubia Casagrande, Waldiceu A. Verri, Jr.

As formatações do artigo seguem as normas da revista Pharmacology biochemistry and behavior (Anexo).

Analgesic effect of vinpocetine: spinal and peripheral inhibition of cytokines production and oxidative stress

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Abstract

Vinpocetine is a nootropic agent used for memory improvement. Recent studies demonstrated some of its anti-inflammatory and analgesic effects. Herein, the analgesic effect and mechanisms of vinpocetine were further addressed. Overt pain-like behavior was assessed by acetic acid and phenyl-p-benzoquinone-induced writhing response and formalin and complete Freund's adjuvant (CFA)-induced paw flinches. Mechanical hyperalgesia was investigated using an electronic anesthesiometer, thermal hyperalgesia using the hot-plate, the recruitment of neutrophils by myeloperoxidase (MPO) activity, cytokines production in spinal cord (L4-L6) and paw skin samples by ELISA, antioxidant activity by the levels of reduced glutathione (GSH). Vinpocetine inhibited the overt pain-like behavior induced by acetic acid (up to 56%), phenyl-p-benzoquinone (81%), formalin (0-5 min-36% and 15-30 min-54%) and CFA (36%). Vinpocetine also inhibited carrageenin-induced MPO activity (up to 69%), mechanical (up to 78%) and thermal (up to 100%) hyperalgesia, IL-1β and TNF-α production in the spinal cord and paw skin (65 and 64%, and 49 and 73%, respectively), and GSH depletion in paw skin and spinal cord (99 and 50%, respectively). In the model of CFA model, vinpocetine inhibited the MPO activity (68%); mechanical (up to 30%) and thermal (up to 100%) hyperalgesia, IL-1β and TNF-α production in paw skin and spinal cord (41 and 53%, and 84 and 79%, respectively), and GSH depletion in paw skin and spinal cord (60 and 61%, respectively). Vinpocetine reduces inflammatory pain by mechanisms related to reduction of cytokine production and oxidative stress.

Key words: Vinpocetine, inflammation, cytokine, oxidative stress, pain, hyperalgesia.

Abbreviations: AST, Aspartate aminotransferase); ALT, Alanine aminotransferase; cAMP, cyclic adenosine monophosphate; CFA, Complete Freund's adjuvant; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); ELISA, Enzyme-linked immunosorbent assay; GSH, Reduced glutathione; HTAB, Hexadecyltrimethylammonium bromide; IL-1β, Interleukin-1 beta; MPO, Myeloperoxidase; PBQ, Pphenyl-p-benzoquinone; TNFα, Tumor necrosis factor alpha.

1 Introduction

Vinpocetine ((3α,16α)-Eburnamenine-14-carboxylic acid ethyl ester) is a synthetic ethyl ester of the alkaloid apovincamine, which is isolated from the leaves of *Vinca minor*, commonly known as the lesser periwinkle (Lörincz et al., 1976). It has been used for more than 20 years in certain European countries and Japan as a protective agent to reduce brain damage resulting from an ischemic infarct (Bönöczk et al., 2000; Feigin e tal., 2001; McDaniel et al., 2003). In fact, the main therapeutic uses of vinpocetine are related to its effect of improving cerebral circulation and metabolism in the management of various types of cerebrovascular circulatory disorders such as cerebral infarction and residual cerebral hemorrhage (Luo et al., 2006), and for the treatment of cognitive disorders and related symptoms (Bönöczk et al., 2000; Feigin et al., 2001; McDaniel et al.,2003). Vinpocetine has also been marketed as a nootropic agent for the improvement of memory (DeNoble et al., 1986).

Regarding the mechanisms of vinpocetine it has been demonstrated that it selectively inhibits phosphodiasterase-1 (van Staveren et al., 2001) resulting in increased cAMP and cGMP levels (Beavo, 1995), an effect that has been related to its vasodilation effects (Jeon et al., 2010). On the other hand, studies have shown that vinpocetine has potent anti-inflammatory actions that are caused by a direct inhibition of the IκB kinase complex (IKK) in a phosphodiesterase blockade-independent manner, resulting in reduced production of major proinflammatory cytokines such as IL-1β and TNF-α (Mizokami et al., 2012). An additional effect of vinpocetine that has not been related to inhibition of phosphodiasterase is the reduction of oxidative stress as shown by the prevention of decrease of reduced glutathione (GSH) levels and malondialdehyde production induced by ethidium bromide intracerebral injection (Abdel-Salam et al., 2011).

There is also evidence that vinpocetine reduces tetrodotoxin Nav1.8 sodium currents in dorsal root ganglion-derived neuronal cell line, which suggests an analgesic effect (Zhou et al., 2003). In agreement that vinpocetine presents analgesic activity; it reduces the number of abdominal contortions induced by acetic acid by an adenosine-dependent mechanism (Abdel-Salam, 2006) as well as formalin-induced overt pain-like behavior (Csillik et al., 2008).

Importantly, inflammatory pain depends on cytokines such as TNFα and IL-1β since they are produced upon inflammatory stimulus and targeting these cytokines with antibodies, soluble receptors or drugs that inhibit their production reduces the nociceptive behavior in a variety of models including antigen-induced arthritis, carrageenin, acetic acid, formalin and complete Freund's adjuvant (Chichorro et al., 2004; Cunha et al., 2005; Ribeiro et al., 2000; Safieh-Garabedian et al., 1995; Verri et al., 2009). These cytokines are also responsible for recruiting neutrophils to the inflammatory foci that further contribute by the production of nociceptive mediators (Cunha et al., 2008). Besides the peripheral contribution of neutrophils, the peripheral inflammation also induces cytokine-dependent activation of neurons, astrocytes and microglia in the spinal cord (Svensson et al., 2003). There is also evidence that oxidative stress contributes to inflammatory hyperalgesia in the carrageenin model (Valério et al., 2009).

Considering the evidence mentioned above, the present study further investigated the analgesic effect of vinpocetine and whether its analgesic mechanism would depend on inhibition of cytokine production and oxidative stress.

2 Material and Methods

2.1 Animals

Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12-12h and keep at 21ºC. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 13278.2011.3). All efforts were made to minimize the number of animals used and their suffering.

2.2 Drugs and Stimuli

Materials were obtained from the sources as follows: vinpocetine (Marjan Indústria & Comércio Ltda, São Paulo, Brazil), carrageenin (FMC Corp, Philadelphia, PA, United States), acetic acid and formaldehyde (Mallinckrodt Baker, S.A., Mexico, Mexico City), complete Freund's adjuvant and phenyl-*p*-benzoquinone (PBQ) (Sigma Chemical Company, St.Louis, MO), and indomethacin (Prodome, Campinas, SP, Brazil).

2.3 Mechanical Hyperalgesia test

Mechanical hiperalgesia was tested in mice as previously reported (Cunha et al., 2004). In a quiet room, mice were placed in acrylic cages (12x10x17 cm) with wire grid floors, 15-30 minutes before the start of testing. Stimulation was performed only when animals were quiet, did not display exploratory movements or defecation, and was not resting

on their paws. In these experiments, an electronic pressure-meter was used. It consists of a hand-help force transducer (electronic anesthesiometer; insight, Riberão Preto, SP, Brazil) adapted with 0.5mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically and the value for the response was obtained by average of three measurements. The animals were tested before and after treatment and the results of flexion-elicited threshold was expressed by delta (Δ) withdrawal threshold in grams (g).

