Universidade Estadual de Londrina

MARIANA MARQUES BERTOZZI

AVALIAÇÃO DOS MECANISMOS DE AÇÃO DO DIOSMIN EM MODELOS MURINOS DE DOR NO CÂNCER E DOR NEUROPÁTICA

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Dissertação de mestrado apresentada a banca de qualificação do programa de pós-graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Patologia Experimental.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior

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Londrina, 26 de abril de 2018.

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Ainda que eu tenha a minha individualidade,
tudo o que eu faço reflete na vida de
alguém ao meu redor.
(Rodolfo Abrantes)

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RESUMO

O número de casos de dores crônicas vem crescendo a cada ano e acomete geralmente a população adulta. A dor associada ao câncer é um sintoma de altíssima queixa entre pacientes com câncer, e debilita ainda mais a vida destes indivíduos. Assim como a dor crônica neuropática, esta é uma síndrome complexa resultante de lesão ou disfunção de fibras nervosas. As terapias farmacológicas disponíveis atualmente para ambos os quadros têm se mostrado insuficientes para o controle destes tipos de dor. Portanto há a necessidade e busca de um novo tipo de abordagem terapêutica. O crescente interesse no estudo de compostos naturais se deve principalmente ao fato de muitos destes apresentarem propriedades benéficas e baixa toxicidade, dentre tais compostos, podemos destacar os flavonoides, os quais têm sido amplamente estudados devido as suas diversas atividades biológicas. O diosmin é um flavonoide, que além de apresentar várias atividades biológicas, é considerado um composto seguro, com boa tolerabilidade e não apresenta efeito tóxico. Desse modo, o objetivo desse estudo foi avaliar o efeito antinociceptivo do flavonoide diosmin em modelos murinos de dor crônica induzida pelas células tumorais de Ehrlich (CTE) e dor crônica neuropática induzida pela constrição do nervo ciático (CCI).Para isto, CEUA/UEL aprovou o uso de camundongos Swiss para realização dos experimentos e os animais foram submetidos ao modelo de CTE ou CCI. Em ambos foram avaliados parâmetros comportamentais como hiperalgesia mecânica e térmica e no modelo de CTE verificamos a espessura da pata para avaliação do efeito dose-resposta do diosmin. Os dois modelos passaram pelo tratamento prolongado e foram avaliados nos mesmos parâmetros comportamentais e após foram feitos testes de toxicidade hepática e gástrica. No modelo de dor no câncer induzida por CTE, foi avaliado o efeito do tratamento com diosmin na dor espontânea, perfil de migração celular e parâmetros envolvendo o estresse oxidativo e expressão de mRNA de citocinas pró-inflamatórias (IL-1β, Il-33 e seu receptor St2, IL-10). No modelo de CCI também foi avaliadaparticipação do diosmin na via do NO/GMPc/PKG/Canais de Potássio ATP Sensíveis, expressão de mRNA de citocinas pró-inflamatórias (IL-1β, TNF-α, IL-33 e seu receptor St2). Considerando que no modelo de CCI utilizamos protocolo de tratamento único e prolongado, os resultados das avaliações comportamentais (hiperalgesia mecânica, térmica e espessura da pata foram satisfatórios em ambos tratamentos e modelos, sendo que a dose de 10mg/kg de diosmin foi a escolhida para os demais testes. Quanto aos resultados referentes à dor no câncer, o tratamento foi capaz inibir a dor espontânea, ter ação no recrutamento neutrofílico (MPO), porém não alterou o padrão de macrófagos (NAG).O tratamento com diosmin apresentou efeito protetor contra o estresse oxidativo induzido pela CTE. Além de reduzir a expressão de mRNA de citocinas envolvidas no processo inflamatório a níveis espinais. Quanto aos resultados referentes à dor neuropática, esses mesmos parâmetros hiperalgésicos foram avaliados após o pré-tratamento com inibidores da via de sinalização NO/GMPc/PKG/Canais de Potássio ATP Sensíveis e foi observada a reversão parcial do efeito antinociceptivo do diosmin. O tratamento com diosmin foi

capaz de inibir a produção de citocinas na medula espinal assim como a ativação das células da glia. Testes de toxicidade corroboraram a segurança e baixo risco de efeitos colaterais desse flavonoide. Dessa forma, foi concluído que o diosmin apresenta efeito benéfico em modelos de dor induzida por CTE e dor induzida por CCI por ativar a via NO/GMPc/PKG/Canais de Potássio ATP Sensíveis, por reduzir o estresse oxidativo, reduzir o recrutamentocelular e inibir a produção de citocinas e ativação das células da glia.

*avaliação feita apenas no modelo de dor no câncer induzida por CTE.

Palavras-chave: Diosmin. Dor crônica. Hiperalgesia.

BERTOZZI, Mariana Marques. **Evaluation of the mechanisms of action of diosmin in murine models of cancer pain and neuropathic pain.** 2018. 96 p. Dissertation. (Master in Experimental Pathology) - Londrina State University, Londrina, 2018.

ABSTRACT

The number of cases of chronic pain is growing every year and usually affects the adult population. Pain associated with cancer is a symptom of high complaint among cancer patients, further debilitating the lives of these individuals. Like chronic neuropathic pain, this is a complex syndrome resulting from injury or dysfunction of nerve fibers. The pharmacological therapies currently available for both frames have been shown to be insufficient for the control of these types of pain. Therefore, there is a need and search for a new type of therapeutic approach.The growing interest in the study of natural compounds is mainly due to the fact that many of these have beneficial properties and low toxicity, among these compounds, we can highlight flavonoids, which have been widely studied due to their diverse biological activities. Diosmin is a flavonoid, which besides presenting various biological activities, is considered a safe compound with good tolerability and does not exert toxic effects.Thus, the aim of this study was to evaluate the antinociceptive effect of flavonoid diosmin in murine models of chronic pain induced by Ehrlich tumor cells (CTE) and chronic neuropathic pain induced by sciatic nerve constriction (CCI).For this, male Swiss mice approved by CEUA / UEL, were submitted to the models CTE or CCI. Behavioral parameters such as mechanical and thermal hyperalgesia and in the CTE model we verified paw thickness to evaluated the dose-response effect of diosmin.Both models underwent prolonged treatment and were evaluated in the same behavioral parameters and after for hepatic and gastric toxicity tests.In the CTE-induced cancer pain model, the effect of diosmin treatment on spontaneous pain, cell migration profile, and parameters involving oxidative stress and mRNA expression of proinflammatory cytokines (IL-1β, IL-33 and its St2 receptor, IL 10). In the CCI model, participation of diosmin in the NO/GMPc/PKG/Potassium Chanels ATP Sensitive pathway, mRNA expression of proinflammatory cytokines (IL 1β, TNF-α, IL-33 and its St2 receptor).The results of the behavioral evaluations (mechanical and thermal hyperalgesia and paw thickness*) were satisfactory in acuteand chronic treatment in both models, and the dose of 10mg/kg of diosmin was chosen for the other tests. Regarding cancer pain results, the treatment was able to inhibit spontaneous pain, acting on neutrophil recruitment (MPO), but did not alter thestandard recruitment of macrophages (NAG).The treatment with diosmin presented a protective effect against the oxidative stress induced by CTE.In addition to reducing the mRNA expression of cytokines involved in the inflammatory process at spinal levels.Regarding neuropathic pain results, these same hyperalgesic parameters were evaluated after pre-treatment with inhibitors of the NO/GMPc/PKG/Potassium Chanels ATP Sensitive pathwayand the partial reversion of the antinociceptive effect of diosmin was observed. Treatment with diosmin was able to inhibit cytokines of the spinal cord as well as the activation of glial cells. Toxicity tests corroborated the safety and low risk of side effects of this flavonoid.In this way, it was concluded that diosmin has a beneficial effect on CTE-induced pain and CCI induced pain by activating the NO/GMPc/PKG/Potassium Chanels ATP Sensitive pathwayby reducing oxidative stress, reducing cellular recruitment and inhibit cytokine production and activation of glial cells.

* assessment made only in the CTE-induced cancer pain model.

Keywords: Diosmin. Chronic pain. Hyperalgesia.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ADTs	Antidepressivos tricíclicos
AINES	Anti- inflamatório não esteroidal
CCI	Constrição do nervo ciático
CTE	células tumorais de Ehrlich
CXCL1	Ligante de quimiocina CXC1
EROs	Espécies reativas de oxigênio
GMPc	Guanilato ciclase solúvel
IFNγ	Interferon gama
IL-1β	Interleucina 1 beta
IL-33	Interleucina 33
IL-6	Interleucina 6
LPS	Lipopolissacarídeo
MAPKs	Proteínas quinases ativadas por mitógenos
NF-κB	Fator de transcrição nuclear kappa B
NO	Óxido nítrico
Nrf2	Fator nuclear eritroide 2 relacionado ao fator 2
OMS	Organização Mundial da Saúde
PAMP	Padrão molecular associado a patógeno
PGE2	Prostaglandina E2
PGI2	Prostaciclina
PKG	Fosfoquinase G
PRR	Receptor de reconhecimento padrão
SNC	Sistema nervoso central
ST2	Receptor de IL-33
TNFR	Receptor do fator de necrose tumoral
TNF-α	Fator de necrose tumoral α

SUMÁRIO

1 INTRODUÇÃO

1.1 Inflamação e dor: Aspectos gerais

A inflamação é uma resposta apropriada e efetiva para a defesa do hospedeiro e manutenção da homeostase. O processo inflamatório é desencadeado por intermédio de agentes externos nocivos e/ou injuria tecidual. Esse processo é benéfico ao organismo pois visa a eliminação do estímulo inflamatório desencadeante e posterior resolução da lesão (FERREIRA et al, 2009; LAWRENCE; WILLOUGHBY; GILROY, 2002).

O processo inflamatório é caracterizado por uma série de eventos vasculares e celulares que permitem a migração sequencial de leucócitos para o sítio da inflamação. A produção de mediadores inflamatórios como as citocinas pró-inflamatórias Interleucina 1 beta (IL-1β), Fator de necrose tumoral alfa (TNF-α), Interferon gama (IFNγ), dentre outros mediadores como espécies reativas de oxigênio (EROs), Prostaglandina E2 (PGE_2), Prostaciclina (PGI_2) e ligante de quimiocina CXC1 (CXCL1) possuem importante papel na reação inflamatória (VERRI, et al., 2006). Estes mediadores participam induzindo a ativação de fatores de transcrição, que possuem papel fundamental na regulação da inflamação, como fator de transrição kappa B (NF-κB) e fator nuclear eritroide 2 relacionado ao fator 2 (Nrf2) (LI et al., 2008; KOBAYASHI et al., 2016). A fase aguda da inflamação é caracterizada pelos sinais cardinais: calor, rubor, edema, dor/hiperalgesia (VALÉRIO, et al, 2009). Entretanto, se ocorrer de forma exacerbada e/ou prolongada pode levar a destruição do tecido, fibrose e eventual perda de função do tecido e/ou órgão afetado (MADERNA; GODSON, 2009).

A dor é a percepção de uma experiência sensorial nociceptiva (“nocere” do latim = ‘causar dano’) com conotação afetiva aversiva e desagradável, que pode estar, ou não, associada a um dano tecidual real ou potencial (FERREIRA et al., 2009). A Associação internacional para o Estudo da Dor (IASP, International Association for the Study of Pain) a classifica como uma experiência multidimensional, na qual estão envolvidos o sinal nociceptivo, reconhecimento e processamento central desses sinais, pelos núcleos supra espinais.

Nociceptores são neurônios aferentes primários responsáveis pelo reconhecimento e transdução de estímulos nocivos, como estímulos térmicos, mecânicos e químicos. A nocicepção é o resultado da ativação de receptores específicos presentes nas terminações nervosas livres do neurônio aferente primário, e posterior comunicação entre o sistema nervoso periférico e o sistema nervoso central (SNC) por vias neuroanatômicas, a fim de que o estímulo chegue ao SNC. A dor inflamatória ocorre pela ação dos mediadores inflamatórios produzidos no local da lesão nas terminações nervosas livres dos nociceptores, este sinal é conduzido por fibras aferentes, a fim de que o estímulo chegue ao SNC e gere uma resposta protetiva (FERREIRA et al., 2009).

A percepção dolorosa é um mecanismo evolutivo, que confere ao indivíduo a capacidade de autopreservação. Essa percepção possibilita a identificação de situações que possam causar danos ou mesmo controlar as consequências de uma lesão ocorrida. No entanto, existem situações nas quais existe a persistência do processo inflamatório e dano tecidual ou neuropatologias que não são reparadas pelo organismo.Por outro lado, mesmo que ocorra a resolução da lesão inicial, a plasticidade neuronal decorrente de doenças constantes mantém o quadro doloroso. Com isso, um estado mal adaptativo do organismo pode ocasionar dor persistente, que ocorre pela combinação de alterações nos eventos básicos da nocicepção associado a disfunções de origem física, emocional, psicológica e social. Neste caso, a dor torna-se crônica e perde o caráter protetor necessário à manutenção da integridade do organismo, como em casos de dor aguda (ALVES, 2009), passando a ser considerada a própria patologia

1.2 Dor Crônica

Diferente do caráter de alerta da dor aguda, a dor crônica está associada a alterações da via fisiológica homeostática, originando a hiperalgesia ou alodínia, quando, respectivamente, um estímulo pouco nocivo ou não nocivo induz a dor. Geralmente a dor cônica se prolonga e é persistente, podendo se estender por meses ou anos, (ASHBURN; STAATS, 1999; LOESER; MELZACK, 1999; SMITH et al., 2014).

Portanto, além de caracterizar um problema de saúde pública, a alta prevalência da dor crônica apresenta desdobramentos socioeconômicos por afetar diretamente a população economicamente ativa. Desta forma, o estudo dos mecanismos de nocicepção é fundamental para o desenvolvimento de terapias mais eficientes, tanto no tratamento da dor crônica, quanto na sua prevenção, uma vez que a cronificação está intimamente relacionada ao processo inflamatório persistente e não resolvido (JI et al., 2014).

A Organização Mundial da Saúde (OMS) estima que cerca de 30% da população mundial apresenta dor crônica (WHO, 2012; JI et al., 2016), o que promove limitação de movimentos simples do cotidiano pelo paciente. Além do mais a dor crônica pode afetar o psicológico do paciente levando a sentimentos de desesperança e isolamento social. Sendo assim, esse quadro compromete a qualidade de vida do indivíduo (OUTCALD et al., 2015; LERMAN et al., 2015; JI et al., 2016).

As dores crônicas são classificadas em dois subgrupos: a dor inflamatória, ligada a uma lesão no tecido; e a dor neuropática, que se relaciona a lesão nos nervos.

1.2.1 Câncer e a dor oncológica

Câncer é um termo genérico para um conjunto de mais de 100 doenças que podem afetar qualquer parte do corpo. Outros termos utilizados são neoplasias ou tumores malignos. O ponto em comum delas é o crescimento rápido e desordenado de células mutadas que invadem os tecidos e órgãos sadios, e ainda podem espalhar-se caracterizando a metástase para outras regiões do corpo (KUMAR et al., 2008).

O câncer engloba um grupo de doenças com morbidade e mortalidade elevadas e prevalência crescente na população. É a segunda maior causa de morte no mundo, sendo responsável por 8,8 milhões de mortes em 2015. Aproximadamente 1 em 6 mortes é decorrente de câncer (OMS, 2017). No Brasil, o câncer é a terceira causa mais frequente de morte em ambos os gêneros (INCA, 2017).

Quando um quadro de câncer é detectado, tanto o médico quanto o paciente têm seu enfoque natural e legítimo na própria doença, porém o câncer, muitas vezes, pode causar danos deixado um quadro coadjuvante, como é o caso das dores resultante do câncer. Aproximadamente 62% dos pacientes com câncer relatam terem dores constantemente e normalmente esse tipo de dor é subtratado (van den BEUKEN-van EVERDINGEN et al., 2007; DEANDREA et al., 2008).

Receber o diagnóstico de câncer ainda é estigmatizante, e o medo pode estar relacionado à presença da dor. Os mitos e preconceitos que são carregados em relação às drogas utilizadas no tratamento e seus efeitos colaterais, os quais geram reflexos emocionais, sociais e físicos, muitas vezes resultam em um tipo de dor psíquica. A dor psíquica, ou sofrimento, tem um papel importante na qualidade de vida do paciente (LOSCALZO, 1996; MAKO et al., 2006; SYRJALA et al., 2014).Ambas, dor física e psíquica, estão intimamente ligadas, o que ressalta a importância da interdisciplinaridade na abordagem do paciente com dor oncológica e requerem uma terapia multimodal.

Os mecanismos fisiopatológicos da dor no câncer ainda não estão completamente compreendidos. Porém, sabe-se que a apresentação clínica da dor no câncer é dependente de 3 fatores: 1) a histopatologia das células tumorais; 2) a localização do carcinoma primário e 3) localização das metástases. Atualmente inúmeros modelos animais experimentais, como indução de câncer neuropático, câncer ósseo, entre outros tem sido introduzido para aprimorar o estudo da dor e compreender os seus mecanismos moleculares envolvidos na geração e cronicidade (SCHMIDT et al., 2010; BURTON et al., 2014).

