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ENVOLVIMENTO DE MASTÓCITOS EM UM MODELO DE DOR PÓS-OPERATÓRIA EM CAMUNDONGOS

TESE DE DOUTORADO

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Santa Maria, RS, Brasil,

2012

ENVOLVIMENTO DE MASTÓCITOS EM UM MODELO DE DOR PÓS-OPERATÓRIA EM CAMUNDONGOS

Por

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Tese apresentada no curso de doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**.

Orientador: Dr. Juliano Ferreira

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Bioquímica Toxicológica

A comissão examinadora, abaixo assinada,
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ENVOLVIMENTO DE MASTÓCITOS EM UM MODELO DE DOR PÓS-OPERATÓRIA EM CAMUNDONGOS

elaborada por

Sara Marchesan de Oliveira

como requisito parcial para obtenção do grau de

Doutor em Bioquímica Toxicológica

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RESUMO

Tese de Doutorado

Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria, RS, Brasil

ENVOLVIMENTO DE MASTÓCITOS EM UM MODELO DE DOR PÓS-OPERATÓRIA EM CAMUNDONGOS

AUTORA: Sara Marchesan de Oliveira

ORIENTADOR: Juliano Ferreira

LOCAL E DATA DA DEFESA: Santa Maria, 28 de fevereiro de 2012

Estudos recentes indicam que praticamente a metade de todos os pacientes submetidos à procedimentos cirúrgicos apresentam um quadro de dor moderada à severa, o que torna importante entender os mecanismos envolvidos na dor pós-operatória para melhor tratá-la. Dados da literatura demonstram que incisões podem causar a degranulação de mastócitos. Assim, o objetivo deste estudo foi investigar o envolvimento dos mastócitos e seus mediadores inflamatórios, serotonina, histamina e triptase, em um modelo de dor pós-operatória em camundongos. A depleção dos mediadores dos mastócitos produzida pelo pré-tratamento repetido com o composto 48/80 (1, 3, 10 e 10 µg/pata), que promove degranulação de mastócitos, preveniu a nocicepção pós-operatória (98 ± 23% de inibição) e reduziu os níveis de histamina e serotonina (88 ± 4 % e 68 ± 10%, de redução, respectivamente) e a atividade da triptase (82 ± 14% de redução) no tecido da pata. Além disso, a cirurgia plantar produziu grande degranulação dos mastócitos, como avaliado por histologia e confirmado pelo aumento dos níveis de serotonina (três vezes maior) e histamina (quinze vezes maior) e pelo aumento na atividade da triptase (duas vezes maior) no perfusato tecidual após a cirurgia. O pré-tratamento com o estabilizador da membrana celular dos mastócitos, cromoglicato (200 µg/pata, i.pl.), preveniu a nocicepção mecânica (inibição de 96 ± 21%) e o aumento dos níveis de histamina (44 ± 10% de inibição) e serotonina (73 ± 5% de inibição), bem como preveniu a liberação de triptase (100% de inibição) induzida pela cirurgia plantar. Finalmente, o tratamento local com os antagonistas dos receptores H_1 (prometazina, 100 µg/pata,

i.pl.), 5-HT_3 (ondansetrona, 10 µg/pata, i.pl.), 5-HT_{2A} (cetanserina, 5 µg/pata, i.pl.) ou PAR-2 (ENMD-1068, 10-100 nmol/pata) ou com o inibidor da triptase (gabexato, 0,01-1 nmol/pata) reduziu parcialmente a dor pós-operatória em camundongos. Assim, os mecanismos de ativação de mastócitos, bem como a liberação dos seus mediadores inflamatórios e conseqüente ativação dos seus respectivos receptores são alvos interessantes para o desenvolvimento de novas terapias para tratar a dor pós-operatória.

Palavras-Chave: alodínia; hiperalgesia; nocicepção; cirurgia; histamina; serotonina; triptase; PAR-2.

ABSTRACT

PhD Thesis

Graduate Course in Biological Sciences: Toxicological Biochemistry

Federal University of Santa Maria, RS, Brazil

INVOLVEMENT OF MAST CELLS IN A MODEL OF POSTOPERATIVE PAIN IN MICE

AUTHOR: Sara Marchesan de Oliveira

ADVISOR: Juliano Ferreira

PLACE AND DATE OF THE DEFENSE: Santa Maria, February, 28nd, 2012.

Recent studies have indicated that nearly half of all surgical patients present a picture of moderate to severe pain what become important to understand the mechanisms involved postoperative pain to be better treat it. Previous studies have shown that incisions can cause mast cell degranulation. Thus, the aim of this study was to investigate the involvement of mast cells and its inflammatory mediators, histamine, serotonin and tryptase in a model of postoperative pain in mice. The depletion of mast cell mediators produced by repeated pre-treatment with compound 48/80 (1, 3, 10 and 10 µg/paw), that promote mast cell degranulation, prevented postoperative nociception (98 ± 23% of inhibition) and reduced histamine and serotonin levels (88 ± 4% and 68 ± 10%, respectively) and tryptase activity (82 ± 14% of reduction) in paw tissue. Furthermore, plantar surgery produced immense mast cell degranulation, as assessed by histology and confirmed by the increased levels of serotonin (three–fold higher) and histamine (fifteen–fold higher) and by increased activity of tryptase (two–fold higher) in the perfused tissue after surgery. Accordingly, pre-treatment with the mast cell membrane stabilizer cromoglycate (200 µg/paw, i.pl.) prevented mechanical allodynia (inhibition of 96 ± 21%) and an increase in histamine (44 ± 10% of inhibition) and serotonin (73 ± 5% of inhibition) levels and prevented the tryptase release (100% of inhibition) induced by plantar surgery. Finally, local treatment with H_1 (promethazine, 100 µg/paw, i.pl.), 5-HT_3 (ondansetron, 10 µg/paw, i.pl.), 5-HT_{2A} (ketanserin, 5 µg/paw, i.pl.) or PAR-2 (ENMD-1068, 10-100 nmol/paw)

receptor antagonists or with the tryptase inhibitor (gabexate, 0.01-1 nmol/paw) partially decreased postoperative nociception in mice. Thus, mast cell activation mechanisms as well as release of mast cells inflammatory mediators and activation of its respective receptors are interesting targets for the development of novel therapies to treat postoperative pain.

Keywords: allodynia; hyperalgesia; nociception; surgery; histamine; serotonin; tryptase; PAR-2.