2.4 Writing Response Tests

The phenyl-p-benzoquinone (PBQ) and acetic acid-induced writhing models were performed as previously described (Verri et al., 2008). PBQ (diluted in DMSO 2% / saline), acetic acid (0.6% v/v, diluted in saline), or vehicle was injected into the peritoneal cavities of mice pre-treated with vinpocetine (1-30 mg/kg, p.o route). Each mouse was placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 20 minutes after stimulus injection. The writhing response consisted of a contraction of the abdominal muscle together with a stretching of hind limbs. The intensity of writhing response was expressed as the cumulative writhing score over 20 minutes.

2.5 Formalin and CFA Test

The number of paw flinches were determined between 0-30 minutes after intraplantar injection of 25µL of formalin 1.5% or CFA (10µL) as previously described (DeNoble et al., 1986; Valério et al., 2009). The period was divided in intervals of 5 minutes, and clearly

demonstrated the presence of first and second phases, which are characteristic of method (DeNoble et al., 1986; Valério et al., 2009). Results were presented as first (0-5min) and second phase (15-30min).

2.6 Hot Plate Test

Mice were placed in a 10 cm-wide glass cylinder on a hot plate (IITC Life Science Inc. Woodland Hills, CA) maintained at 55ºC. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cut-off) was set at 30s to avoid tissue damage (Verri et al., 2005).

2.7 Myeloperoxidade (MPO) Activity

MPO activity was used as an index of neutrophil accumulation (Bradley et al., 1982; Casagrande et al., 2007). Samples were collected in 50 mM K_2PO_4 buffer (pH=6.0) containing 0.5% HTAB (Hexadecyltrimethylammonium bromide), and were homogenized using a PolytronR (PT3100). After the homogenates were centrifuged at 16,100 *g* for 2 min, the resulting supernatant was assayed spectrophotometrically for MPO activity determination at 450 nm (Victor3™ 1420-050 Multilabel Counter (Perkin-Elmer® Precisely) with three readings within 1 min. The MPO activity of the samples was compared with a standard curve of neutrophils. Briefly, a 10 µL sample was mixed with 200 µL 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.015% hydrogen peroxide. The results were presented as the MPO activity (number of neutrophils x 10^4/mg of tissue).

2.8 Dosing of Aminotransferases

Plasma levels of aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) were used as indicators of hepatotoxicity (Fracasso et al., 1990; Ribeiro-Rama et al., 2011). These assays were performed using kits from Labtest® (Lagoa Santa, Minas Gerais, Brazil).

2.9 Cytokine Measurement

Paw skin and spinal cord (L4-L6) samples were homogenized in 500 µl of buffer containing protease inhibitors, and IL-1β and TNF-α levels were determined as described previously (Verri et al., 2007; Verri et al., 2006) by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. The results are expressed as pictograms (pg) of cytokine/ 100 mg of paw or spinal cord tissues.

2.10 GSH measurement

Samples of paw skin and spinal cord were collected and maintained at -80ºC for at least 48 h. The sample was homogenized with 200 µL of 0.02 M EDTA. The homogenate was mixed with 25 µL of 50% trichloroacetic acid and was homogenized three times during 15 min. The mixture was centrifuged (15 min x 1500 g x 4 ºC). The supernatant was added to 200 µL of 0,2 M TRIS buffer, pH 8.2, and 10 µL of 0,01M DTNB. After 5 min, the absorbance was measured at 412 nm against a reagent blank with no supernatant. A standard curve was performed with standard GSH. The results are expressed as GSH per mg of protein (Sedlak et al., 1968).

2.11 Experimental Procedures

Mice received per oral (p.o.) pretreatment with vinpocetine (1, 3, 10 or 30 mg/Kg/saline) 1 h before inflammatory stimulus or post-treatment 1 h after CFA injection. The dose of inflammatory stimuli were determined in our laboratory and based on previously studies (Cunha et al., 2005; Mizokami et al., 2012; Pavao-de-Souza et al., 2012; Valério et al., 2007; Valério et al., 2009; Verri et al., 2009; Zarpelon et al., 2012). The writhing response was evaluated for 20 min after i.p. injection of PBQ (1890 μg/kg) or acetic acid 0.6% (10 ml/kg). The number of formalin (formalin 1.5%, 25 μl/paw) or CFA (10μL/paw)-induced flinches was evaluated for 30 min after stimulus. Mechanical and thermal hyperalgesia was evaluated 1-9 h after carrageenin (100 μg/25 μl/paw) or 1-7 days after CFA (10 μl/paw) injection, neutrophil migration was evaluated in paw skin by MPO activity assay 9 h after carrageenin (100 μg/25 μl/paw) or 7 days after CFA (10 μl/paw) injection. IL-1β and TNFα level was measured in paw skin and spinal cord 3 hours after carrageenin or in the 7^{th} day after CFA injection 3 h after the last treatment with vinpocetine. The level of aspartate aminotransferase (AST), alanine aminotrasferase (ALT) and myeloperoxidase (MPO) activity in the stomach were assessed after 7 days of daily p.o. treatment with vehicle (saline or Tris/HCL buffer, pH 8.0), vinpocetine (30 mg/kg) or indomethacin (1,5 mg/kg diluted in Tris/HCL buffer, pH 8.0) 5 h after the last treatment. All inflammatory stimuli injected i.pl. induced only ipsilateral nociception. Experiments were double blind.

2.12 Statistical Analysis

Results are presented as means ± SEM of measurements made on 5-10 (indicated in the legends) animals in each group in each experiment and are representative of two independent experiments. Two-way analysis of variance (ANOVA) was used to compare the

groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the stimulus injection. The analyzed factors were treatments, time, and time *versus* treatment interaction. When there was a significant time *versus* treatment interaction, one-way ANOVA followed by Tukey's t-test was performed for each time. On the other hand, when the hyperalgesic responses were measured once after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Additionally, comparative statistical analysis between two groups were done using *t* test. Statistical differences were considered to be significant at $P < 0.05$.

3 Results

3.1 Vinpocetine inhibits acetic acid- and phenyl-p-benzoquinone (PBQ)-induced writhing response and formalin and CFA-induced paw flinches.

In the first series of experiments, the analgesic effect of vinpocetine was evaluated in acetic acid-, PBQ-, formalin- and CFA-induced pain-like behaviors. Mice were treated with vinpocetine (1, 3, 10, and 30 mg/kg p.o.) or vehicle (saline) (Fig. 1A) 1 hour before acetic acid injection. Vinpocetine at the doses of 3-30 mg/kg inhibited the writhing response induced by acetic acid. The dose of 1 mg/kg of vinpocetine presented no effect. The dose of 10 mg/kg of vinpocetine was selected for the next experiments on overt pain-like behavior considering that it achieved maximal effect (56% inhibition), which was similar to the effect of 30 mg/kg of vinpocetine. Vinpocetine inhibited the writhing response (81%) induced by phenyl-p-benzoquinone (PBQ) (Fig. 1B), first (36%) and second phases (54%) of formalin-induced flinch (Fig. 1C), and complete Freund's adjuvant (CFA)-induced flinch (36%, Fig. 1D).