Porém, sabe-se que tanto mecanismos da dor inflamatória, quanto da dor neuropática promovam a dor no câncer. Ou seja, é uma dor multifatorial, o que expande a gama de tratamentos alternativos. O crescimento tumoral promove o processo inflamatório tecidual e os mediadores inflamatórios liberados sensibilizam os nociceptores. Ainda, a resposta imunológica do câncer contra o próprio organismo, ou do organismo contra o tumor também parece levar a produção de vários mediadores inflamatórios. Além disso, a proliferação do tumor pode comprimir os nervos periféricos ou centrais, induzindo injúria. Por isso, a dor no

câncer envolve mecanismos semelhantes aos responsáveis pela dor inflamatória e/ou dor neuropática (BASBAUM et al., 2009; SCHMIDT et al., 2010).

Além disso, em modelo murino de dor associado ao câncer induzido pela injeção intraplantar (i.pl.) de células de carcinoma pulmonar de camundongos C57BL/6 observa-se o aumento de citocinas pró-inflamatórias, como TNF-α, IL-1β e IL-6 no tecido plantar (CONSTANTIN et al, 2008). Em especial a sinalização TNF-α/TNFR2 desempenha papel importante na hiperalgesia térmica na dor associado ao câncer, uma vez que o tratamento intraperitoneal (i.p.)diário com o anti-TNFα, Etanercept, ou a utilização de animais C57BL6/C knockout (KO) para TNFR2 inibe a hiperalgesia térmica associada ao câncer (CONSTANTIN et al, 2008). De acordo, as injeções intraplantar (i.pl.), i.p.ou subcutânea (s.c.) de TNF-α induz hiperalgesia térmica e mecânica em camundongos (JUNGER & SORKIN et al. 2000).

1.2.2 Dor neuropática

Segundo a Organização Mundial de Saúde em torno de 1 a 3% da população mundial apresentam algum tipo de dor crônica neuropática (BOWSHER, 1993; DWORKIN et al., 2003; IRVING, 2005). Muitas vezes a dor neuropática é subdiagnosticada e subtratada, e está associada com o sofrimento, incapacidade e redução da qualidade de vida do paciente (CHONG, BAJWA, 2003; TOTH, MOULIN, 2014). A dor neuropática é definida pelo Grupo de Interesse Especial sobre Dor neuropática, *Special Interest Group on Neuropathic Pain* (NeuPSIG) como dor que ocorre como consequência de uma lesão direta ou de doenças que afetam o sistema somatossensorial (IASP, 2012; HAANPÄÄ, TREEDE, 2010).

Após lesão (total ou parcial) do tecido nervoso periférico, ocorre o desencadeamento de uma cascata de sinalização intracelular tanto local nos nociceptores, quanto em mecanismos espinais, envolvendo micróglias, astrócitos e oligodendrócitos. Essas cascatas envolvem a ativação de proteínas quinases ativadas por mitógenos (MAPKs), síntese de mediadores inflamatórios, como IL-1β, TNF-α, PGE_2 e óxido nítrico (NO) que promovem a ativação de células gliais.

Desta forma, a ativação glial na medula espinhal está relacionada com a hiperalgesia e alodinia e parece ser um mecanismo envolvido em neuropatias de diferentes etiologias (MILLIGAN, WATKINS, 2009). Tem sido demonstrado recentemente que astrócitos, micróglias e oligodendrócitos desempenham papéis importantes na transmissão da dor, podendo modular a função sináptica e a excitabilidade neural por diferentes mecanismos (HALASSA et al., 2007; LONGHI-BALBINOT et al; 2016, ZARPELON et al., 2016; PINHO-RIBEIRO et al; 2017, BERTOZZI et al; 2017).

1.3 Terapias Atuais na dor no câncer e na dor neuropática

Há duas etapas para realizar o tratamento da dor no câncer, sendo elas, inicialmente o tratamento primário da própria doença por cirurgia, radioterapia, quimioterapia ou hormonioterapia, e o tratamento farmacológico (MELO et al., 2009).

O manejo da dor nos pacientes com câncer dependerá da intensidade de dor relatada pelos mesmos, que pode variar entre branda e grave(AURET, 2013). Para o tratamento da dor branda no câncer, são utilizados medicamentos não opioides como acetaminofeno e anti-inflamatórios não esteroidais (AINEs) (SCHUG; CHANDRASENA, 2015). No tratamento da dor moderada, são administrados por via oral opioides fracos, como codeína e tramadol, associados com não opióides. Para tratamento de dor grave no câncer, prevê-se a utilização de opióides fortes como a morfina (SCHUG; CHANDRASENA, 2015).

Pelo fato de a dor neuropática ter um ou mais mecanismos envolvidos na sensibilização central ou periférica, esses múltiplos mecanismos e mediadores envolvidos estabelecem diferentes alvos de tratamento nesses casos. Os fármacos comumente utilizados na prática clínica para o tratamento da dor neuropática são os analgésicos opióides, AINEs, antidepressivos tricíclicos (ADTs) e anticonvulsivantes. Entretanto, essas terapias existentes podem apresentar diversos efeitos adversos além de terem baixa eficácia neste tipo de dor (DWORKIN et al., 2003; ATTAL et al., 2010).

1.4 Flavonoide: Diosmin

Durante a evolução, os seres vivos sempre conviveram com a dor, visto que é um mecanismo de autopreservação, como já citado anteriormente, mas também sempre tentaram dominá-la. A utilização de espécies vegetais com fins de tratamento, cura de doenças e alívio de sintomas remetem desde o início da civilização. As plantas são utilizadas pelo homem e pelos animais silvestres com vários objetivos para sua sobrevivência, inclusive fins medicinais.

Os flavonoides são compostos polifenólicos sintetizados como metabólitos secundários por plantas. Os flavonoides desempenham diversas funções como atração de polinizadores e disseminadores de sementes, além da pigmentação em frutas, flores, sementes e folhas. Também têm função importante na sinalização entre plantas e micróbios, na fertilidade de algumas espécies, na defesa como agentes antimicrobianos e na proteção à radiação ultravioleta (PATEL et al.,2013).A fórmula estrutural básicados flavonoides é baseada no núcleo que consiste em dois anéis fenólicos A e B e um anel C (Figura 1).

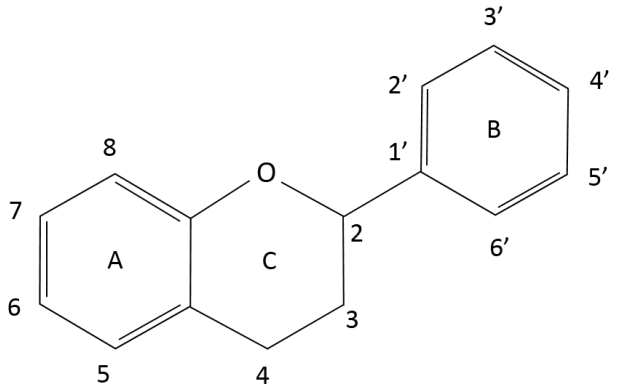

Figura 1- Fórmula estrutural química básica dos flavonoides.

Foi descrito na literatura o potencial terapêutico dos flavonoides devido a atividade anti-hepatotóxica, antitumoral (DUNG et al., 2012), antioxidante (BOUSKELA et al., 1997) e anti-inflamatória (GUAZELLI et al., 2013). Além disso, estes compostos estão amplamente distribuídos na natureza e têm sido foco de diversos trabalhos na área farmacológica.

Sendo assim, a utilização de substâncias naturais com propriedades modulatórias no processo doloroso tem sido considerada uma nova abordagem

terapêutica para dor neuropática e no câncer. Neste sentido, o diosmin tem se apresentado como uma possível nova terapia para quadros dolorosos crônicos.

O diosmin é um flavonoide comum nas plantas rutáceas. A diosmina flavona glicosido (3',5,7-tri-hidroxi-4'-metoxi-flavona-7-ramnoglucosídeo) (Figura 2) é obtida através da desidrogenação do composto glicosilado hesperidina (PATEL et al, 2013).

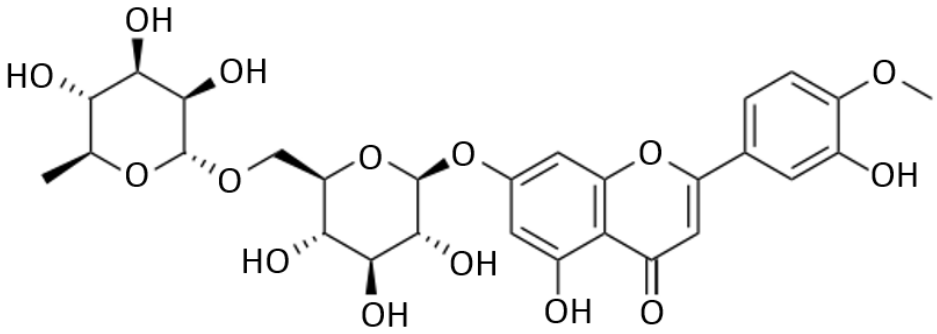

Figura 2-Fórmula estrutural química do diosmin.

O diosmin está presente abundantemente no pericarpo de várias citros (DEL BAÑO et al., 2004; NOGATA et al., 2006; CAMPANERO et al., 2010) e na clínica tem sido utilizado em associação com a hesperidina. Está associação de flavonoides possui efeito de proteção vascular no tratamento de hemorroidas, linfedema, varizes e diferentes tipos de câncer (STRUCKMANN, 1999). Seus efeitos parecem ter relação com sua ação anti-inflamatória e antiapoptótica. Diosmin apresenta efeito anti-inflamatório e antiapoptótico por inibir a produção de TNFα e apoptose induzido pelo LPS em cultura celular de feocromocitoma de rato (PC12) sem induzir toxicidade (DHOLAKIYA and BENZEROUAL (2011).Também apresentou ação reduzindo o estresse oxidativo sistêmico induzido por isquemia seguido de reperfusão em ratos(UNLÜ et al., 2003).Em relação ao tratamento clínico de hemorroidas e alterações vasculares o diosmin apresenta ótima tolerabilidade e é considerado um medicamento seguro e não tóxico (HITZENBERGER, 1997) (EL-SHAFAE e EL-DOMIATY, 2001).

Desta maneira, os mecanismos anti-inflamatórios e antioxidantes do

diosmin demonstrados até o momento apontam, portanto, seu possível efeito em modular casos de dor oncológica e dor neuropática. Considerando então, buscamos avaliar, no presente trabalho, o efeito do diosmin em dois modelos experimentais de dor crônica: (a) modelo de dor no câncer induzida pela inoculação de células tumorais de Ehrlich e (b) modelo de dor neuropática induzida pela constrição do nervo ciático (CCI).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar o efeito analgésico e mecanismos de ação do diosmin em modelo de dor no câncer induzida pelas células tumorais de Ehrlich e em modelo de dor neuropática induzida pela constrição do nervo ciático e em camundongos swiss machos.

2.2 OBJETIVOS ESPECÍFICOS

2.2.1. Modelo de dor no câncer:

- avaliar o efeito do tratamento agudo e crônico com diosmin sobre parâmetros de dor manifesta, hiperalgesia e edema;

- avaliar o efeito do tratamento com diosmin sobre perfil celular inflamatório peritumoral;

- avaliar a correlação entre efeito analgésico do diosmin e diminuição do estresse oxidativo;

- avaliar o efeito do tratamento com diosmin sobre hepatotoxicidade, lesão gástrica induzidas pelas células tumorais de Ehrlich

- comparar os níveis de expressão de mRNA de genes relacionados com a inflamação nos animais induzidos com CTE tratamentos ou não.

2.2.2.Modelo de dor neuropática:

- avaliar o efeito dose e tempo-dependente do diosmin sobre a hiperalgesia mecânica e térmica induzida pela constrição crônica do nervo ciático (CCI) em camundongos;

- avaliar a participação da via de sinalização NO/GMPc/PKG/canais de potássio ATP sensíveis no possível mecanismo antinociceptivo do diosmin

-avaliar o efeito do tratamento prolongado com diosmin sobre a hipealgesia mecânica e térmica

-avaliar a possibilidade de ocorrência de efeitos colaterais gástricos e renais do tratamento prolongado com diosmin

-- comparar os níveis de expressão de mRNA de genes relacionados com a inflamação nos animais induzidos com CCI após o tratamento prolongado com diosmin.

- Correlacionar o tratamento prolongado com diosmin com a ativação das células da glia.

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3 ARTIGO PARA PUBLICAÇÃO (PLANTA MEDICA)

O presente trabalho foi realizado no Laboratório de Dor, Inflamação Neuropatia E Câncer, da Universidade Estadual de Londrina e segue as normas da revista Planta Medica- Journal of Medicinal Plant and Natural Product Research. Os resultados parciais estão descritos no artigo intitulado “Diosmin reduces Ehrlich tumor cells-induced cancer pain in mice”.

Diosmin reduces Ehrlich tumor cells-induced cancer pain in mice.

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ABSTRACT

Cancer pain is an injury which debilitates the life of the patients. Due to lack of safe treatment for this type of pain, there is the necessity to search new type of therapeutic approach. Flavonoids are compounds derived from plants that show several beneficial effects. Thus, this work aims to study the effects of flavonoid diosmin in Ehrlich tumor cells-induced pain model. Spontaneous pain was evaluated on the 8th day after inoculation.In another set of experiments, a prolonged protocol of treatment, the animals were daily treated for 12 days and were evaluated mechanical and thermal hyperalgesia, changes in paw thickness for the dose-response effect of diosmin in male Swiss mice approved by CEUA/UEL. The dose of 10 mg/kg of diosmin was chosen for the next experiments tests to evaluate the mechanisms triggered by diosmin. In the end of experiments, serum samples and stomach tissue were collected to determine hepatic and gastric toxicity tests. Paw tissue and spinal cord were collected to determine cell migration profile, parameters involving oxidative stress and expression of mRNA of inflammatory cytokines were also evaluated. Treatment with diosmin inhibited mechanical and thermal hyperalgesia, and also prevented the paw thickness. Regarding cancer pain results, the treatment was able to inhibit spontaneous pain, acting on neutrophil recruitment (MPO), but did not alter the standard recruitment of macrophages (NAG). The treatment with diosmin presented a protective effect against the Ehrlich tumor cells-induced oxidative stress.In addition to reduce the mRNA expression of cytokines (IL-1β, IL-33 and its St2 receptor, IL-10) involved in the inflammatory process at spinal levels. Toxicity tests corroborated the safety and low risk of side effects of this flavonoid. In this sense, this study demonstrated that the flavonoid diosmin has analgesic effect in the pain model induced by Ehrlich tumor cells through anti-inflammatory and antioxidant mechanisms.

Key words: Ehrlich tumor cells. Chronic pain. Flavonoids. Diosmin.

1. Introduction

Cancer pain is a symptom that affects about 53% of patients who are in any state of the disease [1]. Neuropathic and inflammatory pain play important role in cancer pain releasing inflammatory mediators that interact or activates nociceptors leading to the development of the symptom [2].

The principal mediators known to participate in inflammatory and neuropathic pain are chemokines and cytokines, such as proinflammatory as Tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) [3]. Moreover, these cytokines induce central sensitization and hyperalgesia through three ways: increasing excitatory synaptic transmission, decreasing inhibitory synaptic transmission, and inducing synaptic plasticity in the long term [4].

Also, tumoral cells proliferation increases oxidative stress [5]. In addition, oxidative stress amplifies the pain and inflammatory process [6]. The endogenous antioxidant system neutralizes reactive oxygen species (ROS)-induced oxidative stress in physiological situations of homeostasis. On the other hand, exacerbated production of free radicals (e.g. superoxide anion and hydrogen peroxide) in an inflammatory overcomes the intracellular antioxidant. Thus, oxidative stress induces an imbalance between antioxidants and oxidants leading to inflammatory pain [7] through activation of MAP kinases-, PI3K- and NFκB-induced the production of hyperalgesic mediators [8-9].

Ehrlich tumor cells induce inflammatory pain with the production of IL-1β and TNF-α promoting activation of nociceptors [9]. This is a model of murine mammary adenocarcinoma-induced pain presenting features like those of preoperative breast cancer with spontaneous pain and pain upon examination (pressure of the lump, hyperalgesia) [10-11-12] with the benefit of development in standard Swiss mice.

Nociception signal is transmitted through sensitive afferent neuron, and activated NMDA receptors in the horn of the dorsal root allowing Ca 2^{+} influx and promoting the activation of iNOS which increases peroxynitrite (ONOO-) and superoxide (O2-) formation and accumulation [13-14]. Oxidative stress also trigger and maintain the nociceptive pathways through the activation of glutamatergic signaling[15]. Thus, there is a possibility of having a central activation, since the activity of the rostrolateral ventral medulla is involved with the transmission of pain and activation of N-Methyl-D-aspartate receptors (NMDs), which glutamate acts [15- 16]. All these factors promote the maintenance of tumor-induced hyperalgesia.