LISTA DE ABREVIATURAS

ATP	Trifostato de Adenosina
PAR	Receptor Ativado por Protease
SNC	Sistema Nervoso Central
SNP	Sistema Nervoso Periférico
DRG	Gânglio da Raiz Dorsal
CGRP	Peptídeo Relacionado ao Gene da Calcitonina
NGF	Fator de Crescimento do Nervo
$P2X_3$	Receptor de Purinas
GDNF	Fator Neurotrófico Derivado de Células Gliais
NMR	Núcleo Magno da Rafe
PAG	Substância Cinzenta Periaquedutal
RVM	Medula Rostroventromedial
AMPc	Monofosfato Cíclico de Adenosina
IP3	Inositol Trifosfato
DAG	Dialcilglicerol
AINEs	Antiinflamatórios Não- Esteroidais
ASIC	Canais Iônicos Sensíveis à Ácido
IL	Interleucina
TNF-α	Fator de Necrose Tumoral alfa
PG	Prostaglandina
NK	Neurocinina
TRPV	Receptor de Potencial Transitório Vanilóide
5-HT	5-hidroxitriptamina (serotonina)
H_1R	Receptor para a Histamina
5-HTR	Receptor para a Serotonina
ANOVA	Análise de Variância

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SUMÁRIO

APRESENTAÇÃO

Nos itens **INTRODUÇÃO** e **REVISÃO BIBLIOGRÁFICA** consta uma revisão sucinta da literatura sobre os temas abordados nesta tese.

A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigo e manuscrito científicos, os quais se encontram no item **RESULTADOS**. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprio artigo e representam a íntegra deste estudo.

Os itens **DISCUSSÃO** e **CONCLUSÕES** encontrados no final desta tese, apresentam interpretações e comentários gerais sobre o artigo e manuscrito científicos contidos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA** e **DISCUSSÃO** desta tese.

1. INTRODUÇÃO

1. Introdução

Durante as últimas duas décadas, o tratamento da dor pós-operatória tem sido reconhecido como uma importante questão em relação aos cuidados da saúde e, apesar da introdução de novas normas, diretrizes e ações educativas, os dados mundiais sugerem que a dor pós-operatória continua a ser inadequadamente tratada (Wu e Raja, 2011).

Os principais medicamentos utilizados no tratamento da dor pós-operatória são os opióides e os anti-inflamatórios não esteroidais (AINEs) (Dahl e Kehlet, 2006). Apesar de ocorrer um aumento mundial na utilização de opióides na tentativa de tratar a dor aguda ou crônica, o progresso no tratamento da dor pós-operatória continua limitado (Manchikanti et al., 2010). O aumento da utilização de opióides poderia estar associado com um aumento na incidência de efeitos adversos, incluindo sedação excessiva, depressão respiratória e constipação (Chang et al., 2007). As principais preocupações com o uso dos AINEs em pacientes cirúrgicos são interferência na função plaquetária e na cicatrização, bem como no desenvolvimento de úlcera gastroduodenal (Pilotto et al., 2003; Juhlin et al., 2005). Assim, terapias mais seguras são necessárias para o alívio da dor no período pós-operatório.

Perifericamente, ocorrem sinais de inflamação, como edema, hipertermia e dor (Swarm et al., 2001; Clark et al., 2007) que são mantidos através de eventos vasculares e celulares, assim como a liberação de mediadores inflamatórios por células residentes (Metcalfe et al., 1997; Marchand et al., 2005; Basbaum et al., 2009; Ren e Dubner, 2010). Os mastócitos são células residentes do tecido conjuntivo e após a sua degranulação liberam mediadores inflamatórios como a histamina, a serotonina e a triptase que ativam seus respectivos receptores H_1, 5-HT ou PAR-2 (Di Rosa et al., 1971; Schwartz et al., 1987) promovendo nocicepção (Vergnolle et al., 2001; Kuner, 2010; Ren e Dubner, 2010). Estudos prévios mostraram que os mastócitos estão envolvidos na dor que ocorre em pacientes com doença inflamatória intestinal, pancreatite ou dores de cabeça (Hoogerwerf et al., 2001; Barbara et al., 2007; Levy et al., 2007; De Winter et al., 2011). Assim, acredita-se que eles poderiam desempenhar um importante papel na dor pós-operatória já que eles

degranulam após a incisão da pele ou após procedimento cirúrgico (Weller et al., 2006; The et al., 2007).

Já que a dor pós-operatória permanece inadequadamente tratada, uma compreensão abrangente de seus mecanismos assim como o desenvolvimento de novas abordagens terapêuticas são de fundamental importância para um melhor controle da dor.

2. OBJETIVOS

2. Objetivos

2.1 Objetivo Geral

Investigar o envolvimento dos mastócitos em um modelo de dor pós-operatória em camundongos.

2.2.1 Objetivos Específicos

2.2.1 Avaliar se a depleção dos mediadores inflamatórios ou a estabilização da membrana dos mastócitos são capazes de prevenir a nocicepção pós-operatória em camundongos;

2.2.2 Confirmar histologicamente e bioquimicamente a degranulação e a liberação de mediadores dos mastócitos após a cirurgia;

2.2.3 Verificar o papel dos receptores para histamina H_1, para serotonina 5-HT_3 e 5-HT_{2A} ou ativados por proteases PAR-2 na nocicepção pós-operatória em camundongos.

3. REVISÃO BIBLIOGRÁFICA

3. Revisão Bibliográfica

3.1 Dor e Nocicepção

A dor é classicamente definida pela Associação Internacional para o Estudo da Dor como “uma experiência sensorial e emocional desagradável, associada a uma lesão tecidual real ou potencial, ou descrita em termos de tal lesão” (Merskey e Bogduk, 1994). Entretanto, a incapacidade do paciente de comunicar-se verbalmente não exclui a possibilidade do mesmo estar enfrentando um quadro doloroso, sendo necessário um tratamento adequado para o alívio da dor (Craig, 2006). Mesmo indivíduos com comando da linguagem e habilidades culturais podem enfrentar dificuldades de expressar as complexidades da experiência da dor (Craig, 2009).

A dor é comumente definida como aguda ou crônica. A dor aguda é definida como “dor de início recente e de duração provavelmente limitada. Ela geralmente tem uma relação temporal e causal identificável para a lesão ou doença”. Por outro lado, a dor crônica, “comumente persiste além do tempo de cura de uma lesão e frequentemente pode não ter causa claramente identificável” (Ready e Edwards, 1992; Russo e Brose, 1998).

A capacidade do sistema somatossensorial em detectar estímulos nocivos e estímulos que potencialmente lesionam os tecidos é um importante mecanismo protetor que envolve múltiplos mecanismos centrais e periféricos. Os processos neurais subjacentes à codificação e processamento dos estímulos nocivos são definidos como “nocicepção” (Loeser e Treede, 2008). Além desses efeitos sensoriais, a percepção e a experiência subjetiva da “dor” são multifatoriais e influenciadas por fatores físicos e psicológicos. Fatores psicológicos que influenciam a experiência da dor incluem os processos de atenção e outros processos cognitivos (memória/aprendizado, processamento do pensamento, crenças, humor), respostas comportamentais, e interações com outras pessoas. A dor pode ser um indicador de dano tecidual, mas pode também ser experimentada na ausência de alguma causa identificável (Macintyre, 2010).