3.2 Vinpocetine inhibited carrageenin- induced hiperalgesia and myeloperoxidase (MPO) activity.

Mice were treated with vinpocetine (3, 10 and 30 mg/kg, p.o.) 1 h before carrageenin (100 μg/25 μL) i.pl. injection, and at indicated time points mechanical (Fig. 2A) and thermal (Fig. 2B) hyperalgesia were evaluated followed by sample collection for MPO activity (Fig. 2C) determination. Carrageenin induced significant mechanical hyperalgesia, which was not affected by 3 mg/kg of vinpocetine. On the other hand, 10 mg/kg of vinpocetine significantly inhibited carrageenin-induced mechanical hyperalgesia between 5-9 h with significant differences with the lower dose of vinpocetine tested at 7 and 9 h. The dose of 30 mg/kg of

vinpocetine inhibited carrageenin-induced mechanical hyperalgesia between 3 and 9 h (up to 78%), with significant differences compared to the lower doses of vinpocetine between 5 and 9 h (Fig. 2A). In the sample treatment schedule, vinpocetine also inhibited carrageenin-induced thermal hyperalgesia at 30 mg/kg (up to 100%). Furthermore, 10 mg/kg of vinpocetine significantly inhibited carrageenin-induced thermal hyperalgesia at 5 h with significant differences with the lower dose of vinpocetine and carragennin (Fig. 2B). In the 9th h after carrageenin injection, the MPO activity was reduced by the treatment with 30 mg/kg of vinpocetine (69%) without significant effect with the lower doses of vinpocetine though the dose of 3 mg/kg presented a tendency of reduction (Fig. 2C). The dose of 100 mg/kg of vinpocetine did not present additional effect compared to the dose of 30 mg/kg (data not shown). Taking into account the results of Fig. 2, the dose of 30 mg/kg of vinpocetine was selected for next experiments in the carrageenin and CFA models of hyperalgesia.

3.3 Post-treatment with vinpocetine inhibited CFA-induced hyperalgesia and MPO activity.

Mice were treated with vinpocetine (30 mg/kg, p.o.) 1 h after i.pl. injection of CFA (10μL/paw) plus 3 h before each daily measurement. Vinpocetine inhibited the CFA-induced mechanical (Fig. 3A) hyperalgesia (up to 30%) in all intervals tested and thermal hyperalgesia (up to 100%) between the 3rd - 7th day (Fig. 3B). In the 7th day of treatment after CFA injection it was observed a reduction of MPO activity (68%) by vinpocetine treatment (Fig. 3C).

3.4 Vinpocetine inhibited carrageenin-induced IL-1β and TNFα production.

Mice were treated with vinpocetine (30 mg/kg, *po*) 1h before i.pl. injection of carrageenin, and samples of cutaneous paw skin and spinal cord (L4-L6) were collected 3 h after stimulus injection for IL-1β and TNFα measurement (Fig. 4). Carrageenin induced significant production of IL-1β and TNFα in the paw skin (Figs. 4A and 4B, respectively) and spinal cord (Figs. 4C and 4D, respectively), which was inhibited by vinpocetine (49 and 73% and 65 and 64%, respectively) (Fig. 4).

3.5 Post-treatment with vinpocetine inhibited CFA-induced IL-1β and TNFα production.

Mice were treated with vinpocetine (30 mg/kg, p.o.) 1 h after i.pl. injection of CFA. Mice also received daily treatment with vinpocetine thereafter until the 7th day in which 3 h after treatment samples of cutaneous paw skin and spinal cord (L4-L6) were collected for IL-1β and TNFα measurement (Fig. 5). CFA induced significant production of IL-1β and TNFα in the paw skin (Figs. 5A and 5B, respectively) and spinal cord (Figs. 5C and 5D, respectively), which was inhibited by vinpocetine (41 and 53%, and 84 and 79%, respectively) (Fig. 5).

3.6 Pretreatment and post-treatment with vinpocetine inhibited carrageenin and CFA, respectively,-induced depletion of reduced glutathione (GSH).

Mice were treated with vinpocetine (30 mg/kg, p.o.) 1 h before i.pl. injection of carrageenin or 1h after CFA injection. The CFA group also received daily treatment with vinpocetine. Samples of cutaneous paw skin and spinal cord (L4-L6) were collected 3 h after carrageenin (Figs. 6A and 6B, respectively) or 3 h after the 7th treatment with vinpocetine in

the CFA model (Figs. 6C and 6D, respectively) for GSH measurement (Fig. 4). Carrageenin and CFA induced significant decrease of GSH in the paw skin (Fig. 6A [99%] and 6C [60%], respectively) and spinal cord (Fig. 6B [50%] and 6D [61%], respectively), which was inhibited by vinpocetine.

3.7 Effect of prolonged treatment with vinpocetine on plasma level of aspartate aminotransferase (AST) and alanine aminotrasferase (ALT), stomach MPO activity.

Mice received daily *po* treatment with vehicle (saline or tris[2-amino-2-hydroxymethylpropan-1,3-diol]/HCl buffer, pH 8.0), vinpocetine (30 mg/kg) or indomethacin (1,5 mg/kg diluted in Tris/HCL buffer, pH 8.0) during 7 days. Samples of plasma and stomach were collected 3 h after the last treatment. Vinpocetine did not induce increase of AST (Fig. 7A) and ALT (Fig. 7B) in plasma samples or MPO activity in stomach samples (Fig. 7C). On the other hand, the positive control indomethacin induced significant increase of plasma AST (Fig. 7A) and ALT (Fig. 7B), and stomach MPO activity (Fig. 7C).

4 Discussion

Vinpocetine is a nootropic agent that has been used for the improvement of memory. Nevertheless, there is pre-clinical evidence that it also exerts analgesic effects in acetic acid- (Abdel-Salam, 2006) and formalin (Csillik et al., 2008)-induced overt pain-like behavior. In the present study, we extended the investigation on the analgesic activity of vinpocetine demonstrating that it reduces the nociceptive responses in other models. Importantly, the analgesic mechanism of vinpocetine was related to inhibition of cytokine production and oxidative stress in the inflammatory foci and spinal cord.

Vinpocetine reduced the overt pain-like behavior induced by acetic acid, phenyl-p-benzoquinone, formalin and complete Freund's adjuvant (CFA). Importantly, vinpocetine exerted analgesic effect as pretreatment and post-treatment in the mechanical and thermal hyperalgesia induced by carrageenin and CFA respectively. The nociceptive responses in these models have in common the dependency of cytokines, including TNFα and IL-1β (Chichorro et al., 2004; Cunha et al.,2005; Safieh-Garabedian et al., 1995; Verri et al., 2008; Verri et al., 2006). In fact, there is increased production of these cytokines and targeting these cytokines using antibodies, receptor antagonists, soluble receptors or drugs that inhibit their synthesis reduces the nociceptive responses (Chichorro et al., 2004; Cunha et al.,2005; Safieh-Garabedian et al., 1995; Verri et al., 2008; Verri et al., 2006). Therefore, the present results line up well with the notion that the inhibition of carrageenin- and CFA-induced TNFα and IL-1β production represents an important analgesic mechanism of vinpocetine. Furthermore, it has been shown that vinpocetine inhibits IκB kinase kinase (IKK) resulting in reduced NFκB activation in TNFα- and LPS-induced lung inflammation (Jeon et al., 2010). The inhibition of IKK activation by vinpocetine is independent of phosphodiasterase inhibition

(Jeon et al., 2010), which further corroborates that inhibition of cytokine production is a relevant mechanism of action of vinpocetine.