Diosmin (3,5,7-trihydroxy-4-methoxyflavone-7-ramnoglucosidium) is a flavonoid that belongs to the class of flavones which has demonstrated several beneficial effects both *in vitro* and *in vivo*(17-18-19). Its molecular weight is 608.6 dalton [19-20], and was first isolated from *Scrophularia nodosa*in 1925, and can be found in several citrus fruits, or synthesized from the flavonoid hesperidin [19-21]. Diosmin possesses vascular protective effect and is already used in the clinic in association with hesperidin for the treatment of chronic venous insufficiency, hemorrhoids, lymphedema and varicose veins [22-23].Its various effects have been associated with its antioxidant structure that neutralizes ROS, and its influence on inflammation reducing the synthesis of prostaglandins and thromboxane [24-25]. Studies have shown that diosmin may have a possible effect on the reduction of oral cancer induced in rats, and inhibits the development of various types of cancer. However, this role may be presented by the association or not with other flavonoids, for example, hesperidin [26-27].

Considering the above information, we propose to investigate in the present study the analgesic activity and peripheric and central mechanisms of diosmin in the model of cancer pain in mice induced by Ehrlich tumor cells.

2. Results

2.1 Acute treatment with diosmin inhibitis Ehrlich tumor cells-induced hyperalgesia and paw thickness.

In the first series of experiments, the animals were stimulated with intraplantar injection (i.pl.) of $1x10^6$ tumor cells in the hind paw [11]. On the 8^{th} day after inoculation, mice were treated acutely with diosmin intraperitoneally (i.p.) at doses of 1, 3, 10 and 30 mg/kg. Treatment in the control animals was performed with vehicle (2% DMSO + Saline). Mechanical hyperalgesia (Figure 1A), thermal hyperalgesia (Figure 1B) and assessment of paw thickness (Figure 1C) were performed 1, 3, 5 and 7 hours post-treatment. Ehrlich tumor cells induced mechanical hyperalgesia, thermal hyperalgesia, and increased paw thickness compared to control animals receiving (i.pl.) saline. Only the dose of 10mg/kg (i.p.) of diosmin was able to inhibit Ehrlich tumor cells-induced mechanical hyperalgesia (Figure 1A), thermal hyperalgesia (Figure 1B) and paw thickness (Figure 1C). Thus, the dose of 10mg/kg (i.p.) was chosen for the next experiments tests and the anti-hyperalgesic peak was observed after 5h post-treatment.

2.2 Prolonged treatment with diosmin inhibited mechanical and thermal hyperalgesia, an increase of the paw thickness and spontaneous pain induced by Ehrlich tumor cells.

After determining the best dose of diosmin, we continue for the tests. For this, mice received $1x10^6$ Ehrlich ipl tumor cells and were treated with diosmin (10 mg / kg) for 12 days after

tumor cells stimuli. Treatment in the control animals was performed with vehicle (2% DMSO + Saline). Mechanical hyperalgesia, thermal hyperalgesia, and thickness of the paw were evaluated 5 h post-treatment on 2, 4, 6, 8, 10 and 12 days after tumor cells stimulus. Diosmin treatment inhibited Ehrlich tumor cells-induced mechanical hyperalgesia (Figure 2A), thermal hyperalgesia (Figure 2B), and increased in paw thickness (Figure 2C) compared to the vehicle-treated tumor group. Considering that spontaneous pain reflexes occur in response to tumor cells [11], the spontaneous pain was determined by counting the number of paw agitation (flinches) for 10 minutes (Figure 2D) in mice treated with diosmin (10 mg/kg, i.p.) for 8 days after stimulus with $1x10^7$ tumor cells of Ehrlich. Treatment with diosmin inhibited Ehrlich tumor-cells induced spontaneous pain behavior.

2.3 Diosmin inhibitis Ehrlich tumor cells-induced the activity of the enzyme myeloperoxidase (MPO) but did not alter the activity of N-acetylglucosaminidase (NAG) enzyme

Mice received $1x10^6$ tumor cells and were treated with diosmin (10 mg/kg i.p.) for 12 days. On the 12^{th} day, plantar cutaneous tissue was collected for MPO activity (Figure 3A) and NAG activity (Figure 3B). Treatment with diosmin inhibited Ehrlich tumor cells-induced MPO activity but was not able to change NAG activity.

2.4 Treatment with diosmin prevented by Ehrlich tumor cells-induced oxidative stress in the spinal cord and paw.

Mice received $1x10^6$ tumor cells and were treated with diosmin (10 mg/kg i.p.) for 12 days. On the $12t^h$ day, mice were euthanized and the plantar cutaneous tissue and spinal cord (L4-L6) were harvested to the evaluation of the reduced glutathione (GSH) levels and ABTS

radical scavenge ability. Diosmin treatment demonstrated antioxidant activity preventing consumption of GSH in the paw (Figure 4A) and spinal cord (Figure 4C), and restore the total ability to scavenge the ABTS radical in the paw (Figure 4B) and spinal cord (Figure 4D).

2.5 Treatment with diosmin has a protective effect on the liver and has no deleterious effect on the stomach.

Mice were treated with diosmin (10 mg/kg, i.p.) for 12 days after i.pl. injection of $1x10^6$ tumor cells. On day 12^{th} day, plasma and stomach samples were harvested toassess the levels of the enzymes aspartate aminotransferase (AST) (Figure 5A) and alanine aminotransferase (ALT) (Figure 5B) and MPO activity in the stomach (Figure 5A). Diosmin treatment did not induce increase MPO activity in the stomach. Furthermore, diosmin inhibited Ehrlich tumor cells-increased AST and ALT plasmatic levels demonstrating that the tumor could lead to some liver damage.

2.6 Diosmin does not alter Ehrlich tumor-induced histological modifications.

Mice were treated with diosmin (10 mg/kg, i.p.) for 12 days after i.pl. injection of $1x10^6$ tumor cells. In the 12^{th} day, mice were euthanized and the paw was collected for histological analysis performed with hematoxylin/eosin staining. Figure 6, Panel (a and b) shows the histology of saline i.pl.group, (c and d) tumor animal treated with vehicle, and (e and f) tumor animal treated with diosmin (10 mg/kg i.p.). Arrows indicate intact bone cartilage, presence of skeletal muscle fibers, dermis and epidermis bone/cartilage destruction, and presence of tumor cells.

2.7 Prolonged treatment with diosmin inhibits Ehrlich tumor cells-induced paw tissue and spinal cord up-regulation of pro-Il-1β, Tnf-α, Il-33, Il-10 and St2 receptor mRNA expression.

Mice received diosmin (10 mg/kg, ip) daily treatment from the12th-day post ETC injection. Paw tissue and spinal cord samples were collected 5 h after the last treatment with diosmin. Ehrlich tumor cells induced a significant increase of pro-Il-1β, Il-10, Il-33 and its receptor St2 mRNA expression in the paw tissue and spinal cord (L4-L6) when compared with the saline group (Fig. 7). The prolonged treatment with diosmin reduced the ETC-induced increase of pro-Il-1β (Fig. 7A and B), Il-33 (Fig. 7C and D), St2 (Fig. 7E and F) and Il-10(Fig. 7G and H) mRNA expression in the paw and spinal cord.

3. Discussion

Cancer pain weakens the patient's life, and difficult the treatment [28]. Ehrlich tumor cells-induced cancer pain for displaying painful behaviors such as mechanical hyperalgesia, thermal hyperalgesia, increasing paw thickness and spontaneous pain behavior increasing leukocytes recruitment and oxidative stress [11]. This model has been used to search for new treatment approaches for cancer pain [13]. Herein, we demonstrated that diosmin treatment inhibited Ehrlich tumor cells-induced mechanical hyperalgesia, thermal hyperalgesia, increased paw thickness, spontaneous pain behavior, neutrophils recruitment, oxidative stress in the paw and spinal cord, besides stomach and liver damage.

Diosmin is a flavonoid commonly used in the clinic for treatment of vascular diseases [29]. Concerning to pain, diosmin inhibits the constriction of sciatic nerve-induced mechanical, and thermal hyperalgesia involving the activation of the NO/cGMP/PKG/K_{ATP} channel signaling pathway, inhibiting glial cells activation and inhibition of spinal cord

cytokines [30]. In agreement, our study treatment with diosmin reduced Ehrlich tumor cells-induced mechanical hyperalgesia, thermal hyperalgesia and spontaneous pain sharing some mechanisms. In addition, diosmin was also capable to reduce paw thickness. Thus, this molecule has analgesic and anti-inflammatory activity.

MPO is a heme-enzyme released by leukocytes, mainly neutrophils, in the inflammatory focus [31-32], and it is possible to quantify indirectly neutrophils recruitment [31-32-33]. The present data also showed that diosmin inhibited MPO enzyme activity as demonstrated in other studies [34-35]. In the same line, NAG enzyme is released by the macrophage at the site of inflammation and can be used to indirectly indicate its presence at the site of injury [36-37]. Although macrophages are important resident cells for the innate inflammatory response, they exert characteristic and important functions in the chronic inflammatory response [38]. In the present data, diosmin treatment did not alter Ehrlich-tumor cells-induced NAG activity.

Free radicals are molecules highly reactive/injurious to cellular components because of their unpaired electrons in the molecular electronic configuration [39-40]. These molecules are formed during homeostatic cellular response and are controlled by endogenous antioxidants (eg, endogenous antioxidant glutathione (GSH) and enzymes such as superoxide dismutase (SOD)) [41-42]. On the other hand, under pathological conditions such as in the Ehrlich tumor, ROS promotes an unbalance between antioxidants and oxidants promoting oxidative stress [11-43]. In this sense, depletion of GSH in tissues leads to impairment of the cellular defense against free radicals and may result in oxidative injury [44] as it happens in the tumor. Diosmin treatment inhibited Ehrlich tumor-depleted GSH levels in peripheral and supra-spinal pathways restoring the total antioxidant ability to scavenge the ABTS radical in peripheral and supra-spinal pathways demonstrating its antioxidant effects. Corroborating the

present data, other flavonoids, as Ethyl extract of *Aponogeton undulatus* also inhibits the oxidative stress caused by Ehrlich tumor cells in mice [45]. Thus, diosmin inhibits Ehrlich tumor cells-induced oxidative stress.

The antioxidant activity of diosmin is possibly related to its chemical structure with several hydroxyl radicals (OH) and aromatic rings. This molecular structure confers the ability to donor an electron (H^+) to stabilize the reactive species. In fact, diosmin reestablish antioxidant activity in hindlimb induced-oxidative stress [46]. In addition, diosmin treatment inhibits ethanol-induced gastric oxidative stress inhibiting lipoperoxidation and increasing the level of glutathione (GSH) [47].

One of the main benefits of using flavonoids is its antioxidant effect with low toxic effect [48]. In fact, diosmin appears to exhibit no toxicity [30-47-49]. In animal studies, a flavonoid mixture containing 90%of diosmin and 10%of hesperidin had an LD_{50} of more than 3g/kg. In addition, animal studies have shown the absence of acute, subacute, or chronic toxicity after repeated oral dosing for 13 and 26 weeks using a dose representing 35 times the recommended daily dose [50]. Interestingly, the Ehrlich tumor cells themselves increased AST and ALT levels in plasma demonstrating that the inoculation of peripherally Ehrlich tumor cells can lead to stomach and liver damage. These toxic effects of Ehrlich tumor cells have not been investigated yet. Increases in AST and ALT plasmatic levels could occur both by the systemic inflammatory response, which may contribute to liver inflammation, but also to increased intestinal permeability and increased circulating bacteria with consequent activation of Kupffer cells [51].

The present data also show that prolonged treatment with diosmin did not induce gastric lesions since MPO levels in the stomach of mice treated with diosmin was not elevated compared to the samples of mice who received a usual treatment of clinical drug,

indomethacin. It is known that indomethacin, a cyclooxygenase inhibitor, is a classical nonsteroidal anti-inflammatory drug, that if used for a prolonged period causes gastric lesion as a side effect [52-53-54].

Furthermore, hematoxylin/eosin staining of paw samples revealed no histological differences between mice with tumor prolonged treated with diosmin and vehicle control group. The group without tumor showed normal tissue. The arrows show the presence of epidermis, dermis, skeletal muscle fibers, and intact bone and cartilage. Meanwhile, mice that received Ehrlich tumor cells and were treated with the vehicle or with diosmin presented cartilage destruction, necrotic tissue, intense tumor proliferation, and abundant inflammatory infiltrate.

This data demonstrates that diosmin does not inhibit the growth of Ehrlich tumor cells and therefore does not have an antitumor effect at this analgesic dose. Thus, the role of diosmin is affirmed with anti-inflammatory and antioxidant action, exerting an analgesic effect without affecting tumor growth; thus, it is suitable for the treatment of cancer pain and does not promote tumor growth.

In tumor microenvironment, the relationship between the tumor cells, immune system, stromal cells and inflammatory mediators produced is complex and is the key for the disease outcome [55-56].Inflammatory cytokines and chemokines can be produced by the tumor cells and/or tumor-associated leucocytes and platelets, may contribute directly to malignant progression [57].The inflammatory response shares various molecular targets and signaling pathways with the carcinogenic process, such as apoptosis, increased proliferation rate, and angiogenesis. Furthermore, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and cytokine and chemokine antagonists have been shown to decrease incidence and mortality of several cancers[58-59].

In this sense, our results corroborate the importance of the tumor microenvironment and its potency, since the level of mRNA expression of cytokines involved in the inflammatory process in the paw of animals injected with Ehrlich tumor cells were not down-regulated after prolonged treatment with diosmin. Meanwhile, the treatment was able to inhibited the ETC-induced Il-1β, Il-33, St2 and Il-10 mRNA high expression in the spinal cord at the 12th-day post injection of ETC. Therefore, the analgesic effect promoted by this flavonoid may be by spinal modulation, not for peripheral pathways. This confirms the results of our histological analysis, in which no measured difference in paw of the animal untreated with the animal treated with diosmin.

Concluding, the present data demonstrate that diosmin presents analgesic effect in Ehrlich tumor cells-induced cancer pain inhibition mechanical hyperalgesia, thermal hyperalgesia and spontaneous pain due to mechanisms related to peripheral inhibition of MPO activity and oxidative stress, and in the spinal cord by reducing oxidative stress. Thus, this preclinical study demonstrated that the flavonoid diosmin could be a new conceivable pharmacological approach for the treatment of cancer pain. More studies are needed to determine in which other tumors the diosmin is effective, as well as its clinical applicability.

4. Material and methods

4.1. Animals

The Ethics Committee for Animal Research of the Universidade Estadual de Londrina (UEL) approved all experiments under the process number 8482.2014.86. Experiments were conducted using male Swiss mice (20-25 g) obtained from the Universidade Estadual de Londrina's animal facility that were housed at 22 ±1 ºC under a 12-h light/12-h dark cycle

(lights on at 06:00 a.m.) with access to food and water *ad libitum*. Mice were acclimatized to the laboratory for at least 1 h before testing and were used in only one experiment.

4.2. General Experimental Procedures

Ehrlich's tumor cells are cultivated in vivo, by passages in the peritoneum of Swiss mice in ascitic form. Ten days after the intraperitoneal (i.p.) injection of 0.2 mL of ascitic peritoneal fluid containing Ehrlich tumor cells in mice, the ascitic fluid of tumor cells was harvested and washed in phosphate-buffered saline (PBS, pH 7.4) followed by centrifugation (0,2 RCF, 10 min) three times. The cell viability was determined by 0.5% trypan blue exclusion method in Neubauer chamber. Ehrlich tumor cells were suspended to the final concentrations of 1×10^6 or 1×10^7 in 25 µL of PBS and injected into the subcutaneous tissue of mice, which passes from ascitic form to solid form [11]. The measurement of basal responses to mechanical and thermal stimuli and paw thickness was performed at day 0. Afterwards, mice received intraplantar (i.pl.) injection of Ehrlich tumor cells (1×10^6 or 1×10^7)received the acute treatment with diosmin (1,3,10, or30 mg/kg, i.p.) or vehicle (2% DMSO in saline) on the 8^{th} day after injection of the cells, and mechanical hyperalgesia, thermal hyperalgesia and paw thickness were determined after 1, 3, 5, and 7 h. For chronic treatment, mice were treated with diosmin (10 mg/kg, i.p) 10 min after 1×10^6 cells Ehrlich tumor cells injection followed by daily treatment for 12 days. Mechanical hyperalgesia, thermal hyperalgesia, and paw thickness were evaluated 5 h after treatment with diosmin on 2, 4, 6, 8, 10, and 12 days after the injection of 1×10^6 cells or saline. Also, on the 12^{th} day paw skin, spinal cord [MPO and NAG activity, ABTS radical scavengeassay, and GSH levels, histopathological analyses, expression of pro and anti-inflammatory cytokines], plasma [AST and ATL] and stomach [MPO activity] samples were harvested. In another set of experiments, mice were treated 8

days with diosmin (10 mg/kg, i.p.) or vehicle daily after i.pl. injection with 1×10^7 Ehrlich tumor cells or saline starting 10 min after Ehrlich tumor cells injection. On the 8^{th} day, 5 h after treatment, the overt pain-like behavior was assessed.