A dor pode ser causada por diferentes fatores, como é o caso da dor nociceptiva, da dor inflamatória e da dor neuropática. A dor nociceptiva é um sistema protetor fisiológico essencial para detectar e minimizar o contato com estímulos prejudiciais ou nocivos e continua somente na presença mantida do estímulo nocivo. Esta é a dor que sentimos quando tocamos algo muito quente, frio ou em objetos cortantes, resultado da ativação de receptores sensoriais capazes de traduzir e codificar estímulos nocivos (nociceptores) encontrados em subtipos de fibras C não-mielinizadas e fibras Aδ pouco mielinizadas, as quais são somente ativadas na presença de estímulos intensos. Seu papel protetor demanda de atenção e ação imediata decorrentes do reflexo de retirada do membro afetado pelo estímulo (Woolf e Ma, 2007; Basbaum, 2009; Woolf, 2010).

O segundo tipo de dor também pode ser adaptativa e protetora. A dor inflamatória ocorre em resposta à lesão tecidual e subseqüente resposta inflamatória. Para ajudar na cura e reparação da parte do corpo atingida, o sistema nervoso sensorial passa por uma profunda mudança em sua capacidade de resposta, onde estímulos normalmente inócuos agora produzem dor (alodínia) e respostas a estímulos nocivos são exageradas e prolongadas (hiperalgesia) (Loeser e Treede, 2008; Costigan et al., 2009). A sensibilidade ocorre dentro da área inflamada, podendo atingir áreas não inflamadas, como resultado da plasticidade em nociceptores e em vias nociceptivas centrais (Woolf e Salter 2000; Huang et al. 2006; Hucho e Levine 2007). Devido à sensibilização dos nociceptores após o processo inflamatório, eles já não atuam apenas como detectores de estímulos nocivos, mas podem ser ativados também por estímulos inócuos de baixo limiar (Costigan et al., 2009). Esta dor resulta da ativação de células residentes, como os mastócitos, da infiltração de células inflamatórias, tais como os neutrófilos, macrófagos e da liberação de mediadores inflamatórios, como cininas, aminas, prostanóides, fatores de crescimento e citocinas, que com prótons e trifosfato de adenosina (ATP) compõem uma “sopa inflamatória” que promove redução do limiar e amplificação na resposta dos nociceptores que inervam o tecido inflamado (sensibilização periférica). Além destas alterações periféricas, pode também ocorrer uma resposta aumentada dos neurônios nociceptivos no sistema

nervoso central (SNC) pelos estímulos aferentes normais ou sublimiares (sensibilização central) (Scholz e Woolf, 2001; Loeser e Treede, 2008; Latremoliere e Woolf, 2009; Woolf, 2010).

Finalmente, há a dor que não é protetora, mas mal-adaptativa, conhecida como dor neuropática. Esta resulta de uma lesão ou doença que afeta o sistema somatosensorial, tanto central ou perifericamente, promovendo alteração do processamento nociceptivo. Neste caso, a dor pode ocorrer na ausência de estímulos e respostas para estímulos previamente inócuos e nocivos estão aumentadas (Loeser e Treede, 2008; Treede et al., 2008; Costigan et al., 2009).

Enquanto a dor envolve a percepção de um estímulo aversivo, a nocicepção é um termo fisiológico usado para descrever o processo neural de codificação e processamento do estímulo nocivo. A nocicepção é a progenitora da dor, experiência complexa e subjetiva que, por sua vez, causa o sofrimento. Contudo, a nocicepção não é uma sensação uniforme, e a qualidade da dor e o início das respostas protetoras são determinadas por muitos fatores na medula espinhal e em estruturas supra-espinhais envolvidas na integração e modificação dos sinais nociceptivos (Russo e Brose, 1998).

3.2 Transmissão da dor

A detecção de estímulos nocivos tais como calor, frio ou compressão intensa e de algumas substâncias químicas requer ativação dos nociceptores, os quais são amplamente distribuídos por todo o corpo (pele, músculos, articulações, vísceras, meninges) e transmitem tanto a informação nociceptiva quanto a informação não nociceptiva para a medula espinhal. Essas fibras sensoriais aferentes podem ser de três tipos: fibras Aβ, fibras Aδ e fibras C. As fibras Aβ são sensores táteis mielinizados de grande diâmetro com velocidade de condução muito rápida e respondem à estimulação tátil. As fibras Aδ são mielinizadas e de médio diâmetro com velocidade de condução rápida do estímulo doloroso, enquanto que as fibras C são fibras não mielinizadas de pequeno diâmetro e possuem velocidade de condução lenta do estímulo

doloroso constituindo a maior parte das fibras sensoriais do sistema nervoso periférico (SNP) (Furst, 1999; Julius e Basbaum, 2001; Gold e Gebhart, 2010; Kuner, 2010). Os corpos celulares dos nociceptores que inervam o tronco, os membros e as vísceras são encontrados no gânglio da raiz dorsal (DRG), enquanto que aqueles que inervam a cabeça, a cavidade oral e o pescoço estão no gânglio trigeminal e conduzem as informações nociceptivas até o corno dorsal da medula espinhal e o núcleo trigeminal *pars caudalis* na ponte, respectivamente (Macintyre, 2010). O nociceptor possui quatro principais componentes funcionais, tais como o terminal periférico que transduz estímulos externos e inicia potenciais de ação, o axônio, que conduz os potenciais de ação, o corpo celular do neurônio que controla a identidade e a integridade deste, e o terminal central que forma o elemento pré-sináptico no SNC (Woolf e Ma, 2007).

A subclasse mais numerosa de nociceptor é a fibra-C polimodal, que responde a uma ampla gama de estímulos físicos (calor, frio, pressão) e químicos, embora com graus variados de sensibilidade (Hunt e Mantyh, 2001; Macintyre, 2010). A parte superficial do corno dorsal de medula espinhal (lâminas I e II) é a principal zona de terminação dessas fibras, as quais podem ser divididas em duas grandes classes neuroquímicas de acordo com o seu conteúdo de peptídeos e a localização de seus terminais sinápticos no corno dorsal da medula espinhal. As fibras C peptidérgicas sintetizam peptídeos tais como substância P e o peptídeo relacionado ao gene da calcitonina (CGRP) e expressam alta afinidade para o receptor do fator de crescimento do nervo (TrkA). Essas fibras são altamente nociceptivas e arborizam-se mais superficialmente na lâmina I (zona marginal) e no exterior da lâmina II (substância gelatinosa) dentro do corno dorsal. O outro grupo de fibras, as fibras C não-peptidérgicas (previamente identificadas pelo conteúdo de fosfatase ácida resistente ao fluoreto e de superfície celular com resíduos de α-D-galactose) expressam o receptor de purinas $P2X_3$, sítios de ligação para a isolecitina B4 e receptores para o fator neurotrófico derivado de células gliais (GDNF). Estas fibras terminam no interior da lâmina II, quase que exclusivamente dentro da parte mais profunda da substância gelatinosa do cordão espinhal com um tipo glomerular de terminal sináptico. Ambos os

grupos de fibras respondem a estímulos nocivos semelhantes (Hunt e Mantyh, 2001).