In addition to nociception, TNFα and IL-1β are also relevant cytokines in the recruitment of leukocytes. In this sense, it was observed that vinpocetine inhibited cytokine production and as a consequence the myeloperoxidase (MPO) activity. MPO is mainly expressed by neutrophils and macrophages (Verri et al., 2010) indicating that these cellular populations could be reduced by vinpocetine treatment in the carrageenin and CFA models. Neutrophils contribute to hyperalgesia by further producing nociceptive mediators (Cunha et al., 2008; Verri et al., 2009) as well as macrophages are important resident cells in the detection and amplification of inflammation and nociception (Ribeiro et al., 2011).

Cytokines have close relationship with oxidative stress. For instance, TNFα induces the activation of NADPH oxidase by PKC and MAP kinases mechanisms resulting in superoxide anion production (Kilpatrick et al., 2005). Conversely, superoxide anion activates NFκB to induce cytokine production (Verri et al., 2012). In agreement with this interaction, it was observed that vinpocetine inhibited the decrease of GSH levels induced by carrageenin and CFA in the inflammatory foci and spinal cord. It has also been demonstrated that vinpocetine reduces the oxidative stress in a model of toxic demyelination in rat brain (Valério et al., 2009), however, the relation of oxidative stress and cytokines was not addressed.

In the peripheral inflammation induced by carrageenin and CFA there is also activation of spinal cord cells, including neurons, microglia and astrocytes. It is possible that the peripheral inhibition of cytokine production resulted in the reduction of cytokine production in the spinal cord, but it was not disproved that vinpocetine could be acting peripherally and in the spinal cord. Nevertheless, it is more likely that vinpocetine could be

acting in both sites to inhibit cytokine production since NFκB is activated in both sites in both models (Fehrenbacher et al., 2012; Jeon et al., 2010; Medina, 2010; Ren and Dubner, 2010).

The present data do not intend to disprove other mechanisms of action of vinpocetine, but rather demonstrate this previous unrecognized analgesic mechanism e.g. inhibition of cytokine production and oxidative stress. It has been described that vinpocetine blocks the retrograde axoplasmic transport of NGF, which was proposed as an analgesic mechanism of action (Csillik et al., 2008). Vinpocetine also blocks Nav1.8 sodium currents indicating that it can directly block nociceptor activation (Zhou et al., 2003). In fact, we observed that vinpocetine reduced the first phase of formalin test, which is considered a neuronal phase (Chichorro et al., 2004); therefore, corroborating that the analgesic effect of vinpocetine could dependent on neuronal blockade of nociceptive transmission. Additionally, vinpocetine inhibited carrageenin- and CFA-induced thermal hyperalgesia, demonstrating that it can inhibit inflammatory thermal hyperalgesia and not only supra-spinal-dependent thermal hyperalgesia as observed in naïve animals (Abdel-Salam, 2006; Le et al., 2001).

The daily treatment with vinpocetine during 7 days was capable of reducing CFA-induced mechanical and thermal hyperalgesia, and MPO activity. Importantly, this effectively analgesic treatment schedule did not induce increase of plasma AST and ALT indicating no hepatic lesion as well as did not induce increase of MPO activity in stomach tissue indicating no gastric ulcer formation (Mizokami et al., 2012). The control indomethacin induced significant increase of AST and ALT plasma levels and MPO activity in the stomach as expected for non-steroidal anti-inflammatory drugs that are not selective to cyclooxygenase-2 (Wallace et al., 1995). Therefore, 7 days of treatment with vinpocetine seems a safe analgesic protocol and is compatible with the time profile of most of non-severe inflammatory conditions (Valério et al., 2007). Others have also demonstrated that vinpocetine presents no serious side effect (Kemeny et al., 2005).

Concluding, the effect of vinpocetine was demonstrated in a variety of models from acute overt pain-like behavior to prolonged inflammatory hyperalgesia (CFA model) with a dose-dependent effect. The datas do not intend to disprove other mechanisms of action of vinpocetine, but rather demonstrate this previous unrecognized analgesic mechanism e.g. inhibition of cytokine production and oxidative stress. In fact, we observed that vinpocetine reduced the first phase of formalin test, which is considered a neuronal phase (Chichorro et al., 2004); therefore, corroborating that the analgesic effect of vinpocetine could dependent on neuronal blockade of nociceptive transmission. Additionally, vinpocetine inhibited carrageenin- and CFA-induced thermal hyperalgesia, demonstrating that it can inhibit inflammatory thermal hyperalgesia and not only supra-spinal-dependent thermal hyperalgesia as observed in naïve animals (Abdel-Salam, 2006; Le et al., 2001).

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends

Figure 1. Vinpocetine inhibited overt pain-like behavior. Panel A: mice were treated with vinpocetine (1, 3, 10, and 30 mg/kg/saline, p.o) or vehicle (saline) 1 h before acetic acid injection (0.6% v/v, diluted in saline, 10 ml/kg, p.o.). Panel B-D: mice were treated with vinpocetine (10 mg/kg, p.o.) or vehicle (saline) 1 h before i.p. injection of phenyl-p-benzoquinone (PBQ, 1890 μg/kg diluted in DMSO 2% in saline, panel B), or i.pl. injection of formalin (25 μL of 1.5% formalin in saline, panel C) or CFA (10 μL, panel D). The cumulative number of writhings (writhing score) was evaluated for 20 min (Panels A-B) and total number of flinches (Panels C and D) were evaluated for 30 min. *$P < 0.05$ compared with the saline group, #$P < 0.05$ compared to the vehicle group and $^{f}P < 0.05$ compared to the vehicle group and the dose of 1 mg/kg of vinpocetine. One-way ANOVA followed by Tukey's t test. (n = 6).

Figure 2. Vinpocetine inhibited carrageenin- induced hiperalgesia and myeloperoxidase (MPO) activity. Mice were treated with vinpocetine (3, 10 or 30 mg/kg/saline, p.o.) or vehicle (saline) 1 h before carrageenin (100 μg/25 μL) injection. Mechanical (Panel A) and thermal (Panel B) hyperalgesia were assessed at indicated time points after carrageenin administration using an electronic pressure and hot plate tests, respectively. Myeloperoxidase (MPO) activity was determined in samples collected 9 h after carrageenin injection. *$P < 0,05$ compared to saline group, #$P < 0,05$ compared with carrageenin + vehicle group, $^{f}P < 0,05$ compared to the dose of 3 mg/kg of vinpocetine and $^{ff}P < 0,05$ compared to the dose of 10 mg/kg of vinpocetine and at dose 30mg/kg. One-way ANOVA followed by Tukey's t test. (n = 6).