4.3. Electronic pressure meter test of mechanical hyperalgesia

Mechanical hyperalgesia was tested in mice as previously reported [60]. Briefly, the test consists of evoking a hind paw flexion reflex with a hand-held force transducer (electronic von Frey anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 contact area polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw, and the end point was characterized by the removal of the paw. The intensity of the hyperalgesia was quantified as the change in pressure applied by subtracting the mean of the 3 values obtained after the injection of tumor cells from the mean of the three values observed before injection of tumor cells.

4.4. Hot plate test

Mice were placed in the hot plate apparatus (EFF 361; Insight, Ribeirao Preto, SP, Brazil) maintained at 52 °C [61]. The first ipsilateral hind paw flexion reflex (nociceptive behavior) was registered. The response latency was recorded before and after Ehrlich tumor cells injection. The maximum latency (cut-off) was set at 20 s to avoid tissue damage.

4.5. Paw Thickness or Tumor Growth

Paw thickness was determined before and at indicated time points (at 48 h intervals) after the injection of Ehrlich tumor cells using an analog caliper. Paw thickness/tumor growth was presented as Δ mm [11].

4.6. Overt Pain-Like Behavior Evaluation

Mice received 1×10^7 cells/paw in 25 μL and were placed in clear glass compartments at room temperature. After an acclimation period of 10 min, mice were observed during 10 min, and the cumulative number of flinches was determined [11].

4.7. MPO and NAG activity assays

MPO and NAG kinetic-colorimetric assays were performed as indirect indicators of neutrophil and macrophage recruitment to paw tissue, respectively, and stomach tissue for only MPO activityThe samples were collected on the 12^{th} day after stimulus 5h after treatment with diosmin and homogenized in ice-cold K_2HPO_4 buffer (400 μL, 50 mM, pH 6.0) containing HTAB (0.5% weight/volume) using a tissue-tearor (Biospec, Bartlesville, OK, USA). Samples were centrifuged (16100 *g* x 2 min x 4 °C) and the supernatants were used for both assays. The MPO activity assay was performed as previously described [44]. Briefly, 50 mM phosphate buffer, pH 6.0 containing 0.167 mg/ mL o-dianisidine dihydrochloride and 0.015 % hydrogen were added to samples and the absorbance was read at 450nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The values obtained were compared to a standard curve of neutrophils and results are expressed as MPO activity (neutrophils x 10^6/mg of tissue). The NAG activity assay was performed as previously described [45]. Samples were diluted in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na2HPO4, pH 4.5) in a proportion of 5:1. p-nitrophenyl-N-acetyl-β-glucosaminide dissolved in citrate/phosphate buffer was added to samples followed by addition of 0.2 M glycine buffer (pH 10.6). The absorbance was read at 405nm (MultiskanGO, Thermo Scientific). The values obtained were compared to a standard curve of macrophages and results are expressed as NAG activity (macrophages x 10^6/mg of tissue).

4.8. GSH measurement

Paw skin and spinal cord (L4-L6) samples were harvested and maintained at -80°C for at least 48 h. Samples were 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 for 15 min. The mixture was centrifuged (15 min x 1500 g x 4°C). 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 nmol per mg paw or spinal cord [62].

4.9. ABTS radical scavenge assay

ABTS radical scavenging assaywas used to evaluate the tissue antioxidant capacity. Both tests were adapted to a 96-well microplate format as previously described [63]. Paw skin and spinal cord (L4-L6) samples were harvested 5 h after treatment with diosmin and homogenized with a tissue-tearor in ice-cold KCl buffer (500 µL, 1.15% w/v). Samples were centrifuged (835 *g*, 4°C, 10 min) and supernatants were used in both assays. ABTS radical scavenging assaywas performed as an indicator of the sample's ability to scavenge the free radical ABTS. Diluted ABTS solution was added to samples and the absorbance was measured at 730 nm after 6 min of incubation at 25 °C (Multiskan GO, Thermo Scientific). The results for both samples were equated against a standard Trolox curve (0.02-20 nmol) and expressed as nmol Trolox eq. per mg of tissue.

4.10. Hepatotoxicity

Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), was used as an indicator of hepatotoxicity [64]. These assays were performed using a diagnostic kit from Labtest (Lagoa Santa, Minas Gerais, Brazil) in samples harvested in the 12^{th} days after Ehrlich tumor cells injection.

4.11. Histopathological Analyses

Twelve days after the injection of the Ehrlich tumor cells, mice were euthanized and the paw was removed and decalcified in a solution (1:1) of Chloridric Acid (HCl 8% and Formic Acid (CH_2O_2) 8% during 48 hours. Samples were embedded in paraffin, sectioned into 5 μm section, and stained with hematoxylin and eosin for light microscopic analysis [11].

4.12. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR)

Paw skin and spinal cord (L4-L6) samples were collected at the 12^{th} day post-injection of Ehrlich tumor cells. Samples were homogenized in TRIzol® reagent (Life Technologies). Total mRNA was isolated according to manufacturer's directions. RNA purity was confirmed by the 260/280 ratio [65]. All reactions were performed following cycling conditions: 50° C for 2 min, 95°C for 2 min, followed by 60 cycles of 95° C for 15 s and 60° C for 30 s. RT-qPCR was performed in a LightCycler Nano Instrument (Roche, Mississauga, ON, USA) sequence detection system by using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, USA).Table 1 presents the primer sequences. The GAPDH mRNA expression was used as a reference gene to normalize data.

4.13. Statistical Analysis

Results are presented as means ± SEM of measurements made on six mice in each group per 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 administration or enforcement of the stimuli. The factors analyzed were treatment, time, and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's τ-test was performed on each occasion. Statistical differences were considered to be significant at $p < 0,05$.

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Interest conflicts

The authors declare no conflict of interest.

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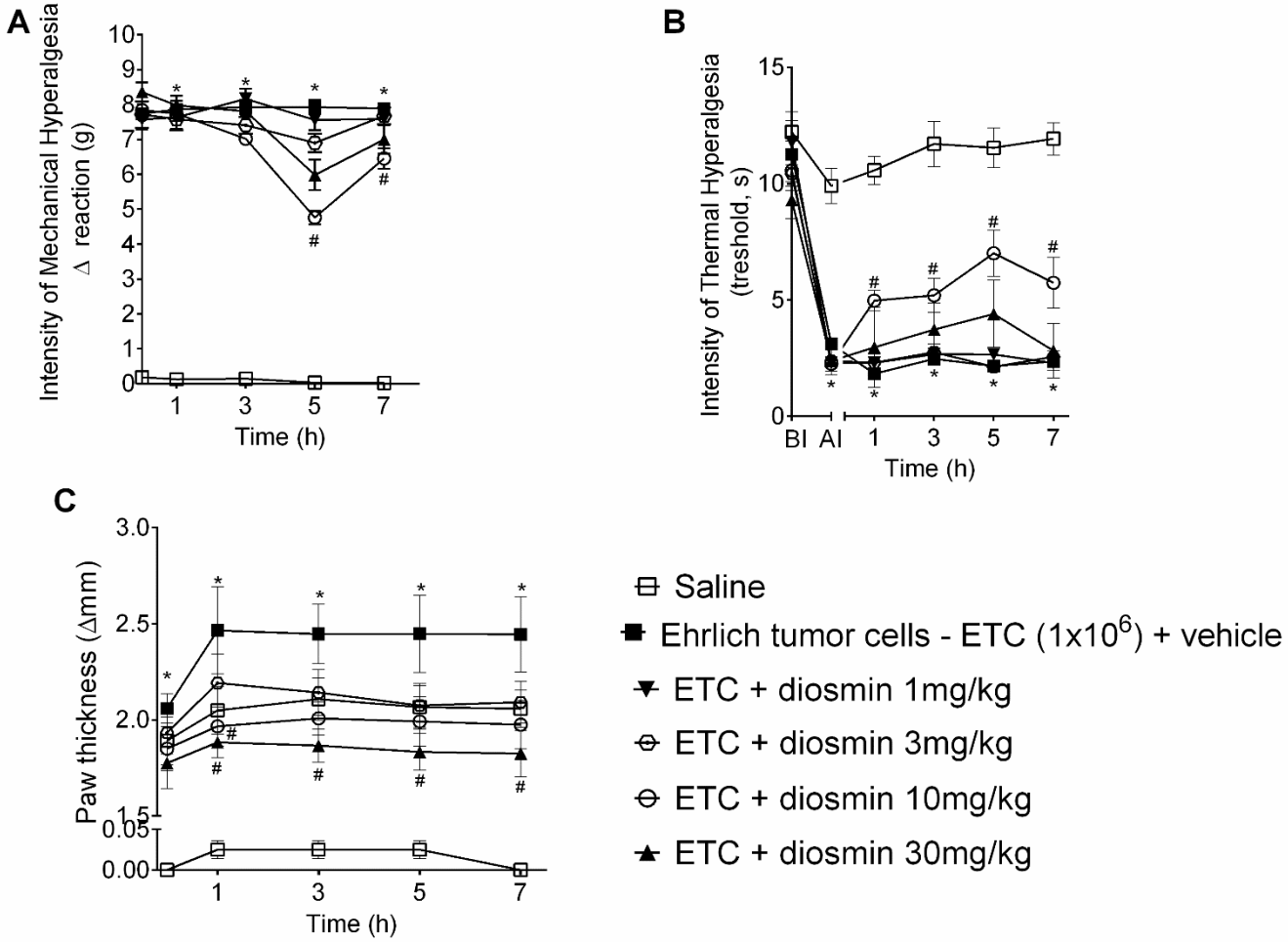


Figure 1: Diosmin inhibits Ehrlich-induced mechanical and thermal hyperalgesia and increased paw thickness on the 8th day. Mice were treated acutely with diosmin (1, 3, 10, 30 mg / kg, intraperitoneal [i.p.]) on the 8th day after intraplantar (i.pl.) injection with $1x10^6$ Ehrlich tumor cells. Mechanical hyperalgesia (Panel A), thermal hyperalgesia (Panel B), and paw thickness (Panel C) were evaluated 1, 3, 5 and 7 h after Ehrlich tumor cells stimulus. Results are presented mean ± SEM of 6 animals per group and experiment and are representative of 2 experiments. * P <0.05 compared to the group saline; #P <0.05 compared to the tumor group (Two-way ANOVA followed by Tukey's post-test).

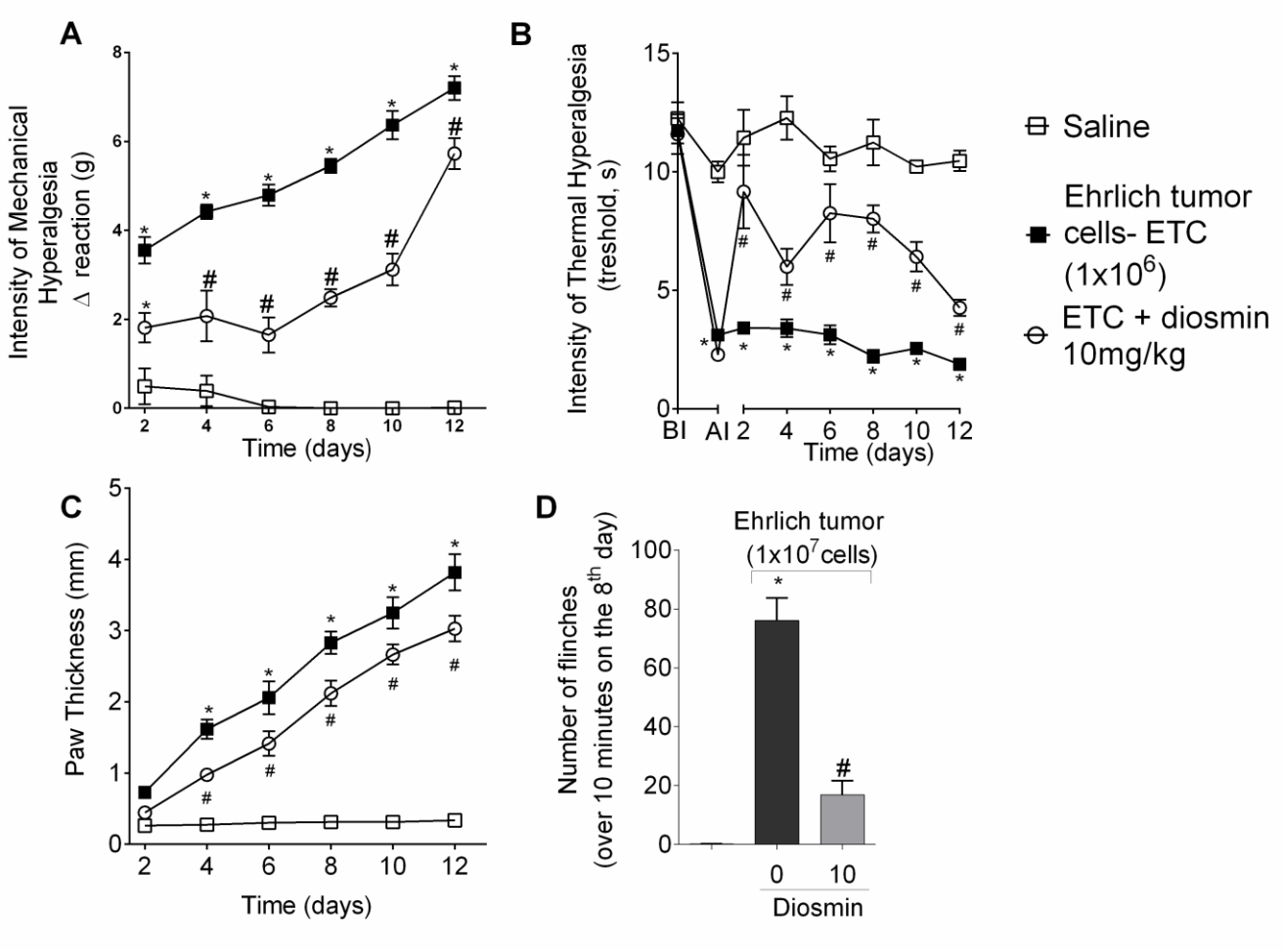


Figure 2: Prolonged treatment with diosmin inhibits mechanical and thermal hyperalgesia, growth thickness paw and paw flinches induced by Ehrlich tumor cells. Mice were treated with diosmin (10 mg/kg, ip) for 12 days or 8 days after i.pl. injection with Ehrlich tumor cells at $1x10^6$ or $1x10^7$ i.pl., respectively. Diosmin inhibited mechanical hyperalgesia (Panel A), thermal hyperalgesia (Panel B), and increased of paw thickness (Panel C) and number of paw flinches (Panel D). Results are presented mean ± SEM of 6 animals per group and experiment, and are representative of 2 experiments. * P <0.05 compared to the saline group; #P <0.05 compared to the tumor group (Two-way ANOVA followed by Tukey's post-test).