A sinapse dos nociceptores no corno dorsal em direção aos neurônios de segunda ordem ocorre predominantemente dentro da lâmina II da medula espinhal. Os neurônios de segunda ordem cruzam a medula espinhal para ascender no trato espinotalâmico com fibras terminais predominantemente localizadas no tálamo. Uma vez que o sinal tenha alcançado o tálamo, neurônios de terceira ordem conduzem estes sinais através de axônios subseqüentes da cápsula interna para o córtex somatossensorial, incluindo o giro pós-central, onde os aspectos discriminativos-sensoriais da dor, como por exemplo, o local e o tipo de estímulo doloroso são identificados. Além da localização somatossensorial, as fibras do núcleo inter-laminar e medial do tálamo irradiam para o giro cingulado anterior e tornam-se envolvidas nos componentes emocionais ou afetivos da dor (Russo e Brose, 1998).

A via espino-talâmica córtico-límbica converge para as mesmas estruturas límbicas e subcorticais que são diretamente acessadas por outra via ascendente da dor, a via espino-parabraquial-amigdalóide (Bernard et al., 1989; Bernard e Besson, 1990). A via espino-parabraquial-amigdalóide origina-se da lâmina I da medula espinhal e conecta-se com a área parabraquial. Os neurônios da área parabraquial, que são ativados especificamente por estímulos nociceptivos, enviam projeções para a amígdala, hipotálamo, substância cinzenta periaquedutal (PAG) e medula oblonga ventrolateral (Gauriau e Bernard, 2002). Na amígdala, as projeções nociceptivas da área parabraquial atingem primeiramente a cápsula lateral do núcleo central (Bernard et al., 1993; Jasmin et al., 1997).

Além de desempenhar importante papel na interpretação da informação nociceptiva ascendente, as estruturas supra-espinhais também estão envolvidas na modulação dos circuitos descendentes que controlam a dor. As vias descendentes originam-se no tronco cerebral e outras estruturas como hipotálamo, córtex, tálamo, núcleo magno da rafe (NMR), PAG e estruturas adjacentes da medula rostroventromedial (RVM), que exercem importante papel na integração e modulação das mensagens nociceptivas no corno dorsal da medula espinhal (Millan, 2002; Vanegas e Schaible, 2004). Os mecanismos

descendentes modulam a resposta nociceptiva por exercer suas ações em nociceptores presentes nas fibras aferentes primárias, bem como em neurônios intrínsecos do corno dorsal, como interneurônios excitatórios, interneurônios inibitórios e neurônios de projeção (Millan, 2002).

Uma das descobertas mais interessantes a respeito do circuito modulatório da dor é que este pode tanto facilitar quanto inibir a transmissão nociceptiva (Julius e Basbaum, 2001; Porreca et al., 2002). Por exemplo, na RVM estão presentes dois tipos de neurônios, as chamadas células “liga” (on) e as células “desliga” (off), as quais estão envolvidas na modulação nociceptiva. É proposto que as células on medeiam a facilitação da condução de estímulos nociceptivos quando ativadas, e as células off medeiam a inibição da transmissão nociceptiva, provocada pela estimulação da PAG. De maneira geral, a PAG deve excitar as células off e inibir as células on na medula rostroventromedial (Fields et al., 2006). Logo, o balanço entre a ativação dessas duas subpopulações de neurônios determina a resposta a um estímulo nociceptivo periférico. No entanto, em situações de dor persistente, alterações na neuroplasticidade podem resultar em uma estimulação facilitatória sustentada, o que ocasiona respostas persistentes e exageradas à dor (Ren e Dubner, 2002; Porreca et al., 2002). Além da modulação descendente da informação nociceptiva envolver uma série de estruturas cerebrais, como mencionado anteriormente, os sistemas de neurotransmissores também estão envolvidos nesta conexão. Todos os neurotransmissores envolvidos na inibição descendente (tais como opióides endógenos, serotonina, noradrenalina) parecem inibir a excitação de neurônios de segunda ordem na presença de estímulo nocivo (Furst, 1999; Fields, 2006). O entendimento dos mecanismos envolvidos na transmissão do sinal doloroso tem progredido muito nos últimos anos, em grande parte devido a um aprimoramento na compreensão dos mecanismos envolvidos na fisiologia das fibras aferentes e no processo de neurotransmissão no corno dorsal da medula espinhal.

Diversos mediadores têm sido propostos na gênese e transmissão da dor, tais como metabólitos do ácido araquidônico (prostaglandinas e leucotrienos), aminas (histamina e serotonina), fatores de crescimento, citocinas, peptídeos (cininas, CGRP, substância P), aminoácidos excitatórios

(glutamato e aspartato) entre outros, são responsáveis pela multiplicidade de eventos que ocorrem durante a transmissão da dor, tanto no SNP quanto no SNC (Furst, 1999; Millan, 1999; Hill, 2001). Alguns mediadores atuam através da interação com receptores acoplados à proteína G, desencadeando a formação de segundos mensageiros, como o monofosfato cíclico de adenosina (AMPc), trifosfato de inositol (IP_3), diacilglicerol (DAG), aumento de cálcio intracelular e ativação de canais iônicos. Outros mediadores da nocicepção podem ativar diretamente canais iônicos, os quais são capazes de alterar a permeabilidade da membrana celular à íons (Dray e Perkins, 1993; Woolf e Costigan, 1999; Woolf e Salter, 2000).

3.3 Tratamento Farmacológico da Dor

A dor é um problema de saúde pública e compostos com ação analgésica possuem um papel central no seu tratamento. Uma vez que a dor associada a diferentes condições patológicas representa o sintoma que mais causa sofrimento aos pacientes, diferentes abordagens terapêuticas têm sido utilizadas com o objetivo de atenuá-la (Brennan et al., 2007; Tracey e Mantyh, 2007).

Os principais medicamentos utilizados na clínica para produzir analgesia são os AINEs e os opióides. No entanto, sabe-se que esses tratamentos causam efeitos colaterais que muitas vezes limitam o seu uso (Dahl e Kehlet, 2006). Os opióides causam sedação, prurido, náuseas, vômitos, constipação, depressão respiratória, tolerância entre outros. Os efeitos de tolerância são apresentados como um decréscimo na efetividade da analgesia opióide. Essa tolerância ocorre razoavelmente rápido tanto para o efeito analgésico bem como para os efeitos adversos, exceto para a constipação, a qual não é modificada com o uso contínuo dos opióides (Chang et al., 2007). Além disso, os efeitos adversos relacionados ao uso dos opióides, principalmente em pacientes cirúrgicos, também estão associados com internação hospitalar prolongada e custos mais elevados, por isso tratamentos farmacológicos que reduzem o uso de opióides podem ser custo-efetivos (Philip et al., 2002;

Oderda et al., 2007). As principais preocupações com o uso dos AINEs em pacientes cirúrgicos são a interferência na função plaquetária e na cicatrização, bem como no desenvolvimento de úlcera gastroduodenal (Pilotto et al., 2003; Juhlin et al., 2005). Assim, terapias mais seguras são necessárias para o alívio da dor pós-operatória.