Figure 3. *Post-treatment with* vinpocetine inhibited CFA-induced hyperalgesia and MPO activity. Mice were treated with vinpocetine (30 mg/kg, p.o.) 1 h after Compete Freund's adjuvant (CFA) (10 μl/paw) injection. i.pl route. Mechanical (Panel A) and thermal (Panel B) hyperalgesia were assessed 3 h after treatment with vinpocetine in the indicated days after CFA injection using an electronic pressure and hot plate tests, respectively. Myeloperoxidase (MPO) activity was determined in samples collected 3 h after the last treatment with vinpocetine in the 7th day. *P < 0,05 compared to saline group, and $^{\#}P$ < 0,05 compared to CFA + vehicle group. One-way ANOVA followed by Tukey's t test. (n = 6).

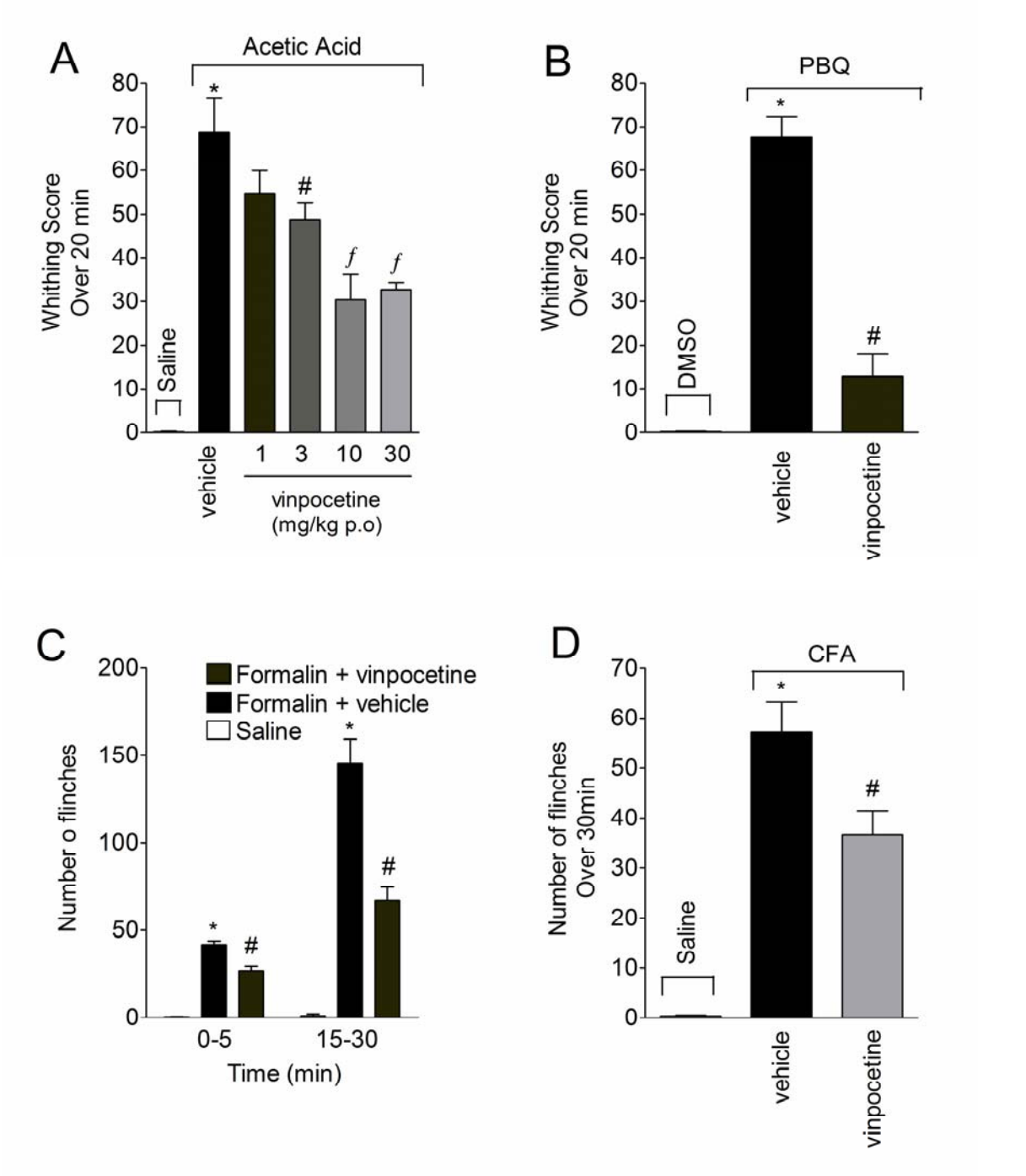
Figure 4. Vinpocetine inhibited carrageenin-induced IL-1β and TNFα production. Mice were treated with vinpocetine (30 mg/kg, p.o.) or vehicle 1 h before i.pl injection of carrageenin, and after 3 h paw skin (Panels A and B) and spinal cord (Panels C and D) samples were collected for the determination of IL-1β (Panels A and C) and TNFα (Panels B and D) production. *P < 0.05 compared to saline group, and $^{\#}P$ < 0.05 compared to carrageenin + vehicle group. One-way ANOVA followed by Tukey's t test. (n = 5).

Figure 5. *Post-treatment with* vinpocetine inhibited CFA-induced IL-1β and TNFα production. Mice were treated with vinpocetine (30 mg/kg, p.o.) or vehicle 1 h after and everyday in the following 7 days after i.pl injection of CFA. Three hours after the last treatment with vinpocetine, paw skin (Panels A and B) and spinal cord (Panels C and D) samples were collected for the determination of IL-1β (Panels A and C) and TNFα (Panels B and D) production. *P < 0.05 compared to saline group, and $^{\#}P$ < 0.05 compared to CFA + vehicle group. One-way ANOVA followed by Tukey's t test. (n = 5).

Figure 6. *Pretreatment and post-treatment with* vinpocetine inhibited carrageenin- and CFA, respesctively,-induced decrease of reduced glutathione (GSH). Panels A-B: Mice were treated with vinpocetine (30 mg/kg, p.o.) or vehicle 1 h before i.pl injection of carrageenin, and after 3 h paw skin (Panels A) and spinal cord (Panels B) samples were collected for the determination of GSH levels. Panels C-D: Mice were treated with vinpocetine 1 h after and everyday in the following 7 days after i.pl injection of CFA. Three h after the last treatment with vinpocetine, paw skin (Panels C) and spinal cord (Panels D) samples were collected for the determination of GSH levels. $*P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to CFA + vehicle or CFA + vehicle groups. One-way ANOVA followed by Tukey's t test. (n = 5).

Figure 7. Effect of prolonged treatment with vinpocetine on plasma level of aspartate aminotransferase (AST) and alanine aminotrasferase (ALT), stomach MPO activity. Mice received daily p.o treatment with vinpocetine (30mg/kg), indomethacin (1,5 mg/kg diluted in Tris/HCL buffer, pH 8.0) or vehicle. In the 7th day, 3 h after the last treatment, mice were terminally anesthetized and blood samples were collected for the determination of the levels of AST (Panel A) and ALT (Panel B). Stomach samples were collected for MPO activity determination (Panel C). There was no difference between vehicles of vinpocetine and indomethacin. $*P < 0.05$ compared to saline group. One-way ANOVA followed by Tukey's t test. (n = 10).