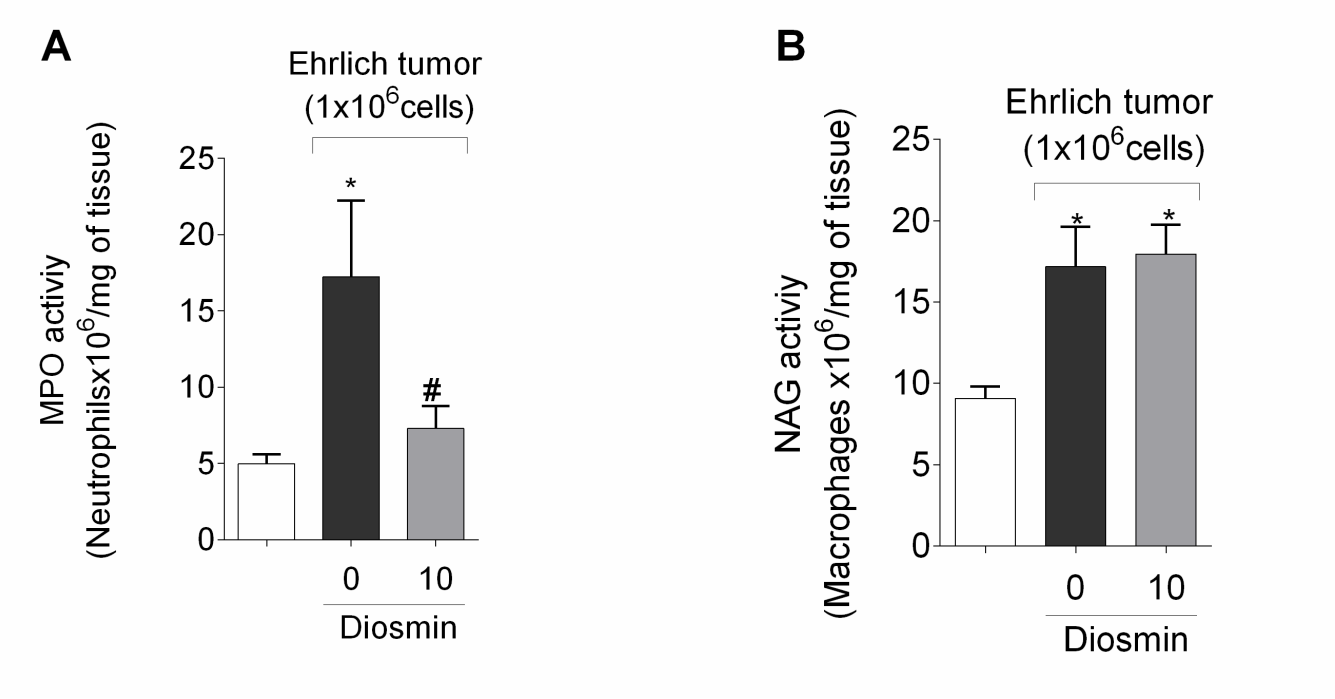


Figure 3: Diosmin inhibits Myeloperoxidase activity and do not alter the N-acetylglucosaminidase activity induced by Ehrlich tumor cells. Mice were treated with diosmin (10 mg/kg, i.p.) for 12 days after Ehrlich tumor cells injection ($1x10^6$, i.pl.). Diosmin inhibited the activity of the MPO enzyme (Panel A) and had no effect on NAG (Panel B). Results are presented as mean ± SEM of 6 animals per group and experiment and are representative of 2 experiments. * $P < 0.05$ compared to the saline group; #$P < 0.05$ compared to the tumor group (One-way ANOVA followed by Tukey's post-test).

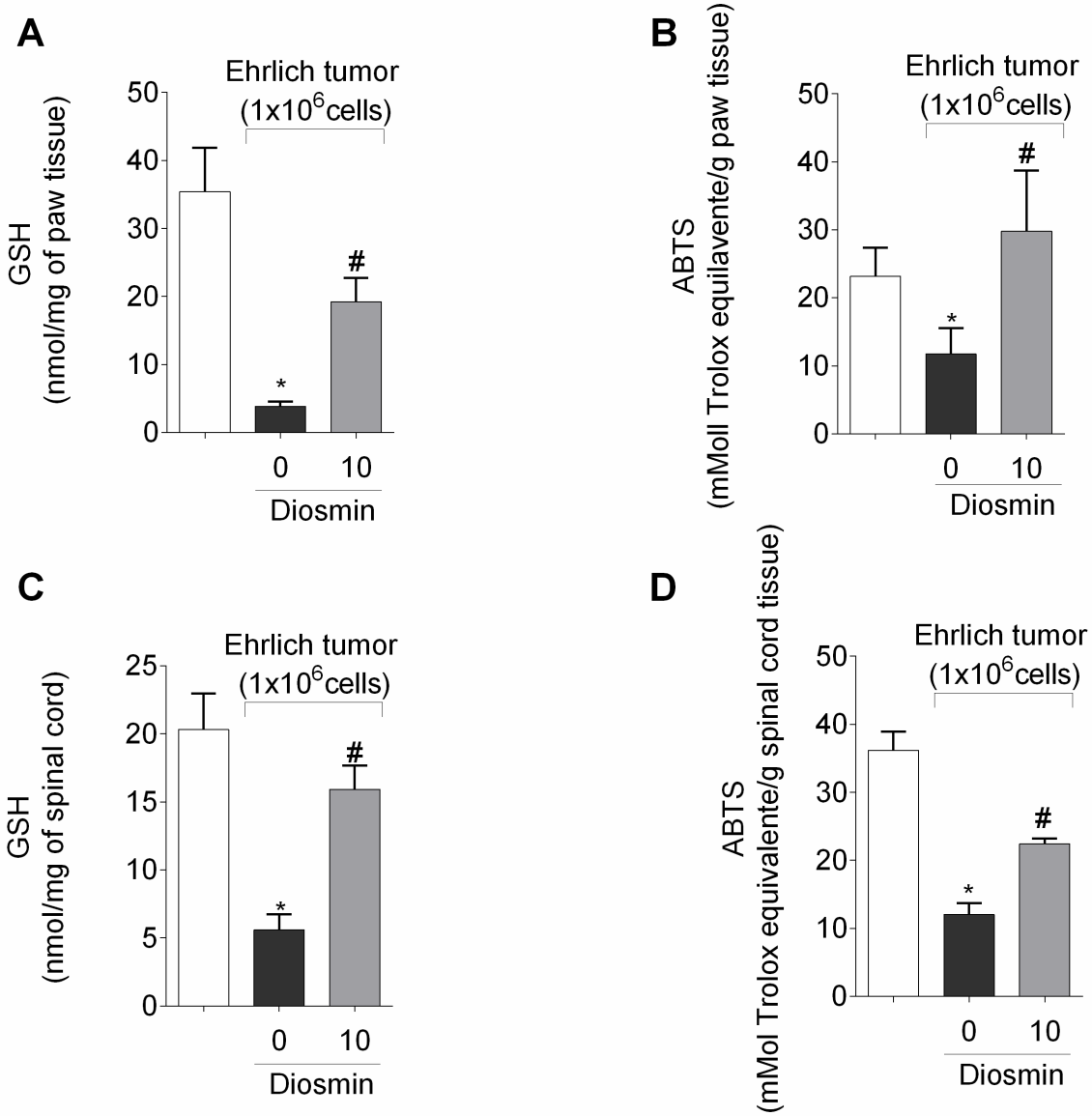


Figure 4: Diosmin inhibits the oxidative stress induced by Ehrlich tumor cells. Mice were treated with diosmin (10 mg/kg, ip) for 12 days after Ehrlich tumor cells injection ($1x10^6$, ipl). Diosmin inhibited GSH depletion in paw (Panel A) and spinal cord (Panel C) and restored ABTS radical scavenging ability in paw (Panel B) and spinal cord (Panel D). Results are presented mean ± SEM of 6 animals per group and experiment and are representative of 2 experiments. * P <0.05 compared to the saline group; #P <0.05 compared to the tumor group (One-way ANOVA followed by Tukey's post-test).

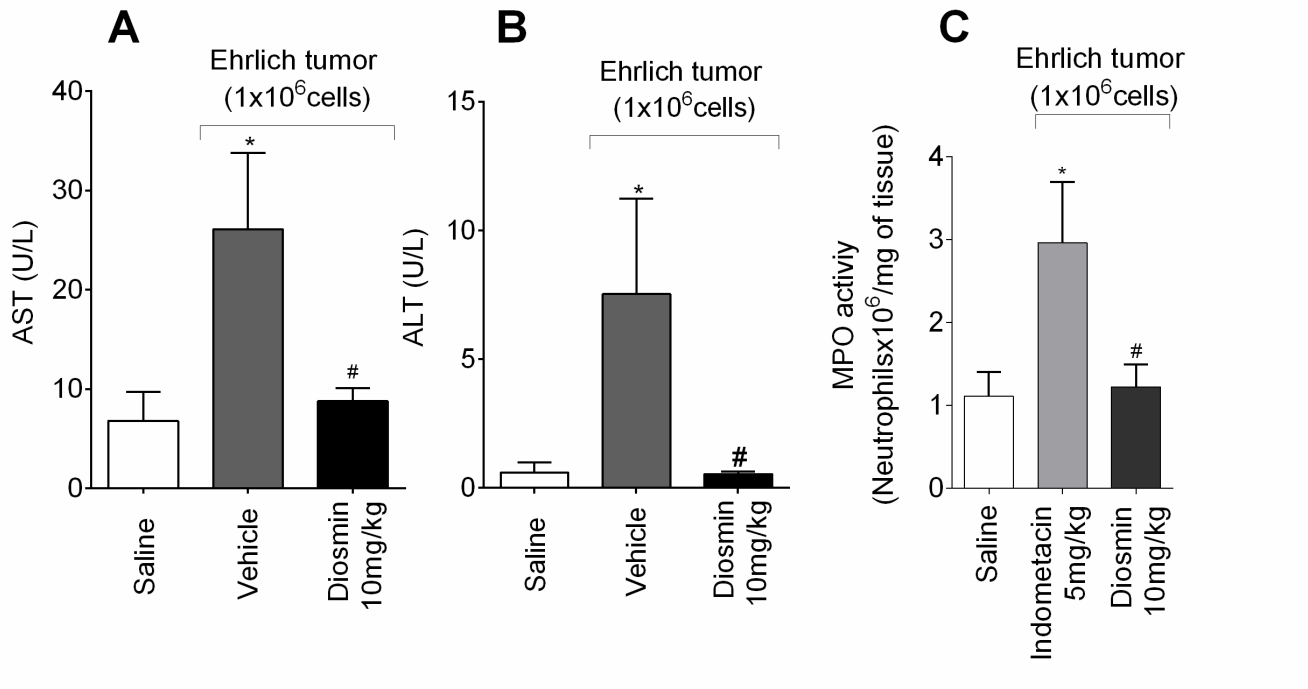


Figure 5: Diosmin exhibits a protective effect on hepatotoxicity or gastric damage induced by Ehrlich tumor cells. Mice were treated with diosmin (10 mg/kg, ip.) for 12 days after Ehrlich tumor cells injection ($1x10^6$, i.pl.) did not alter the amount of AST (Panel A) and ALT (Panel B) enzymes and had no gastric side effect (Panel C). Results are presented mean ± SEM of 6 animals per group and experiment and are representative of 2 experiments. * P <0.05 compared to the saline group; #P <0.05 compared to the indometacin group (One-way ANOVA followed by Tukey's post-test).

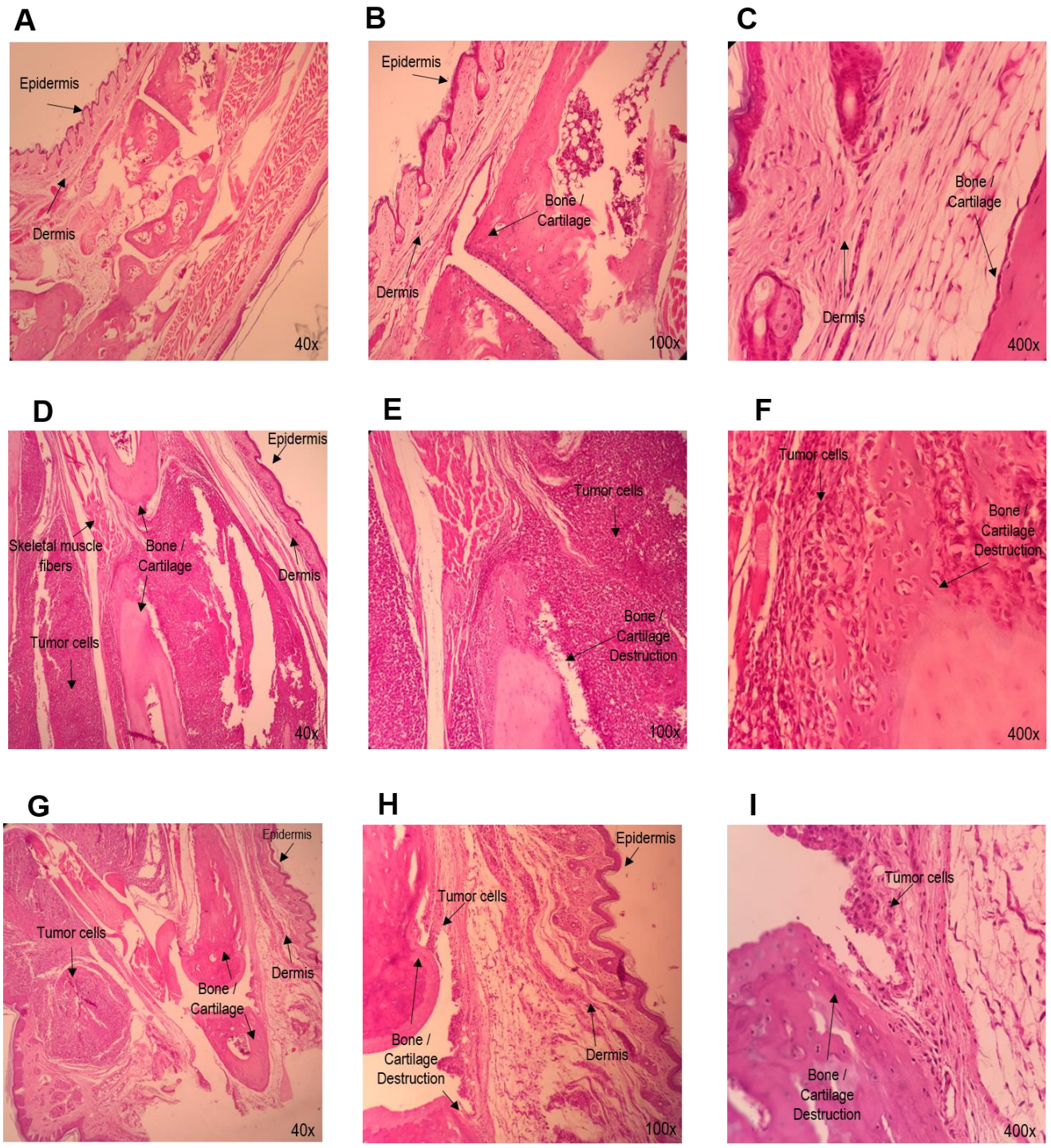
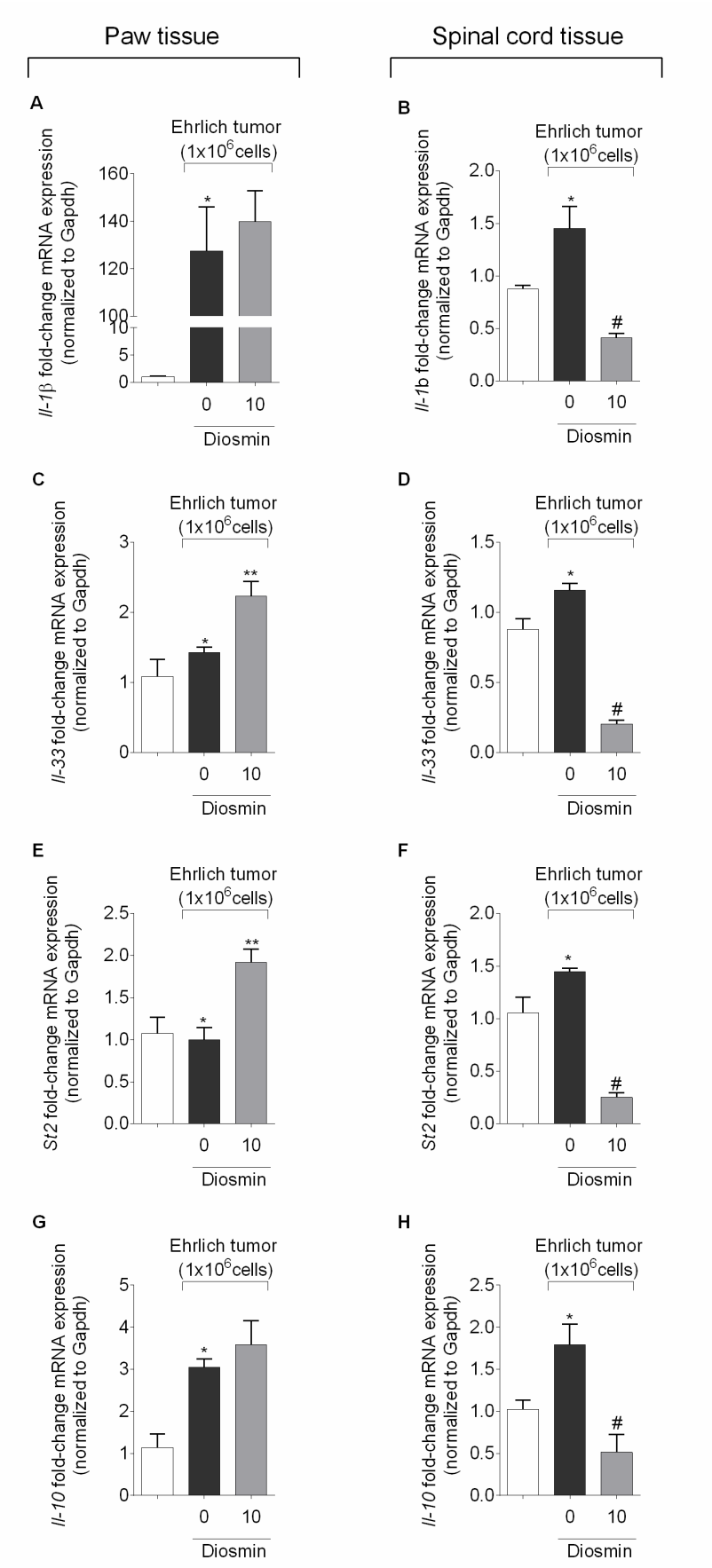


Figure 6: Diosmin does not alter Ehrlich tumor-induced histological modifications. Treatment with diosmin (10 mg/kg, i.p.) was performed for 12 days after i.pl. injection of $1x10^6$ tumor cells. In the 12th day, mice were euthanized and the paw was collected. All sections were stained with Hematoxylin and Eosin (magnification 40x, 100x and 400x) and the figure is representative of all experiments. Panel (A, B, C) shows the histology of saline

i.pl. group, (D, E, F) tumor animal treated with vehicle, and (G, H, I) tumor animal treated with diosmin (10 mg/kg i.p.). Arrows indicate intact bone cartilage, presence of skeletal muscle fibers, dermis and epidermis bone/cartilage destruction, and presence of tumor cells.