3.4 Dor pós-operatória

Recentemente, foi estimado que a cada ano 234,2 milhões de cirurgias são realizadas no mundo todo. Entretanto, este número é provavelmente subestimado devido à dados incompletos e também a intervenções não registradas. Além disso, a distribuição mundial de intervenções cirúrgicas é desigual, sendo que os países desenvolvidos são responsáveis pela maioria das cirurgias não-cardíacas e das informações sobre as mesmas, enquanto que em países em desenvolvimento essas informações são escassas (Weiser et al., 2008). Um estudo realizado por Yu e colaboradores (2010) relatou que no período entre 1995-2007 foram realizadas 32.659.513 operações não cardíacas no Brasil. Um aumento de 20,42% foi observado no número de cirurgias deste período até os dias de hoje, onde quase 3 milhões de operações são realizadas anualmente.

A dor pós-operatória é uma causa comum de dor persistente e de hiperalgesia em humanos e é um dos maiores problemas enfrentados pelos pacientes após as cirurgias. Apesar da ampla gama de fármacos analgésicos utilizados, aproximadamente metade dos pacientes cirúrgicos experimentam dor moderada a intensa, além de relatarem alívio inadequado da dor (Dolin et al., 2002; Pogatzki-Zahn et al., 2007; Nossaman et al., 2010). No período pós-operatório imediato, o quadro clínico é dominado por dor não evocada, ou seja, dor espontânea no local da cirurgia e nos tecidos circundantes. Movimentos do paciente, respiração, tosse ou a aplicação de um toque ou pressão no local da ferida podem também causar dor, sendo esta denominada dor evocada. Além disso, a hipersensibilidade causada por estímulos está presente tanto no tecido lesionado (hiperalgesia primária) como no tecido circundante não lesionado

(hiperalgesia secundária), podendo prejudicar a recuperação pós-operatória (Moiniche et al., 1997; Kehlet et al., 2006; Alkaitis et al., 2010).

Modelos de dor pós-incisional têm sido muito úteis para investigar a fisiopatologia da dor pós-operatória (Brennan et al., 1996; Pogatzki et al., 2002). Um dos melhores modelos caracterizados é o desenvolvido por Brennan et al. (1996), onde a incisão da pata traseira de ratos provoca o desenvolvimento dor espontânea e dor causada por estímulos, o que é evidenciado em pacientes submetidos a procedimentos cirúrgicos (Pogatzki-Zahn et al., 2007). Do mesmo modo, Pogatzki e Raja (2003) padronizaram este mesmo modelo de dor pós-operatória em camundongos, conduzindo à estados de hiperalgesia na pata operada.

A lesão tecidual produzida pela cirurgia desencadeia uma cascata complexa de eventos e muitos destes facilitam a atividade periférica do nociceptor (Costigan e Woolf, 2000). O dano tecidual direto provoca a liberação do conteúdo intracelular, incluindo prótons e ATP. Dados da literatura demonstram que a ativação de canais iônicos sensíveis à ácido (ASIC) está envolvida na dor pós-operatória, já que os íons de hidrogênio promovem uma redução do pH tecidual local, o que está relacionado com a dor pós-operatória decorrente da incisão da pele e/ou do músculo (Woo et al., 2004; Deval et al., 2011). Evidencias também sugerem o envolvimento do ATP na dor pós-operatória, uma vez que a administração sistêmica ou local de um antagonista de receptores P2 é capaz de reduzir a alodínia mecânica causada pela incisão da superfície plantar da pata de ratos (Tsuda et al., 2001).

Ao serem ativadas, as células inflamatórias liberam seus mediadores inflamatórios, como os macrófagos que liberam interleucinas (IL-1β, IL-6, IL-8, entre outras) e fator de necrose tumoral alfa (TNF-α). A manipulação intestinal durante uma cirurgia abdominal convencional resulta em liberação de IL-6 e IL-8, assim como um aumento no recrutamento dos leucócitos no local operado. Contrariamente, nenhum dos mediadores está em níveis elevados, quando uma cirurgia minimamente invasiva é realizada, como a laparoscopia, o que pode explicar em parte uma recuperação mais rápida dos pacientes (The et al., 2007). O período pós-operatório está associado com uma produção aumentada de várias citocinas pró-inflamatórias, conhecidas por aumentar a sensibilidade

dolorosa. O TNF-α, o interferon-gama, a IL-1β, a IL-6 e a IL-8, entre outras, são encontradas em níveis elevados após um trauma cirúrgico (Buvanendran et al., 2006; Clark et al., 2007; Carvalho et al., 2008), embora existam contradições, pois Zahn et al. (2004) demonstrou que o TNF-α parece não estar envolvido na dor pós-operatória em ratos.

Dentre os eicosanóides, a PGE_2 é liberada em maior quantidade após um trauma cirúrgico (Funk et al., 2001), tanto nos tecidos periféricos como no SNC (Buvanendran et al., 2006; Kroin et al., 2006), contribuindo assim para a dor pós-operatória.

A substancia P também parece estar envolvida na dor pós-operatória, uma vez que antagonistas seletivos do receptor NK-1, administrados sistemicamente antes da cirurgia, são capazes de prevenir o desenvolvimento de hiperalgesia em ratos e camundongos (Gonzalez et al., 1998; Sahbaie et al., 2009). De acordo, dados clínicos também mostraram que um antagonista do receptor NK_1 foi capaz de prevenir a dor pós-operatória em humanos (Dionne et al., 1998).

O fator de crescimento do nervo (NGF) é um importante mediador da dor patológica e evidencias sugerem o seu envolvimento na dor pós-operatória, já que níveis elevados deste são encontrados após a cirurgia plantar em ratos. Do mesmo modo, o comportamento de dor espontânea e hiperalgesia térmica após a cirurgia plantar parecem ser dependentes do NGF (Zahn et al., 2004; Banik et al., 2005; Wu et al., 2007; Wu et al., 2009).

Além dos mediadores inflamatórios acima mencionados e a ativação dos seus respectivos receptores, acredita-se que outros receptores estejam envolvidos na nocicepção pós-operatória, como por exemplo, o receptor de potencial transitório vanilóide 1 (TRPV1). Estudos demonstraram que a administração prévia de uma alta dose de capsaicina intra-dérmica foi capaz de prevenir o desenvolvimento de alodínia e hiperalgesia mecânica após a cirurgia plantar em ratos (Pospisilova e Palecek, 2006). Do mesmo modo, um antagonista do receptor TRPV1 foi capaz de reduzir a hiperalgesia térmica, mas não mecânica, induzida pela cirurgia plantar em ratos (Wu et al., 2008).

Durante a lesão pós-operatória ocorre degranulação dos mastócitos com liberação dos mediadores inflamatórios, os quais podem ativar seus receptores

alvo e causar dor. Evidências clínicas indicam que pacientes submetidos à cirurgias minimamente invasivas (por exemplo, laparoscopia) estão associados a uma recuperação mais rápida com um menor grau de degranulação dos mastócitos, quando comparados à cirurgias convencionais (por exemplo, laparotomia) (The et al., 2007).