1 Figure 1



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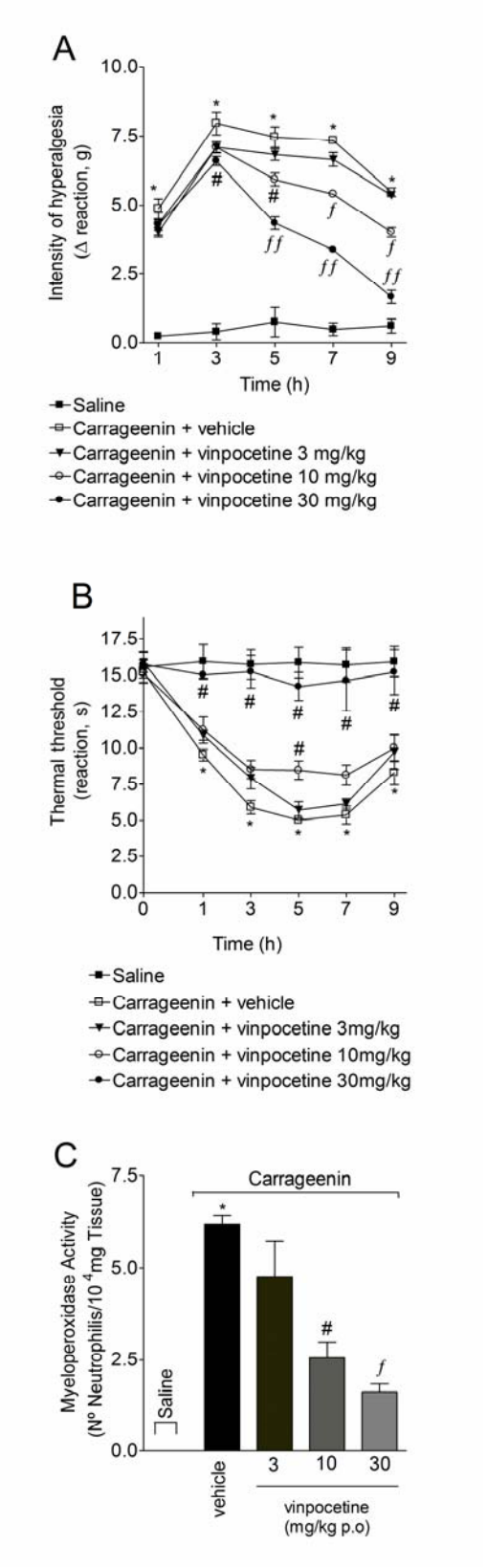
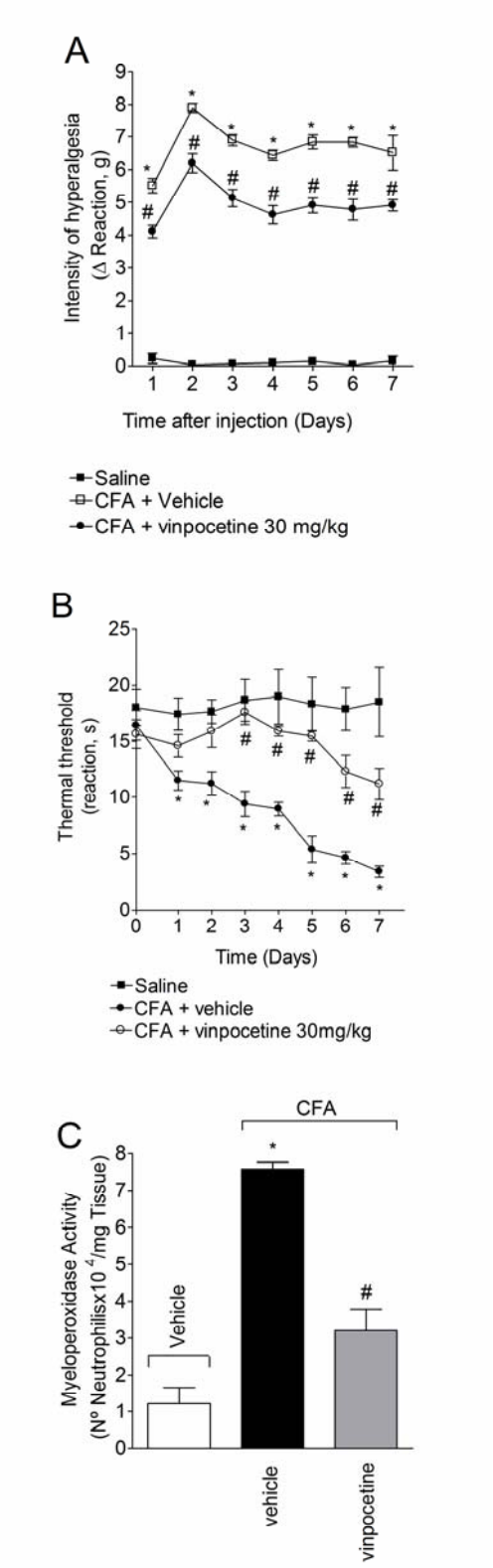
1 Figure 2


A
Intensity of hyperalgesia (Δ reaction, g)
10.0
7.5
5.0
2.5
0.0
1
3
5
7
9
Time (h)
Saline
Carrageenin + vehicle
Carrageenin + vinpocetine 3 mg/kg
Carrageenin + vinpocetine 10 mg/kg
Carrageenin + vinpocetine 30 mg/kg
B
Thermal threshold (reaction, s)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
1
3
5
7
9
Time (h)
Saline
Carrageenin + vehicle
Carrageenin + vinpocetine 3mg/kg
Carrageenin + vinpocetine 10mg/kg
Carrageenin + vinpocetine 30mg/kg
C
Myeloperoxidase Activity (Nº Neutrophilis/10 4 mg Tissue)
7.5
5.0
2.5
0.0
Carrageenin
Saline
vehicle
3
10
30
vinpocetine (mg/kg p.o)