Paw tissue
Spinal cord tissue
A
B
C
D
E
F
G
H
Ehrlich tumor
(1x10^6cells)
Il-1β fold-change mRNA expression
(normalized to Gapdh)
Il-1b fold-change mRNA expression
(normalized to Gapdh)
Il-33 fold-change mRNA expression
(normalized to Gapdh)
St2 fold-change mRNA expression
(normalized to Gapdh)
Il-10 fold-change mRNA expression
(normalized to Gapdh)
0
10
Diosmin

1

Figure 7. Prolonged treatment with diosmin inhibits Ehrlich tumor cells-induced spinal cord up-regulation of pro-Il-1β, Il-33, St2 receptor and Il-10 mRNA expression. Mice that received the injection of $1x10^6$ tumor cells have the paw tissue and spinal cord was collected after 12 days of daily treatment with diosmin (10 mg/kg, i.p.) for determining the expression pro-Il-1β (Fig. 7A and B), Il-33 (Fig. 7C and D), St2 (Fig. 7E and F) and Il-10 (Fig. 7G and H)mRNA expression by RT-qPCR. Results are expressed as mean ± SEM, n=6 mice per group per experiment. *$p < 0.05$ compared to saline group; # $p < 0.05$ compared with tumor group. One-way ANOVA followed by Tukey's post-test.

Table l: Primer sequences for RT-qPCR

Gene	Sense	Antisense
Gapdh	5’ CAT ACC AGG AAA TGA GCT TG 3’	5’ ATG ACA TCA AGA AGG TGG TG 3’
Pro-IL-1β	5’ GAAATGCCACCTTTTGACAGTG 3’	5’ TGGATGCTCTCATCAGGACAG 3’
Tnf-α	5’ TCTCATCAGTTCTATGGCCC 3’	5’GGGAGTAGACAAGGTACAAC 3’
Il-33	5’ TCCTTGCTTGGCAGTATCCA 3’	5’ TGCTCAATGTGTCAACAGACG 3’
St2	5’ GCAATTCTGACACTTCCCATG 3’	5’ ACGATTTACTGCCCTCCGTA 3’
Il-10	5’ GCTGGACAACATACTGCTAAC 3’	5’ CTGGGGCATCACTTCTACCA3’

4 ARTIGO PUBLICADO (CHEMICO-BIOLOGICAL INTERACTIONS)

O presente trabalho foi realizado no Laboratório de Dor, Inflamação Neuropatia E Câncer, da Universidade Estadual de Londrina e publicado na revista Chemico-biological Interactions. O presente artigo intitula-se “Diosmin reduces chronic constriction injury-induced neuropathic pain in mice”. (PMID: 28625489).

Diosmin reduces chronic constriction injury-induced neuropathic pain in mice

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ABSTRACT

Injury or dysfunction of somatosensory system induces a complex syndrome called neuropathic pain, which still needs adequate pharmacological control. The current pharmacological treatments were in part developed from natural compounds. Flavonoids are natural polyphenolic molecules presenting varied biological activities and low toxicity. The flavonoid diosmin is a safe compound with good tolerability and low toxicity. This study evaluated the antinociceptive effect of diosmin in the sciatic nerve chronic constriction injury (CCI)-induced neuropathic pain model. Male Swiss mice were submitted to CCI and 7 days after, diosmin at 1 or 10 mg/kg was administrated intraperitoneally. Mechanical (electronic analgesimeter) and thermal (hot plate) hyperalgesia were evaluated 1-24 h after treatment. The role of the NO/cGMP/PKG/KATP channel signaling pathway in the analgesic effect of diosmin was evaluated using the pretreatment with L-NAME (an inhibitor of NOS), ODQ (an inhibitor of soluble guanylate cyclase), KT5823 (an inhibitor of PKG), or glibenclamide (an ATP-sensitive K+ channels blocker). Single treatment with diosmin inhibited in a dose-dependent manner CCI-induced mechanical and thermal hyperalgesia by activating the NO/cGMP/PKG/KATP channel signaling pathway and inhibiting spinal cord cytokine (*Il-1β* and *Il-33/St2*) and glial cells activation (microglia - *Iba-1*, oligodendrocytes – *Olig2*) mRNA expression markers. Daily treatment during 7 days with diosmin inhibited CCI-induced mechanical and thermal hyperalgesia by inhibiting spinal cord cytokine (*Il-1β, Tnfα,* and *Il-33/St2*) and glial cells activation (astrocytes – *Gfap, Iba-1*, and *Olig2*) markers mRNA expression. In conclusion, diosmin inhibits neuropathic spinal cord nociceptive mechanisms suggesting this flavonoid as a potential therapeutic molecule to reduce nerve lesion-induced neuropathic pain.

Key words: Neuropathy; hyperalgesia; oxidative stress; flavonoids.

1. Introduction

The perception of pain was conserved during evolution. A reasonable explanation is that pain perception is essential to self-preservation, identification of dangerous situations and controlling the consequences of an injury that has already occurred. However, tissue damage, chronic inflammation, and neuropathology can induce maladaptive nociceptive neuron plasticity causing pathological pain. To this end, there are changes in basic events of nociception associated to physical, emotional, psychological, and social dysfunctions. As a result, pain becomes the disease itself and loses the protective character necessary to maintain body integrity [1].

Neuropathic pain is caused by nociceptor neuron maladaptation due to injury or dysfunction of somatosensory system. Hyperalgesia, allodynia, and overt pain characterize chronic neuropathic pain conditions [2,3]. For instance, peripheral nerve injury induces peripheral inflammation and primary sensory neurons sensitization and activation as well as activation of second order nociceptive neurons in the spinal cord. Moreover, peripheral nerve injury induces the activation of spinal cord glial cells including astrocytes, microglia, and oligodendrocytes [4–6]. In fact, the spinal cord regulates the peripheral input to the central nervous system. Once activated, glial cells release pro-inflammatory mediators that activate and sensitize nociceptive neurons in the spinal cord, enhancing nociceptive neurotransmission. Glial cells release cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-33, which induce nociceptors sensitization and activation by actions on nociceptors or glial cells [6,7]. This complex interaction between neuronal and non-neuronal cells increases the responsiveness of spinal cord nociceptive neurons (central sensitization), decreases the activation threshold of nociceptive neurons (peripheral sensitization) and induces spontaneous firing of nociceptors [8–10].

The commonly used drugs in clinical practice to the treatment of neuropathic pain are the opioid analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), tricyclic antidepressants (TCAs), and anticonvulsants. However, the existing therapies present various adverse effects, and more importantly, present limited efficiency in controlling neuropathic pain [11,12]. In this sense, novel therapies are necessary. Molecules derived from natural sources have been important to the development of the current therapeutic approaches, which includes the NSAIDsand analgesic prototype acetyl salicylic acid as well as the opioid morphine [13,14]. Phenolic compounds grouped as flavonoids have been a focus as potential analgesics [15]. Flavonoids inhibit pain in inflammatory, cancer, and neuropathic conditions [3,16,17]. Diosmin is a semi-synthetic flavonoid derived from hesperidin [18,19]. Diosmin is under clinical use as a vascular-protecting agent and has been used safely for the treatment of chronic venous insufficiency, hemorrhoids, varicose veins, venous stasis in the legs leading to edema, and hemorrhage [20–23]. Clinical trials have been using doses of diosmin between 500-2,000 mg daily for up to one year. Therefore, clinical evidence suggests that diosmin is a safe and well-tolerated drug [21,22,24]. Thus, diosmin is already in clinical use, and considering it belongs to the flavonoid group of molecules, we envisage its potential application as an analgesic.

Diosmin inhibits alloxan-induced diabetic neuropathy by modulating nuclear transcription factor kappa B (NF-kB)-dependent signaling pathways resulting in reduced oxidative stress makers [25]. Diosmin reduced renal damage markers, kidneys edema, proteinuria, and creatinine, uric acid and urea serum levels in urolithiasis [26]. In lipopolysaccharide-induced acute lung inflammation model; diosmin decreased the recruitment of neutrophils, monocytes, lymphocytes and total leukocytes. Hence, diosmin down-regulated

the expression of T cell markers CD4+ and CD8+, and the expression of IL-1β and TNF-α, and NF-kB activation [27].

Recently, it has been shown that diosmin improves the anti-hyperalgesic effect of hesperidin in chronic constriction injury (CCI)-induced mechanical and thermal hyperalgesia in rats using the dynamic plantar analgesimeter and Hargreaves apparatus, respectively [28]. Given that this study was designed to determine whether diosmin potentiates the anti-hyperalgesic effect of hesperidin, the selected regimen of treatment demonstrated the anti-hyperalgesic effect of diosmin without addressing its analgesic mechanisms [28]. The present study was designed to determine whether diosmin presents analgesic effect in CCI-induced neuropathic pain in mice and its spinal cord mechanisms.

2. Material and methods

2.1. Animals

The Ethics Committee for Animal Research of the Universidade Estadual de Londrina (UEL) approved all experiments under the process number 28995.2014.39. Experiments were conducted using male Swiss mice (20-25 g) obtained from the Universidade Estadual de Londrina's animal facility that were housed at 22 ± 1 ºC under a 12-h light/12-h dark cycle (lights on at 06:00 a.m.) with access to food and water *ad libitum.* Mice were acclimatized to the laboratory for at least 1 h before testing and were used in only one experiment.

2.2. Chronic constriction injury (CCI) of the sciatic nerve

Mice were anesthetized and maintained with volatile anesthetic isoflurane (3% in O_2). Surgical procedures were performed as previously described [29] with modifications [6]. Briefly, the distal portion of the sciatic nerve was tied with surgical thread (catgut 4-0). In sham-operated

mice, the sciatic nerve was exposed without ligation. The wound was closed and covered with iodine solution.

2.3. Electronic pressure meter test of mechanical hyperalgesia

Mechanical hyperalgesia was tested in mice as previously reported [30]. Briefly, the test consists of evoking a hind paw flexionreflex with a hand-held force transducer (electronic von Frey analgesimeter; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 contact area polypropylene tip. The investigator was trainedto apply the tip perpendicularly to the central area of the hind paw, and the end point was characterized by the removalof the paw. The intensity of the hyperalgesia was quantified as the change in pressure applied by subtracting the mean of the three values obtained after the surgery from the mean of the three values observed before surgery.

2.4. Hot plate test

Mice were placed in the hot plate apparatus (EFF 361; Insight, Ribeirao Preto, SP, Brazil) maintained at 52 °C [31]. The first ipsilateral hind paw flexion reflex was considered the nociceptive endpoint. The response latency was recorded before the surgery and at 7^{th} (single treatment) or at the 7^{th} to 14^{th} days post-surgery (prolonged treatment). The maximum latency (cut-off) was set at 20 seconds to avoid tissue damage [32].

2.5.Myeloperoxidase activity (MPO)

The neutrophil migration to stomach tissue was indirectly evaluated by the MPO activity colorimetric assay [33]. Briefly, samples were collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and were homogenized using

Ultra-Turrax® (IKA T10 Basic, CQA Química, Paulínea, SP). Then the homogenates were centrifuged at 16,100 *g* for 2 minutes at 4°C. Fifteen μL of the resulting supernatant was mixed with 200 μL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.015% hydrogen peroxide and was assayed spectrophotometrically for MPO activity determination at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The results of MPO activity were expressed as the number of neutrophils per g of tissue by using a standard curve of neutrophils. Neutrophils for the standard curve were from the peritoneal cavity of Swiss mice, 6 hours after i.p. stimulus with thioglycolate broth (1 mL, 5%, Becton Dickinson, MD, USA). The number of neutrophils was determined by total counts in Neubauer chamber and differential counts in slides stained by Rosenfelt method. We obtained 96% of neutrophils in a pool of 10 mice. Neutrophils for the standard curve were suspended in K_2HPO_4 buffer containing HTAB and stored at -80°C until use.

2.6. Nephrotoxicity

Serum levels of urea and creatinine were used as indicators of nephrotoxicity [34]. These assays were performed using a diagnostic kit from Labtest (Lagoa Santa, Minas Gerais, Brazil).

2.7. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR)

Spinal cord (L4-L6) samples were collected at the 7th or at the 14th day post-surgery. Samples were homogenized in TRIzol® reagent (Life Technologies). Total mRNA was isolated according to manufacturer's directions. RNA purity was confirmed by the 260/280 ratio [35]. RT-PCR and quantitative PCR were performed using GoTaq® 2-Step RT-qPCR System (Promega) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). Table 1

presents the primer sequences. The *β-actin* mRNA expression was used as a reference gene to normalize data [36].

2.8. Experimental protocols

Mice were kept under the standard conditions described above (item 2.1.), being daily controlled, and their general condition of nourishment, cleanliness, and locomotion were evaluated. For determination of the best analgesic dose of diosmin, mice received intraperitoneal (i.p.) treatment with diosmin at the dose of 1 or 10 mg/kg (diluted in 2% dimethyl sulfoxide [DMSO] in saline) at the 7th day post-surgery. To evaluate the involvement of the nitric oxide/ cyclic guanosine monophosphate/ protein kinase G/ ATP-sensitive potassium channels (NO/cGMP/PKG/K^+ATP) signaling pathway in the diosmin antinociceptive effect, mice received the administration of one of the following inhibitors before diosmin treatment (10 mg/kg): L-NAME (90 mg/kg, i.p.), ODQ (0.3 mg/kg, i.p.), KT5823 (0.5 μg/mouse, i.p.) or glibenclamide (0.3 mg/kg, i.p.). These inhibitors at these doses were selected in previous studies of our research group and do not present an effect *per se*[37]. The mechanical and thermal hyperalgesia were measured 1 to 7 hours after diosmin treatment. Spinal cord (L4-L6) was collected at the 7th day post-surgery (single treatment with diosmin) or at the 14th day post-surgery (daily treatment with diosmin starting at 7th and up to the 14th day post-surgery) for determination of urea and creatinine serum levels, myeloperoxidase activity in the stomach, and spinal cord hyperalgesic mediators (*IL-1β,Tnfα, Il-33*, and *St2*) and glial cell activation (glial fibrillary acidic protein [*Gfap*], ionized calcium-binding adapter molecule 1 [*Iba-1*], and oligodendrocyte transcription factor [*Olig2*]) by RT-qPCR.

2.9. Statistical analysis

Results are presented as mean ± standard error of mean (SEM) of measurements made on six mice in each group per experiment and are representative of two separate experiments. Two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's *post hoc* was used to compare all groups and doses at all times when responses were measured at different times after the stimulus injection. Differences between responses at single time point were evaluated by one-way ANOVA followed by Tukey's *post hoc*. Statistical differences were considered significant when $P \leq 0.05$.

3. Results

3.1. Single treatment with diosmin reduces CCI-induced mechanical and thermal hyperalgesia.

In the first series of experiment, the analgesic effect of single diosmin treatment was evaluated. Mice were treated with diosmin (1 or 10 mg/kg, ip) once at the 7th-day post-surgery. Diosmin at 1 mg/kg did not modify CCI-induced mechanical hyperalgesia while the dose of 10 mg/kg significantly decreased CCI-induced mechanical hyperalgesia (Fig. 1A). The treatment with diosmin (10 mg/kg, i.p.) also reduced the CCI-induced thermal hyperalgesia (Fig. 1B). Therefore, the dose of diosmin of 10 mg/kg was selected for the following experiments.

3.2. Prolonged treatment with diosmin reduces CCI-induced mechanical and thermal hyperalgesia without causing gastric lesion or renal damage.

After that, the analgesic effect of diosmin was evaluated in a prolonged treatment regimen. Mice were treated with diosmin (10 mg/kg, ip) between the 7th and 14th day post-surgery. The prolonged treatment with diosmin promoted a sustained reduction of mechanical (Fig. 2A) and thermal hyperalgesia (Fig. 2B) induced by CCI. Importantly, diosmin did not induce an increase myeloperoxidase in gastric tissue (Fig. 2C), indicating absence of gastric lesion, or increase in the serum levels of urea (Fig. 2D) and creatinine (Fig. 2E), indicating absence of

renal damage. Therefore, the prolonged treatment with diosmin did not induce the commonly adverse effects observed with conventional non-steroidal anti-inflammatory drugs (VALERIO *et al.,* 2007).