3.5 Mastócitos, seus medidores e alvos de ativação

Os mastócitos subdividem-se basicamente em 3 tipos, os mastócitos do tecido conjuntivo que contém triptase e quimase e tendem a ser abundantes na derme (>99%); os mastócitos das mucosas que contém mais triptase e pouca quimase e tendem a ser localizadas na mucosa intestinal e a terceira e pequena população de mastócitos que expressa quimase e catepsina G (Metcalfe et al., 1997; Metcalfe, 2008).

Os mastócitos são derivados de células-tronco hematopoéticas e normalmente circulam em sua forma imatura, passando por diferenciação e/ou maturação em tecidos vascularizados ou cavidades serosas em que finalmente vão residir (Metcalfe et al., 1997; Kawakami et al., 2002; Galli et al., 2005). Em mamíferos e outros vertebrados, os mastócitos maduros normalmente residem perto do epitélio, de vasos sanguíneos e de nervos enquanto que nas vias aéreas e no trato gastrointestinal eles residem perto de células musculares lisas e das glândulas produtoras de muco. Os mastócitos são especialmente localizados perto das superfícies expostas ao meio ambiente, incluindo a pele, vias aéreas e no trato gastrointestinal, onde patógenos, alérgenos e outros agentes ambientais são freqüentemente encontrados (Metcalfe et al., 1997; Kawakami et al., 2002; Galli et al., 2005).

Os mastócitos são células de vida longa que proliferam após a estimulação adequada; o recrutamento aumentado e/ou retenção, e maturação local promovem a expansão do número de mastócitos, bem como alterações locais na sua distribuição, como por exemplo, em tecidos com inflamação persistente (Metcalfe et al., 1997; Galli et al., 2005). Em todos os vertebrados, os mastócitos aparecem como células granuladas, sendo que a dimensão da

célula e o conteúdo de grânulos podem expressar variações consideráveis. Eles armazenam em seus grânulos uma série de compostos biologicamente ativos, como a histamina, a serotonina e a triptase (Metcalfe et al., 1997; Galli et al., 2005; Kushnir-Sukhov et al., 2007). Uma variedade de estímulos, químicos, físicos ou patogênicos pode ativá-los conduzindo à sua degranulação e liberação desses mediadores, os quais podem mediar ações pró-inflamatórias ou imunorregulatórias, permitindo uma resposta adequada à diversas respostas imunológicas e patológicas (Galli et al., 2005). Além disso, a capacidade dos mastócitos de secretar várias moléculas biologicamente ativas pode iniciar e/ou promover inflamação aguda ou de longa duração. A liberação dos mediadores dos mastócitos também ocorre pela estimulação com o composto 48/80 (uma poliamina extraída do veneno de abelhas), que após administrações repetidas promove redução dos níveis desses mediadores (Di Rosa et al., 1971).

Dentre os mediadores inflamatórios liberados pelos mastócitos, a histamina, a serotonina e a triptase podem produzir nocicepção ligando-se aos receptores H_1, 5-HT_{2A}, 5-HT_3 ou PAR-2 respectivamente, que estão presentes em nociceptores (Fox et al., 1997; Martin et al., 1998; Ren e Dubner, 2010). Assim, a degranulação dos mastócitos parece estar envolvida em diversos processos dolorosos patológicos, como salientado separadamente para cada mediador e seu receptor alvo, conforme será comentado à seguir.

Durante as respostas inflamatórias periféricas os mastócitos são a principal fonte de histamina e outros mediadores que sensibilizam os nociceptores e produzem resposta nociceptiva (Leon, 1994; Ren e Dubner, 2010). A resposta a alguns agonistas altamente seletivos do receptor H_1 (H_1R) da histamina (Malmberg-Aiello et al., 1998) e a capacidade de alguns antagonistas seletivos ou não seletivos deste receptor de reduzir a dor induzida por histamina em modelos experimentais (Raffa et al., 2001) é consistente com a evidencia que a histamina desempenha um importante papel em processos dolorosos. O H_1R é um receptor acoplado à proteína G e está localizado por todo o SNP e no SNC, podendo mediar respostas nociceptivas em humanos e animais (Haas et al., 2008).

Foi verificado em camundongos deficientes do H_1R respostas dolorosas significativamente reduzidas em testes de nocicepção térmica, nocicepção mecânica e nocicepção química, quando comparados aos camundongos selvagens. Estes dados indicam que a histamina desempenha um importante papel na dor somática e visceral através de H_1R (Mobarakeh et al., 2000). Outros trabalhos relatam que a administração intraplantar de antagonistas do H_1R é capaz de reduzir a primeira e a segunda fase nociceptiva induzidas por formalina em roedores (Parada et al., 2001; Olsen et al., 2002). Além disso, Liu et al. (2007) demonstrou que a administração intraplantar do veneno de um escorpião na pata de ratos promove degranulação de mastócitos, nocicepção espontânea e hiperalgesia mecânica, enquanto que a co-administração intraplantar de um antagonista do H_1R juntamente com o veneno do escorpião é capaz de bloquear as respostas nociceptivas espontânea e mecânica.

Além dos estudos pré-clínicos, estudos clínicos demonstram que pacientes previamente tratados com antagonistas do H_1R requerem doses menores de morfina para promover analgesia após procedimentos cirúrgicos (Bellville et al., 1979; Chia et al., 2004). Além disso, dados da literatura sugerem que a histamina liberada dos mastócitos está envolvida com a dor abdominal relatada pelos pacientes com síndrome do intestino irritável (Barbara et al., 2004; Barbara et al., 2007; Cenac et al., 2007).

Outro mediador inflamatório é a serotonina (5-HT), a qual pode ser liberada de plaquetas, mastócitos residentes ou basófilos que infiltram uma área de lesão tecidual (Dray, 1995; Levine e Reichling, 1999). Uma vez liberada, a 5-HT pode interagir com uma série de subtipos de receptores expressos em nociceptores aferentes primários, incluindo os receptores $5\text{-}HT_3$ ($5\text{-}HT_3R$) e $5\text{-}HT_{2A}$ ($5\text{-}HT_{2A}R$) que estão envolvidos na sensibilização das fibras aferentes primárias na periferia (Martin et al., 1998; Tokunaga et al., 1998; Okamoto et al., 2002).

A expressão de receptores em aferentes primários, que transmitem estímulos sensoriais e nociceptivos da periferia para o SNC, torna-os alvo interessante para investigação da percepção da dor (Walstab et al., 2010). O $5\text{-}HT_3R$ é um canal iônico ativado por ligante e está presente tanto no SNC quanto no SNP (Machu et al., 2011), estando envolvido na êmese, na dor e em

distúrbios gastrointestinais (Walstab et al., 2010). Já o 5-HT_{2A}R é um receptor metabotrópico e está amplamente distribuído em tecidos periféricos (Bradley et al., 1986) e também está envolvido em processos dolorosos (Sasaki et al., 2006).