2

3

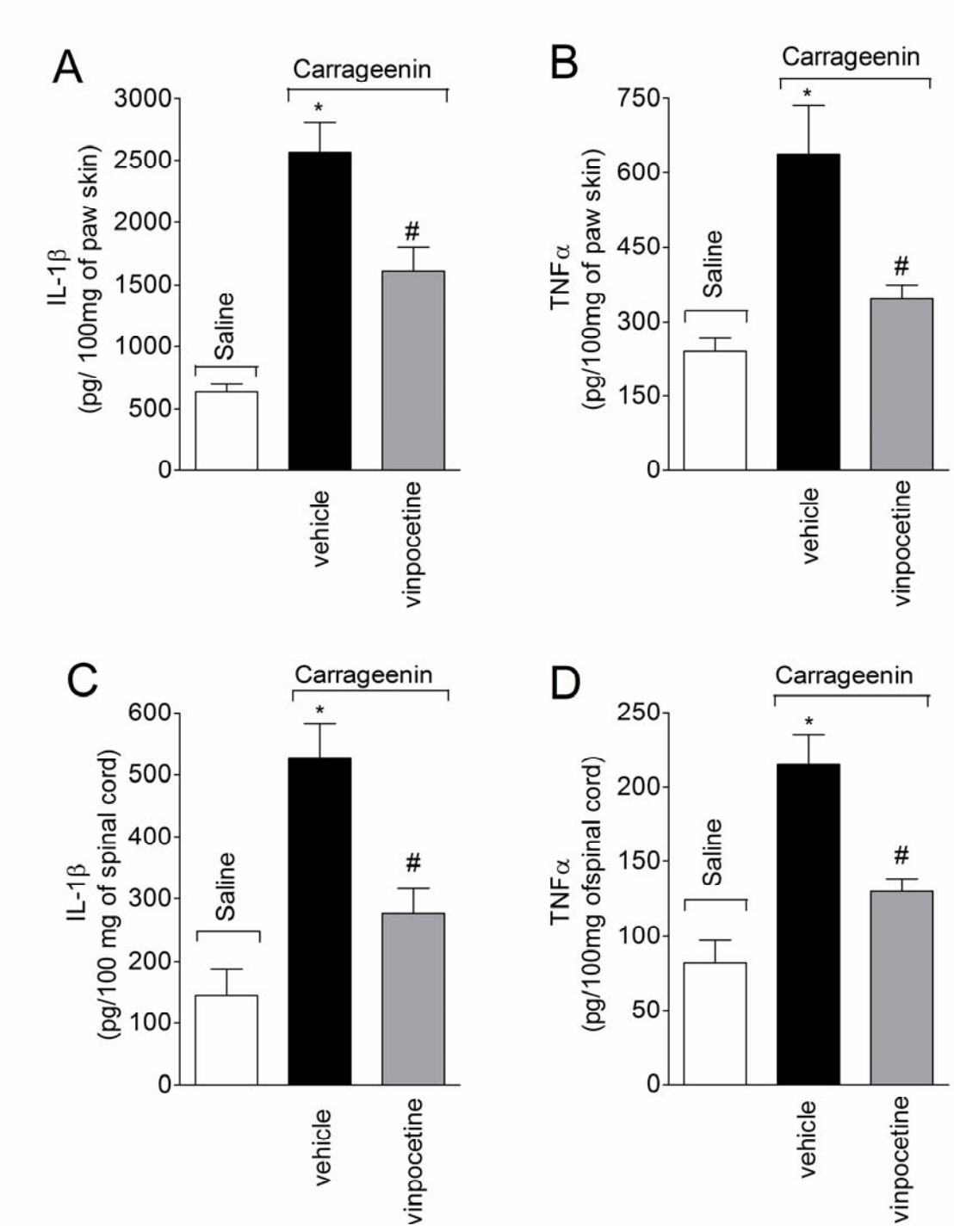
1 Figure 3


A
Intensity of hyperalgesia (Δ Reaction, g)
Time after injection (Days)
Saline
CFA + Vehicle
CFA + vinpocetine 30 mg/kg
B
Thermal threshold (reaction, s)
Time (Days)
Saline
CFA + vehicle
CFA + vinpocetine 30mg/kg
C
CFA
Myeloperoxidase Activity (Nº Neutrophilisx10 -4/mg Tissue)
Vehicle
vehicle
vinpocetine

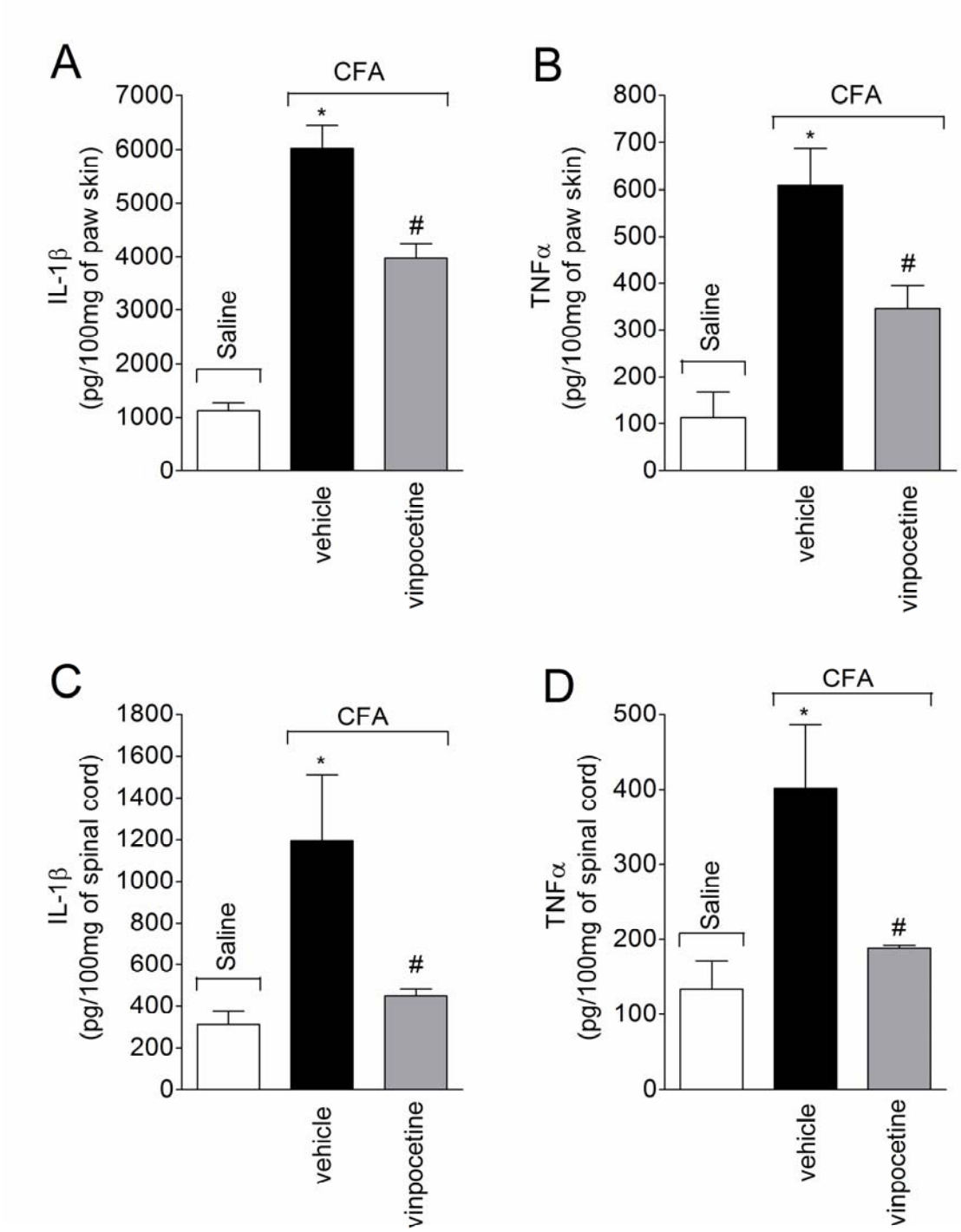
2

3

Figure 4


A
Carrageenin
IL-1β
(pg/ 100mg of paw skin)
3000
2500
2000
1500
1000
500
0
Saline
*
#
vehicle
vinpocetine
B
Carrageenin
TNFα
(pg/100mg of paw skin)
750
600
450
300
150
0
Saline
*
#
vehicle
vinpocetine
C
Carrageenin
IL-1β
(pg/100 mg of spinal cord)
600
500
400
300
200
100
0
Saline
*
#
vehicle
vinpocetine
D
Carrageenin
TNFα
(pg/100mg ofspinal cord)
250
200
150
100
50
0
Saline
*
#
vehicle
vinpocetine