3.3. Diosmin reduces CCI-induced mechanical and thermal hyperalgesia by activating the NO/cGMP/PKG/ATP-sensitive potassium channels signaling pathway.

In order to investigate the activation of the NO/cGMP/PKG/ATP-sensitive potassium channels signaling pathway, mice were pretreated with one of the different inhibitors of nitric oxide signaling (NO) pathway separately prior to the treatment with diosmin at the 7th day post-surgery: a) L-NAME (90 mg/kg, i.p., nitric oxide synthase [NOS] inhibitor), 1 hour before treatment with diosmin; b) ODQ (0.3 mg/kg, s.c., soluble guanylate cyclase inhibitor), 30 minutes before treatment with diosmin; c) KT5823 [0.5 µg/animal, i.p., protein kinase G (PKG) inhibitor], 5 minutes before treatment with diosmin; or d) glibenclamide (GLY) (0.3 mg/kg, i.p., ATP-sensitive potassium channels inhibitor), 45 minutes before treatment with diosmin (Fig. 3). Mechanical and thermal hyperalgesia were evaluated 0 to 7 h after diosmin treatment. The pretreatment with L-NAME (Fig. 3A and 3B), ODQ (Fig. 3C and 3D), KT5823 (Fig. 3E and 3F), or GLY (Fig. 3G and 3H) diminished diosmin inhibition of CCI-induced mechanical and thermal hyperalgesia, respectively.

3.4. Effect of single and prolonged treatment with diosmin in CCI-induced spinal cord up-regulation of pro-*Il-1β, Tnf-α, Il-33, and St2 receptor mRNA expression.*

Mice received diosmin (10 mg/kg, ip) single treatment at 7th-day post-surgery or daily treatment from the 7th to the 14th-day post-surgery. Spinal cord samples were collected 5 h after the last treatment with diosmin. CCI induced a significant increase of *pro-Il-1β*, *Tnf-α*, *Il-33*,

and *St2* (IL-33 receptor) mRNA expression in the spinal cord (L4-L6) at the 7th and 14th days after surgery compared with the sham group (Fig. 4). A single treatment with diosmin at the 7th day post-CCI surgery reduced the *pro-Il-1β* (Fig. 4A), *Il-33* (Fig. 4C), and *St2* (Fig. 4D) mRNA expression without affecting the *Tnf-α* mRNA expression (Fig. 4B). The prolonged treatment with diosmin reduced the CCI-induced increase of *pro-Il-1β* (Fig. 4A), *Tnf-α* (Fig. 4B), *Il-33* (Fig. 4C), and *St2* (Fig. 4D) mRNA expression in the spinal cord.

3.5. Diosmin reduces CCI-induced spinal cord glial cells activation.

Mice received diosmin (10 mg/kg, ip) single treatment at the 7th-day post-surgery or prolonged daily treatment starting at the 7th and up to the 14th-day post-surgery. Spinal cord samples were collected 5 h after the last treatment with diosmin. CCI induced a significant increase in mRNA expression of the spinal cord (L4-L6) glial cells activation markers at the 7th and 14th days post-surgery compared to the sham group (Fig. 5). A single treatment with diosmin at the 7th day post-CCI did not reduce CCI-induced *Gfap* mRNA expression (Fig. 5A), but significantly reduced *Iba-1* (Fig. 5B) and *Olig2* mRNA expression (Fig. 5C). These results indicate that single treatment with diosmin reduces CCI-induced microglia (*Iba-1*) and oligodendrocytes (*Olig2*) activation in the spinal cord. In the prolonged treatment protocol, diosmin reduced the CCI-induced increase of *Gfap, Iba-1*,and *Olig2* mRNA expression (Fig. 5A, B, and C, respectively). Therefore, prolonged treatment with diosmin reduced CCI-induced spinal cord activation of astrocytes, microglia, and oligodendrocytes, respectively.

4. Discussion and conclusions

Diosmin is a flavonoid under clinical use for the treatment of diseases affecting the circulatory system [38]. Recent evidence demonstrated that hesperidin reduces CCI-induced

mechanical and thermal hyperalgesia in rats and that this analgesic effect is improved by diosmin co-treatment [28]. However, the antinociceptive mechanisms of diosmin remained to be investigated. The present data corroborates that diosmin inhibits CCI-induced mechanical and thermal hyperalgesia, however, in the present data a mouse model of neuropathic pain was used instead of rats. Importantly, diosmin inhibited CCI-induced hyperalgesia by activating the analgesic pathway NO/cGMP/PKG/ATP-sensitive potassium channels signaling as well as by inhibiting the spinal cord mRNA expression of hyperalgesic cytokines and glial cells activation markers.

The first round of experiments demonstrated that diosmin inhibited in a dose-dependent manner the CCI-induced mechanical hyperalgesia and thermal hyperalgesia. The present data shows analgesic effect of diosmin at the dose of 10 mg/kg whilst previous data observed significant analgesia only at the dose of 100 mg/kg [28]. Differences in species (mouse versus rat), model (one nerve ligations versus three nerve ligations) and time-points of evaluation (7 and 14 days versus 15 days post-surgery) might have influenced the results (present data; [28], respectively). Nevertheless, the analgesic effect of diosmin is consistent in the CCI model in mice (present data) and rats [28]. The present data also shows that at the prolonged treatment regimen, diosmin did not induce gastric lesions or renal damage, further corroborating the safety of this molecule in pharmacologically active doses. In fact, diosmin presents a safe profile in its current clinical use [39–41].

Evidence demonstrates that the activation of the NO/cGMP/PKG/ATP-sensitive potassium channels signaling pathway inhibits mechanical and thermal hyperalgesia in inflammatory and neuropathic pain models [37,42,43]. The opening of ATP-sensitive potassium channels inhibits neuronal depolarization and consequently the neurotransmission of the nociceptive information [44]. It is noteworthy that this signaling pathway accounts to the

analgesic mechanisms of drugs that have been used in clinical settings such as morphine [45], dipyrone [46], and diclofenac [47]. This clinical evidence supports that the activation of NO/cGMP/PKG/ATP-sensitive potassium channels signaling pathway is an important analgesic mechanism. Furthermore, bypassing the opioid receptor using nitric oxide and nitroxyl donors also activates this pathway starting by soluble guanylate cyclase activation generating cGMP [42,48]. Corroborating the present data, other flavonoids also inhibit hyperalgesia in inflammatory [43], neuropathic [3], and cancer [17] models by activating the NO/cGMP/PKG/ATP-sensitive potassium channels signaling pathway [49,50]. Thus, this is a common analgesic mechanism of flavonoids.

Neuropathic pain arises from damaging the peripheral and central nervous system [51]. The peripheral nerve injury induces a local inflammatory response with the participation of neutrophils, macrophages, mast cells, and T cells. These immune cells communicate with nociceptor neurons by means of nociceptive molecules including cytokines and other hyperalgesic molecules that cause nociceptor neuron sensitization and activation [9,10,52]. The peripheral activation of nociceptor neurons induces their depolarization and communications with second order neurons in the spinal cord as well as the release of the chemokine CX3CL1, which activates glial cells [53,54]. Glial cells such as astrocytes, microglia, and oligodendrocytes communicate with each other and with nociceptor neurons further inducing nociceptor neuron sensitization and activation [7,55]. For instance, spinal cord oligodendrocytes are activated in CCI-induced neuropathic pain and release IL-33, which via the ST2 receptor induces astrocyte and microglia activation in the spinal cord. IL-33/ST2 signaling activates intracellular signaling pathways such as phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin (mTOR), mitogen-activated protein kinases (MAPK), and nuclear factor κB (NFκB), which contribute to the production of IL-1β and TNFα in the

spinal cord [6]. The hyperalgesic cytokines IL-1β and TNFα induce central sensitization and hyperalgesia by increasing excitatory synaptic transmission, decreasing inhibitory synaptic transmission, inducing long-term synaptic plasticity and reducing nociceptor neuron hyperpolarization [52,56–58]. There is also evidence that TNFα and IL-1β-induced enhancement of synaptic transmission occurs indirectly by activation of glial cell cytokine receptors [59]. Given that nociceptor sensory neurons express TNFα [60] and IL-1β [61] receptors, these cytokines can activate these specialized cells. Recent evidence has demonstrated that dorsal root ganglia (DRG) sensory neurons express ST2 receptor, and therefore, can be activated by IL-33 as well [62]. Thus, consistent evidence supports that inhibiting the production or action of hyperalgesic cytokines, and glial cells activation in the spinal cord represent promising therapeutic approaches to reduce neuropathic pain. Importantly, at the 7th day post-CCI, apoptotic sensory neurons are increased in the L4 and L5 segments from the ipsilateral dorsal horn [63]. The apoptosis of these spinal cord sensory neurons and inhibitory interneurons decreases spinal inhibitory mechanisms (analgesic mechanism) contributing to neuropathic pain [64]. Interestingly, diosmin reduces lipopolysaccharide (LPS)-induced TNF-α production and apoptosis in PC12 cells (neuronal lineage cells) [65]. The latter mechanism accounts to the inhibition of neuronal DNA fragmentation, reduction of caspase-3 expression, and increase of the antiapoptotic protein Bcl-2, which suggest that diosmin confers neuronal protection [65].

In the present study, CCI induced an increase of *Il-1β, Tnfα, Il-33,* and *St2* mRNA expression at the 7th and 14th-day post-surgery. At these time points, CCI also increased the mRNA expression of markers of astrocytes (*Gfap*), microglia (*Iba-1*), and oligodendrocytes (*Olig2*) activation as previously observed [37]. Diosmin inhibited the CCI-induced *Il-1β, Tnfα, Il-33, St2Iba-1*, and *Olig2* mRNA expression in the spinal cord at the 7th-day post-surgery,

which corroborates the analgesic effect of single treatment with this flavonoid. The prolonged treatment with diosmin from the 7th day up to the 14th-day post-surgery inhibited the CCI-induced *Il-1β, Tnfα, Il-33*, *St2*, *Gfap*, *Iba-1*, and *Olig2* mRNA expression in the spinal cord. Thus, diosmin targeted the production of hyperalgesic molecules and activation of glial cell in single and prolonged treatment protocols with a more pronounced effect with prolonged treatment. Lining up with these results, the prolonged treatment with diosmin presented an anti-hyperalgesic effect in the thermal test that also increased over time. Of note, the contribution of spinal cord oligodendrocytes to neuropathic pain was only recently demonstrated [6] and diosmin reduces the activation of spinal cord oligodendrocytes as observed by reduced *Olig2* and *Il-33* mRNA expression. Of note, this prolonged treatment protocol did not induce an increase of stomach tissue myeloperoxidase activity or serum urea and creatinine levels, which indicates diosmin does not induce gastric and renal tissue damage, respectively, as non-selective cyclooxygenase inhibitors [66].

Concluding, the present data shows that diosmin inhibits CCI-induced neuropathic pain. The analgesic effect of diosmin depends on activating the NO/cGMP/PKG/ATP-sensitive potassium channels signaling pathway and the inhibition of the spinal cord mRNA expression of hyperalgesic cytokines and glial cells activation. Therefore, clinical trials investigating its analgesic effect in humans seem conceivable given that diosmin is already in clinical use to the treatment of other diseases with a safe profile [20-24, 39-41].

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Figure captions

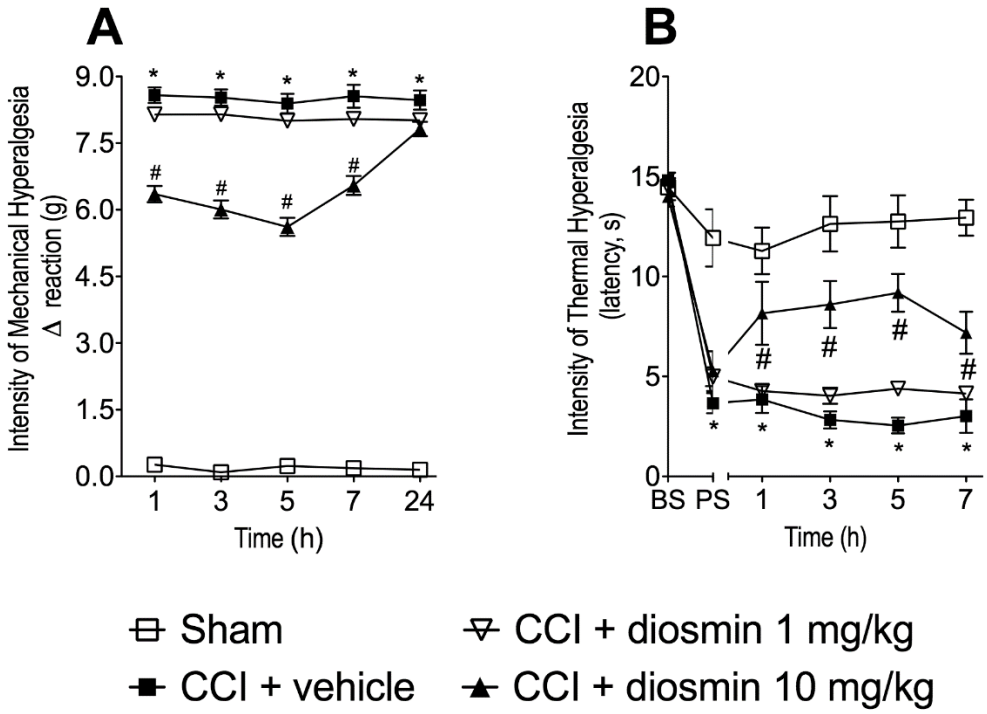

Figure 1.Single treatment with diosmin reduces CCI-induced mechanical and thermal hyperalgesia. Mice were treated with diosmin (1 or 10 mg/kg, i.p.) at the 7thday post-surgery. Mechanical (Panel A) and thermal hyperalgesia (Panel B) were evaluated 1, 3, 5, 7, and 24 h after diosmin treatment. Sham-operated mice, the sciatic nerve was exposed without ligation and was used as control group. n=6, representative of two independent experiments. *P<0.05 compared to sham group and #P<0.05 CCI + diosmin 10 mg/kg compared to CCI + vehicle group. Two-way ANOVA followed by Bonferroni's test. BS (before surgery); AS (after surgery).

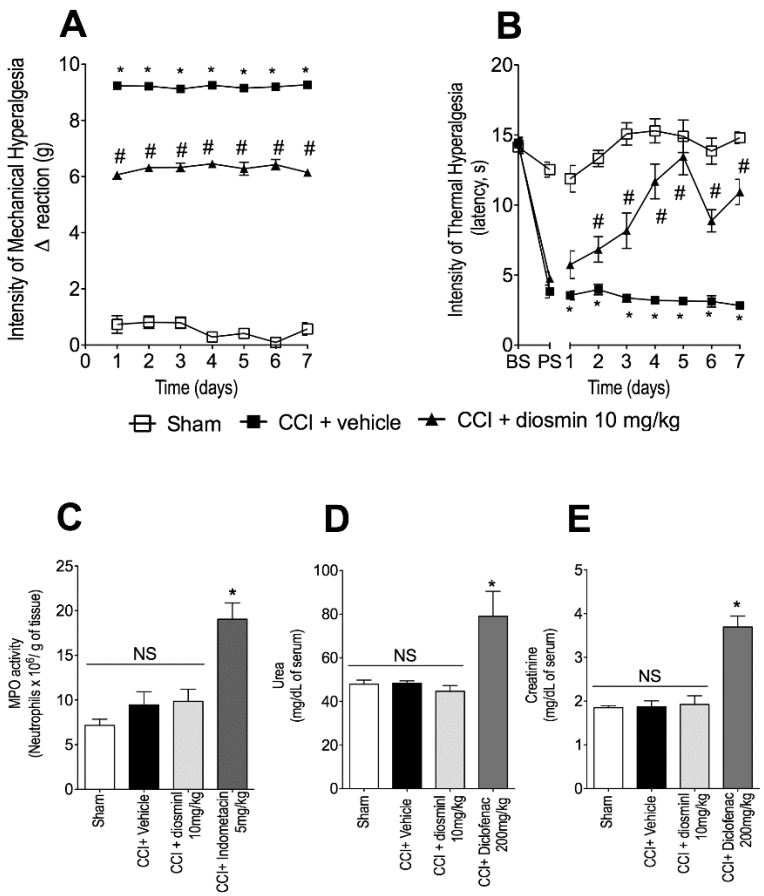

Figure 2.Prolonged treatment with diosmin reduces CCI-induced mechanical and thermal hyperalgesia without causing gastric lesions or renal damage. Mice received prolonged treatment (daily treatment starting at the 7th to 14th day post-surgery) with diosmin (10 mg/kg, i.p.). Mechanical (Panel A) and thermal (Panel B) hyperalgesia were evaluated daily 5 h after treatment with diosmin (Two-way ANOVA followed by Bonferroni's test). Myeloperoxidase activity was determined in gastric tissue samples (C), and urea (D) and creatinine (E) levels were determined in serum samples. Sham-operated mice, the sciatic nerve was exposed without ligation and was used as control group. n=6, representative of two independent experiments. *P<0.05 compared to sham group and #P<0.05 CCI + diosmin 10 mg/kg compared to CCI + vehicle group. One-way ANOVA followed by Tukey's test. BS (before surgery); AS (after surgery).