A injeção periférica de 5-HT provoca respostas nociceptivas (Sufka et al., 1991) as quais são atenuadas por antagonistas seletivos dos 5-HT_{2A} ou 5-HT_3R (Richardson et al., 1985; Sufka et al., 1992). Estudos comportamentais em camundongos deficientes do 5-HT_3R confirmam o envolvimento deste receptor na nocicepção, a qual está ausente após a administração intraplantar de serotonina nestes (Zeitz et al., 2002). A inibição do 5-HT_3R pela ondansetrona foi capaz de prevenir o desenvolvimento da dor crônica em ratos (Suzuki et al., 2004). Além disso, o receptor 5-HT_3 parece estar envolvido na nocicepção induzida por formalina (Kayser et al., 2007). Além do 5-HT_3R, dados da literatura demonstram que os 5-HT_{2A}R estão envolvidos na alodínia mecânica induzida por lesão térmica na pata de ratos (Sasaki et al., 2006). Em humanos, a serotonina está envolvida na dor que ocorre em pacientes com síndrome do intestino irritável (Klooker et al., 2010; Cenac et al., 2007).

O terceiro mediador salientado aqui é a triptase, que é a protease mais proeminente liberada pelos mastócitos em seres humanos (Schwartz et al., 1987), embora outras serina proteinases foram identificadas em mastócitos de ratos (Lutzelschwab et al., 1997) e camundongos (Reynolds et al., 1990). Dessas serina proteinases, apenas a triptase é conhecida por ser um potente ativador do receptor ativado por protease 2 (PAR-2) (Fox et al., 1997). O PAR-2 é um receptor acoplado à proteína G expresso em neurônios aferentes primários nociceptivos (Vergnolle et al., 2001). Para ativar PAR-2, a triptase cliva a porção N-terminal extracelular do PAR, deixando livre uma nova seqüência N-terminal. Esta seqüencia N-terminal irá se ligar há segunda alça do PAR-2 desencadeando uma cascata de eventos intracelulares (Vergnolle et al., 2009).

Vergnolle et al. (2001) demonstrou, usando várias abordagens, a complexidade funcional dos processos de ativação de aferentes sensoriais durante a estimulação nociva. Agonistas PAR-2 promovem hiperalgesia mecânica, como faz a administração intraplantar de tripsina e triptase em

camundongos selvagens, mas não em camundongos deficientes PAR-2. Em animais tipo selvagem, doses sub-inflamatórias de peptídeos que ativam PAR-2 demonstram ser hiperalgésicas. O envolvimento direto de neurônios nociceptivos que expressam PAR-2 na hiperalgesia foi demonstrado pela imunorreatividade aumentada do Fos nas lâminas I e II da medula espinhal após a injeção intraplantar de peptídeos que ativam PAR-2 ou triptase.

O PAR-2 foi identificado pela primeira vez como alvo potencial para o desenvolvimento de terapias para a artrite reumatóide, mas apesar do PAR-2 estar envolvido no processo inflamatório que acomete a maioria dos pacientes com artrite, não há estudos que demonstrem a sua participação no processo doloroso induzido por artrite (Ferrell et al., 2003; Kelso et al., 2006; Ferrell et al., 2010). Contudo, vários estudos sugerem a participação do PAR-2 na dor inflamatória em outros órgãos, o que sugere o seu envolvimento na amplificação de sinais dolorosos (Vergnolle et al., 2001b, 2003a,b; Cenac e Vergnolle, 2005; Dale e Vergnolle, 2008).

Além do envolvimento do PAR-2 em doenças artríticas, foi demonstrado sua participação em pacientes com SII após a liberação de triptase (Bárbara et al., 2002). Foi observado um aumento da atividade proteolítica no meio de cultura de biópsias de pacientes com síndrome do intestino irritável em comparação com biópsias de pacientes controles. Além disso, quando o sobrenadante de biópsias de pacientes com síndrome do intestino irritável foi administrado no cólon de camundongos do tipo selvagem, ele causou hiperalgesia e alodínia somática e visceral. Entretanto, estes efeitos pró-nociceptivos foram inibidos por inibidores das serinas proteases, bem como por um antagonista PAR-2 e esteve ausente em camundongos deficientes do PAR-2 (Cenac et al., 2007). Isto demonstra o envolvimento do PAR-2 e de proteases na dor visceral humana.

Otimizar o tratamento da dor após a cirurgia é importante para aumentar o conforto do paciente e acelerar a recuperação. A etiologia da dor pós-operatória é ainda mal compreendida, e os mecanismos envolvidos são provavelmente múltiplos, incluindo lesão tecidual relacionada com a própria incisão, inflamação secundária, e danos aos nervos causados pela retração do

tecido durante a cirurgia, o que poderia explicar a natureza persistente e a alta incidência de dor após as cirurgias (Flatters, 2008).

Os pacientes que tem um bom controle da dor apresentam melhor qualidade de vida e maior satisfação com a sua experiência, além de reduzida morbidade e mortalidade após a cirurgia (Kehlet et al., 2006; Gandhi et al., 2011). Por isso compreender os mecanismos que promovem a dor pós-operatória assim como abordar novos alvos terapêuticos para combatê-la são de grande importância para aumentar o conforto do paciente e acelerar a sua recuperação.

Ao serem ativadas, as células inflamatórias como os mastócitos liberam seus mediadores inflamatórios, histamina, serotonina e triptase. Estudos prévios têm demonstrado que os mediadores dos mastócitos estão envolvidos em alguns processos dolorosos (Barbara et al., 2007; Levy et al., 2007; De Winter et al., 2011) e devido ao fato de que eles degranulam dentro de horas seguida da incisão tecidual (Egozi et al., 2003), ou lesão pós-operatória onde há manipulação do tecido operado (The et al., 2007), acredita-se que os mastócitos também podem contribuir para o desenvolvimento da dor pós-operatória via ativação dos receptores alvo - H_1, 5-HT_{2A}, 5-HT_3 e PAR-2 - os quais estão envolvidos em diferentes processos inflamatórios e dolorosos.

4. RESULTADOS

4.1 ARTIGO E MANUSCRITO CIENTÍFICOS

Os resultados inseridos nesta tese apresentam-se sob a forma de artigo e manuscrito científicos, os quais se encontram aqui estruturados. Os itens Introdução, Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no artigo e no manuscrito. O artigo está disposto na mesma forma a qual foi publicado na revista científica European Journal of Pharmacology. O manuscrito está disposto da mesma maneira que será submetido à revista Anesthesiology.