1 Figure 5



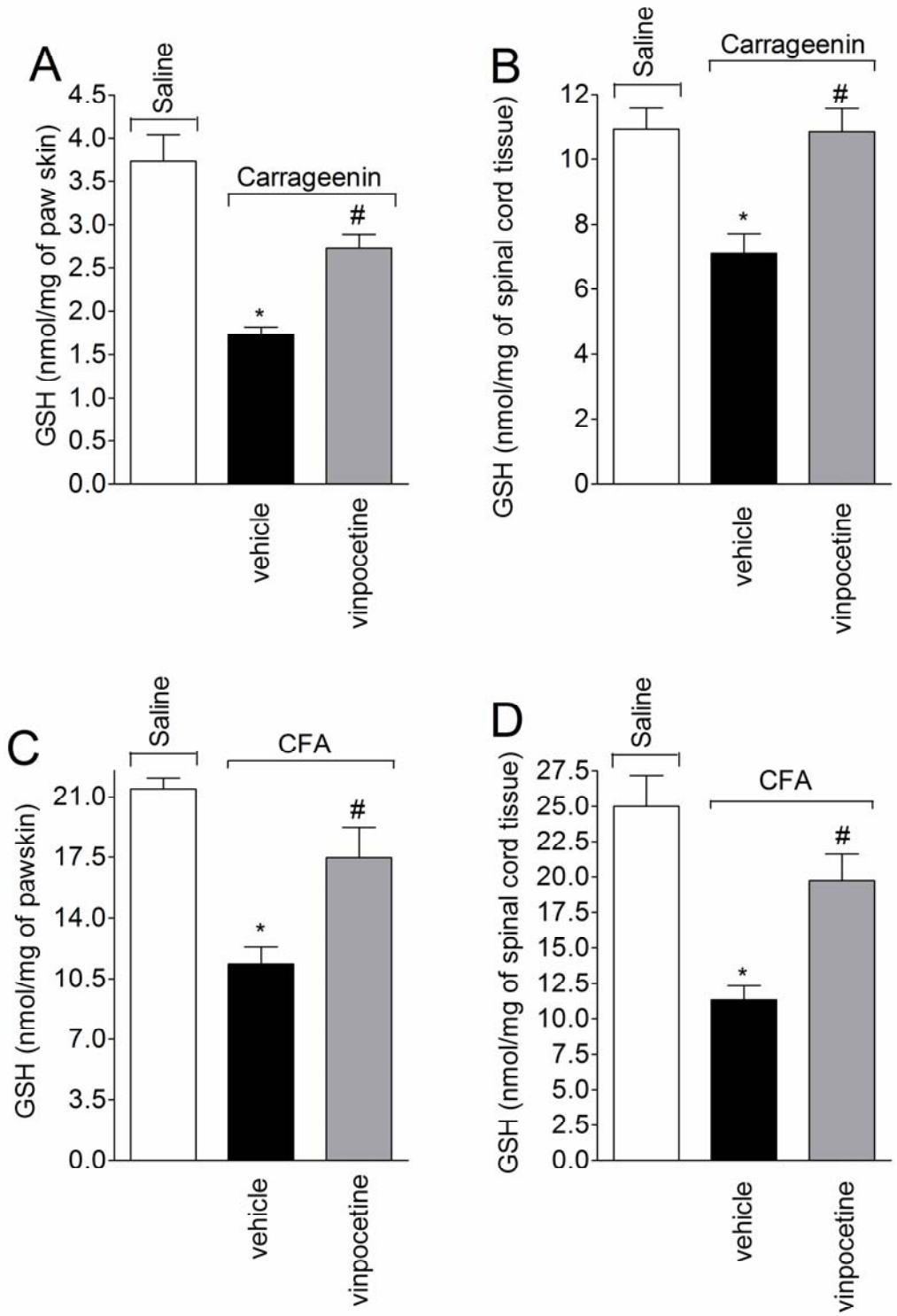
2

3

4

5

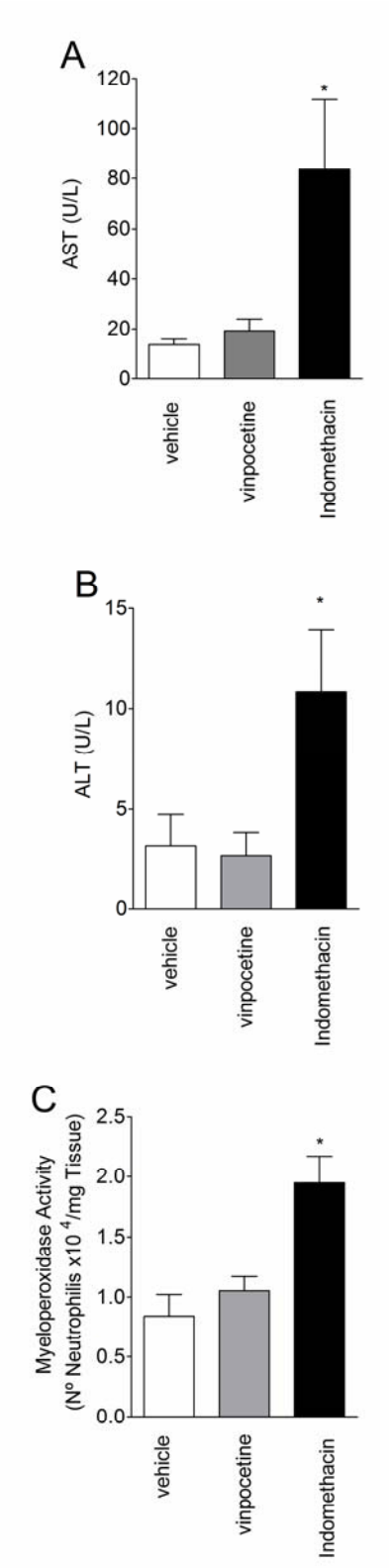
1 Figure 6


A
Saline
Carrageenin
GSH (nmol/mg of paw skin)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
#
vehicle
vinpocetine
B
Saline
Carrageenin
GSH (nmol/mg of spinal cord tissue)
12
10
8
6
4
2
0
*
#
vehicle
vinpocetine
C
Saline
CFA
GSH (nmol/mg of pawskin)
21.0
17.5
14.0
10.5
7.0
3.5
0.0
*
#
vehicle
vinpocetine
D
Saline
CFA
GSH (nmol/mg of spinal cord tissue)
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
#
vehicle
vinpocetine

2

3

1 Figure 7


A
AST (U/L)
120
100
80
60
40
20
0
*
vehicle
vinpocetine
Indomethacin
B
ALT (U/L)
15
10
5
0
*
vehicle
vinpocetine
Indomethacin
C
Myeloperoxidase Activity
(Nº Neutrophilis x10 -4 /mg Tissue)
2.5
2.0
1.5
1.0
0.5
0.0
*
vehicle
vinpocetine
Indomethacin

2

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ANEXO

ANEXO A

Preparation and Submission of Manuscripts

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