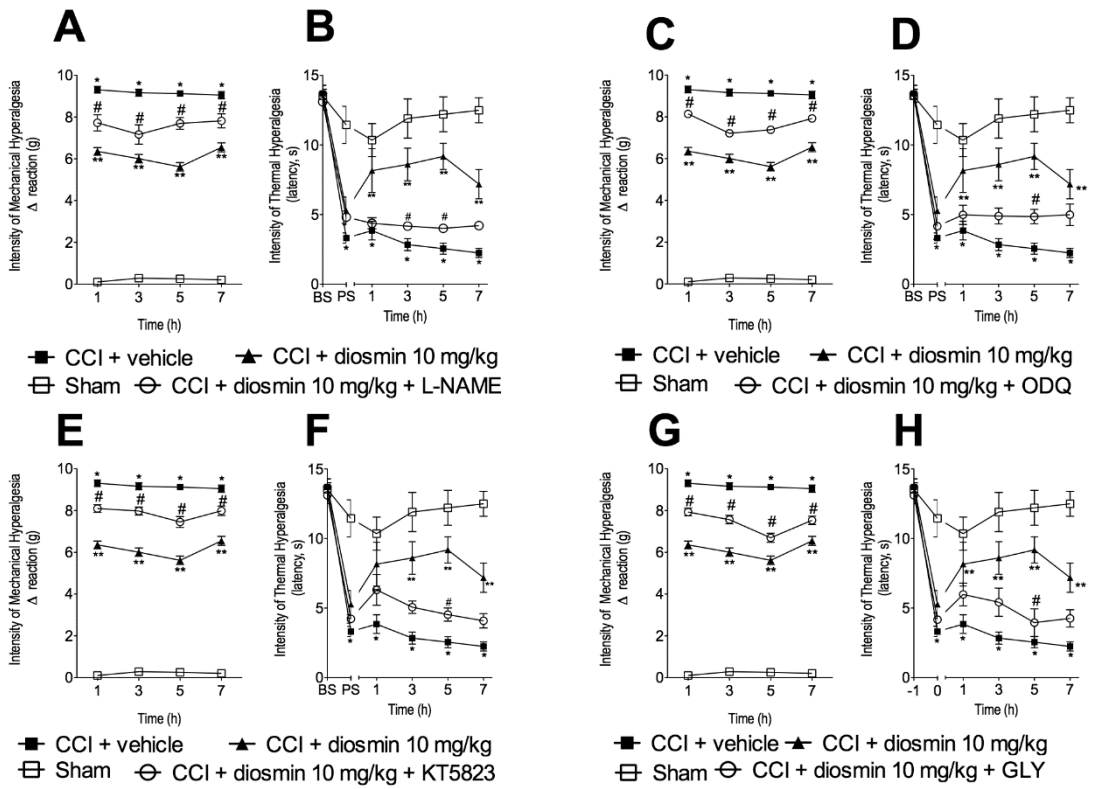

Figure 3. Diosmin reduces mechanical and thermal hyperalgesia by activating the NO/cGMP/PKG/ATP-sensitive potassium channels signaling pathway. Mice were pretreated with one of the different inhibitors of NO signaling pathway separately at 7th day post-surgery: L-NAME (NOS inhibitor, 90 mg/kg, i.p., 1 h before diosmin, Panel A and B); or ODQ (soluble guanylate cyclase inhibitor, 0.3 mg/kg, i.p., 30 min before diosmin, Panel C and D); or KT5923 (PKG inhibitor, 0.5 µg/mouse, i.p., 5 min before diosmin, Panel E and F); or glibenclamide (ATP-sensitive potassium channel inhibitor, 0.3 mg/kg, i.p., 45 min before diosmin, Panel G and H). Mechanical and thermal hyperalgesia were evaluated 1, 3, 5, and 7 h after treatment with diosmin (10 mg/kg, i.p.). Sham-operated mice, the sciatic nerve was exposed without ligation and was used as control group. n=6, representative of two independent experiments. *P<0.05 compared to sham group and **P<0.05 compared to CCI + vehicle group and **P<0.05 compared to diosmin 10 mg/kg group + NO signaling pathway inhibitor. Two-way ANOVA followed by Bonferroni's test. BS (before surgery); AS (after surgery).

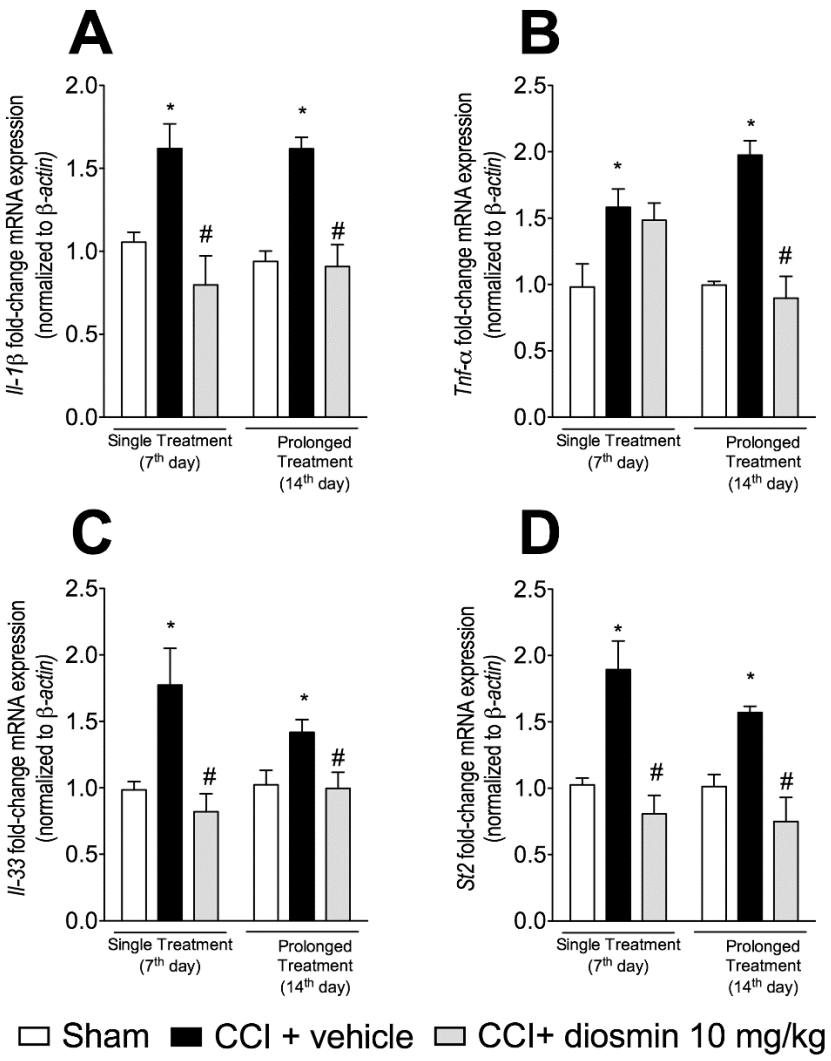

Figure 4. Single treatment with diosmin reduces CCI-induced spinal cord up-regulation of *pro-Il-1β, Tnf-α, Il-33,* and *St2* receptor mRNA expression. Spinal cord was collected 5 h after single treatment at the 7th day post-surgery or daily treatment starting at the 7th and up to the 14th day post-surgery with diosmin (10 mg/kg, i.p.) for determination of *pro-Il-1β* (Panel A), *Tnf-α* (Panel B), *Il-33* (Panel C), and *St2* receptor (Panel D) mRNA expression by RT-qPCR. Sham-operated mice, the sciatic nerve was exposed without ligation and was used as control group. n=6, representative of two independent experiments. **P*<0.05 compared to sham group and #*P*<0.05 compared to CCI + vehicle group. One-way ANOVA followed by Tukey's test.

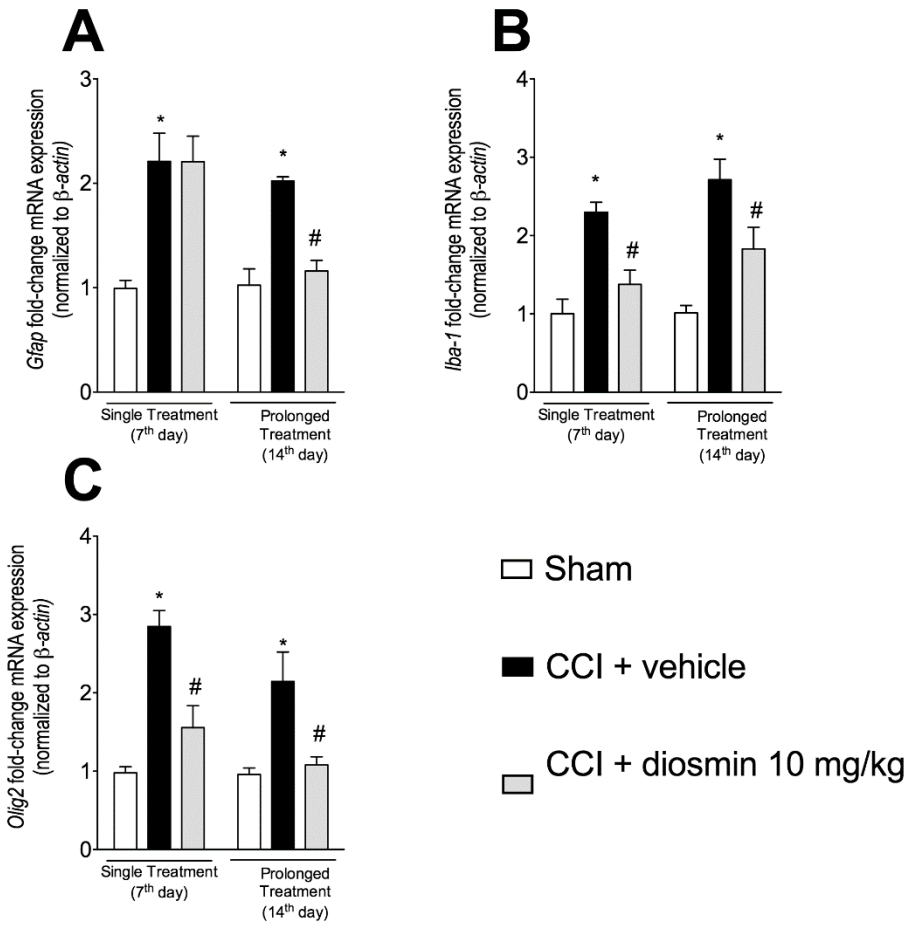

Figure 5. Diosmin reduces CCI-induced spinal cord glial cell activation. Spinal cord was collected 5 h after diosmin (10 mg/kg, i.p.) single treatment at the 7thday post-surgery or after the last treatment of a daily treatment protocol starting at the 7th day and up to the 14th day post-surgery for determination of *Gfap* (Panel A), *Iba-1* (Panel B), and *Olig2* (Panel C) mRNA expression by RT-qPCR. Sham-operated mice, the sciatic nerve was exposed without ligation and was used as control group. n=6, representative of two independent experiments. *P<0.05 compared to sham group and #P<0.05 compared to CCI + vehicle group. One-way ANOVA followed by Tukey's test.

5 CONCLUSÃO

A compreensão dos mecanismos envolvidos na nocicepção torna possível o desenvolvimento de estratégias terapêuticas para o alívio da dor. Apesar do grande número de publicações e do grande conhecimento sobre os mecanismos fisiológicos relacionados à percepção de estímulos dolorosos, ainda existem muitas questões sem resposta, principalmente em casos clínicos de dor crônica.

No presente estudo foi demonstrado que o flavonoide diosmin apresenta papel anti-hiperalgésico, antioxidante e anti-inflamatório em modelos de dor associada ao câncer e dor neuropática. O tratamento com diosmin reduziu a hiperalgesia mecânica, térmica e edema de maneira dose dependente. Modificou o perfil de recrutamento celular envolvidas no processo inflamatório, atuando por vias semelhantes à de analgésicos já descritos. Além de ser capaz de diminuir a expressão de citocinas pró-inflamatórias e atuar com poder antioxidante nos dois modelos sem induzir efeitos colaterais.

Apesar de o desenvolvimento deste trabalho ser apenas uma peça diante do grande estudo que estão desenvolvendo dentro da comunidade científica, os resultados apontam uma nova abordagem terapêutica com diosmin. Evidenciando seus efeitos anti-inflamatórios, antioxidantes e analgésicos visando a melhora da qualidade de vida de pacientes que sofrem de dores crônicas tanto oncológicas como neuropáticas.

ANEXOS

Anexo A

Parecer de aprovação do Comitê de Ética em Experimentação Animal (CEUA) da Universidade Estadual de Londrina (UEL).

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 087/2014 **Londrina, 11 de Julho de 2014**

Prezado Pesquisador,

A CEUA/UEL reunida em 10 de Junho de 2014 avaliou o projeto de pesquisa intitulado "**Avaliação do efeito antinociceptivo dos flavonoides naringenina e diosmin em modelo de dor induzida pelas células tumorais de Ehrlich em camundongos.**", registrado sobe o processo CEUA **nº 8482.2014.86**, pesquisa do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade, julgando-o **aprovado** para execução entendendo-se que os princípios éticos, postulados pelo Conselho Nacional de Controle de Experimentação Animal, estão respeitados.

Serão utilizados 1248 camundongos Swiss machos, com peso aproximado de 20-25g, provenientes do Biotério Central-UEL. O projeto tem como objetivo avaliar o efeito antinociceptivo e mecanismo de ação dos flavonoides naringenina e diosmin em modelo de dor induzida pelas células tumorais de Ehrlich em camundongos. Os camundongos receberão células tumorais de Ehrlich e serão tratados diariamente com a naringenina ou diosmin. Os animais receberão o tumor e no 8° dia após a inoculação serão tratados com as diferentes doses de naringenina ou diosmin e avaliados nos teste de hiperalgesia mecânica, hiperalgesia térmica e edema. No final do 12° dia os animais serão eutanasiados e terão o tecido da pata e medula coletados para dosagem de citocinas, recrutamento celular, estresse oxidativo, avaliação da ativação de MAP quinases e do fator de transcrição NFκB. Também será avaliado o efeito dos flavonoides no crescimento tumoral. Os protocolos experimentais estão aprovados para execução em 36 meses.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo de pesquisa aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente,

Prof. Dr. João Waine Pinheiro
Vice-coordenador da CEUA/UEL

Ilmo. Sr.
Prof. Dr. Waldiceu Aparecido Verri Junior
Departamento de Ciências Patológicas
Centro de Ciências Biológicas
Com cópia para Sra Égle Maria de Sousa (Chefe da DCA/PROPPG), Diretor(a) do Centro de Ciências Biológicas e Diretor (a) do Biotério Central

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Hospital Universitário/Centro de Ciências da Saúde: Av. Robert Koch, 60 - Vila Operária – Fone (043) 381-2000 PABX – Fax 337-4041 e 337-7495 - Caixa Postal 791 - CEP 86038-440
LONDRINA - PARANÁ - BRASIL

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 238/2015 **Londrina, 10 de Novembro de 2016.**

Prezado Pesquisador,

Certificamos que o adendo referente ao projeto intitulado **"Avaliação do efeito antinociceptivo e atividade antioxidante dos diosmin em modelo de dor neuropática induzida pela constrição do nervo ciático em camundongos"**, protocolo CEUA **nº28995.2014.39**, sob a responsabilidade de **Waldiceu Aparecido Verri Junior**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **08/11/2016**.

O adendo solicita o acréscimo de metodologias e para isso será necessária a utilização de 60 animais, que se justifica pelo fato de haver a necessidade da avaliação da ativação de células da glia em tecido medular por meio da técnica de imunofluorescência.

Vigência do Projeto	2015 a 2017
Espécie/linhagem	Camundongo heterogênico / Swiss
Nº de animais	60 (acrescentados aos anteriormente aprovados, total=660)
Peso/Idade	20-25g / Indeterminada
Sexo	Machos
Origem	Biotério Central / UEL

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente,

Profa. Dra. Glaura Scantamburlo Alves Fernandes
Coordenadora da CEUA/UEL

Ilmo. Sr.
Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador do Projeto
Departamento de Ciências Patológicas / Centro de Ciências Biológicas
Com cópia para Coordenação do Biotério Central / UEL, Chefe do Departamento de Ciências Patológicas e Diretor(a) do Centro de Ciências Biológicas.