4.1.1 Artigo: Involvement of mast cells in a mouse model of postoperative pain

Sara Marchesan Oliveira, Carine Cristiane Drewes, Cássia Regina Silva, Gabriela Trevisan, Suelen Lucio Boschen, Camila Guimaraes Moreira, Daniela de Almeida Cabrini, Claudio Da Cunha, Juliano Ferreira

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Neuropharmacology and Analgesia

Involvement of mast cells in a mouse model of postoperative pain

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ABSTRACT

Recent studies have indicated that nearly half of all surgical patients still have inadequate pain relief; therefore, it is becoming increasingly more important to understand the mechanisms involved in postoperative pain in order to be better treated. Previous studies have shown that incisions can cause mast cell degranulation. Thus, the aim of this study was to investigate the involvement of mast cells in a model of postoperative pain in mice. The depletion of mast cell mediators produced by pre-treatment with compound 48/80 (intraplantar (i.pl.)) widely (98 ± 23% of inhibition) and extensively (up to 96 h) prevented postoperative nociception and reduced histamine and serotonin levels (88 ± 4% and 68 ± 10%, respectively) in operated tissue. Furthermore, plantar surgery produced immense mast cell degranulation, as assessed by histology and confirmed by the increased levels of serotonin (three-fold higher) and histamine (fifteen-fold higher) in the perfused tissue, 1 h after surgery. Accordingly, pre-treatment with the mast cell membrane stabilizer cromoglycate (200 μg/paw, i.pl.) prevented mechanical allodynia (inhibition of 96 ± 21%) and an increase in histamine (44 ± 10% of inhibition) and serotonin (73 ± 5% of inhibition) levels induced by plantar surgery. Finally, local treatment with H_1 (promethazine, 100 μg/paw, i.pl.), 5-HT_3 (ondansetron, 10 μg/paw, i.pl.) or 5-HT_{2A} (ketanserin, 5 μg/paw, i.pl.) receptor antagonists partially decreased postoperative nociception in mice, but when co-administered together it completely reversed the mechanical allodynia in operated mice. Thus, mast cell activation mechanisms are interesting targets for the development of novel therapies to treat postoperative pain.

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1. Introduction

A common cause of acute inflammatory pain is the postoperative pain (Brennan et al., 1996; Zahn et al., 2005). Recently, it was estimated that every year, 234.2 millions of surgeries are performed around the world, and they are associated with mortality and morbidity rates reaching up to 10 and 16%, respectively (Weiser et al., 2008). Effective postoperative analgesia improves the satisfaction of the patients and decreases morbidity and mortality after surgery (Kehlet et al., 2006). Until recently, clinical treatment of postoperative pain has been based on the utilization of preventive techniques and pain control after the incision is made (Dahl and Kehlet, 2006; Moiniche et al., 2002). The main drugs that are clinically used to produce postoperative analgesia are nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, ketamine, peripheral local anesthetics and gabapentin. However, it is known that these drugs can cause collateral effects that often limit their use (Dahl and Kehlet, 2006). Furthermore, studies have demonstrated that 50–70% of surgical patients still experienced moderate to severe postoperative pain and that a significant number of these patients reported inadequate pain relief (Dolin et al., 2002; Nossaman et al., 2010; Pogatzki-Zahn et al., 2007).

The mechanisms of postoperative pain involve activation, modulation and modification on the peripheral, spinal and cerebral levels (Vadivelu et al., 2010; Wilder-Smith and Arendt-Nielsen, 2006). Peripherally, the place of incision presents signs of inflammation including edema, local hyperthermia, hyperemia and nociception (Clark et al., 2007; Swarm et al., 2001). Inflammatory processes are maintained through vascular and cellular events, such as the release of inflammatory mediators by resident cells (Basbaum et al., 2009; Marchand et al., 2005; Metcalfe et al., 1997; Ren and Dubner, 2010). Mast cells are resident cells of connective tissue that participate in the immune response and are mainly found in the subcutaneous tissue and mucosa (Brown et al., 2008). Among the inflammatory mediators released by mast cells, histamine and serotonin can produce nociception by binding to the H_1 and 5-HT_2 or 5-HT_3 receptors, respectively, which are present in primary afferent neurons (Bileviciute, et al., 1998,

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George et al., 2005; Kuner, 2010; Ren and Dubner, 2010; Wei et al., 2005; Zeitz et al., 2002).

Reinforcing the role of mast cell mediators in painful processes, previous studies have shown that mast cells are involved in producing the pain that occurs in patients with inflammatory bowel disease or headaches (Barbara et al., 2007; De Winter et al., 2011; Levy et al., 2007). Apart from these painful conditions, mast cells might also be involved in postoperative pain because they degranulate and are drastically decreased in number within hours following tissue incision (De Winter et al., 2011; Egozi et al., 2003). Because mast cells appear to be involved in the inflammatory processes that occur after incision, the aim of the present work was to investigate the involvement of mast cells in a mouse postoperative pain model.

2. Materials and methods

2.1. Animals

The experiments were conducted using male Swiss mice (25–35 g) that had free access to food and water and were maintained in a temperature-controlled room (22 ± 2 °C) under a 12-h light–dark cycle before and after surgery. Animals were acclimatized to the laboratory for at least 2 h before experiments and were used only once. The experiments were performed with the approval of Ethics Committee of the Universidade Federal de Santa Maria (process number 45/2010) and were carried out in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.2. Drugs

Promethazine and ondansetron were obtained from Cristália (São Paulo, Brazil). Ketanserin tartarate was obtained from Tocris Cookson (Ellisville, USA). Compound 48/80, sodium cromoglycate, lidocaine, perchloric acid, phthaldialdehyde and metronidazole were obtained from Sigma Chemical Company (St. Louis, USA). All compounds were diluted in a phosphate-buffered solution (PBS; 10.71 mM K_2HPO_4, 6.78 mM NaH_2PO_4, 120.4 mM NaCl, pH 7.4). We have used PBS to avoid that pH alterations in solution could interfere with the nociceptive test (Meotti et al., 2010). For the histological procedures, we used paraffin, formaldehyde, ethanol, acetic acid, xylol and toluidine blue obtained from Merck (Rio de Janeiro, Brazil).

2.3. Postoperative pain model

The postoperative pain model was carried out according to the procedure described for rats (Brennan et al., 1996) adapted for mice (Pogatzki and Raja, 2003). Mice were anesthetized with 2% halothane via a nose cone. After anti-septic preparation of the right hind paw with 10% povidone–iodine solution (PVPI), a 5 mm longitudinal incision was made with a number 11 blade through the skin and fascia of the plantar foot. The incision was started 2 mm from the proximal edge of the heel and extended toward the toes. The underlying muscle was elevated with a curved forceps, leaving the muscle origin and insertion intact. The skin was apposed with a single mattress suture of 6.0 nylon.

For the behavioral assessment of mechanical allodynia, mice were placed individually in clear plexiglass boxes (7 × 9 × 11 cm) on elevated wire mesh platforms to allow access to the ventral surface of the hind paws. Before the test procedures, the animals remained in the box for approximately 1.5 h for behavioral accommodation. The mechanical stimulus was applied in the plantar surface. The mechanical threshold was determined before and after incision with flexible nylon von Frey filaments using the Up-and-Down method, which consisted of six applications of a particular filament applied once every 3–4 s. Testing was initiated with the 0.4 g filament in the middle of the series. The response was defined as a withdrawal of the stimulated paw. In the absence of a response to a particular filament, the next strongest filament was utilized, and in the case of a response, the next weakest filament was presented (Chaplan et al., 1994). Mechanical allodynia was considered as a decrease in the threshold when compared with the same paw before surgery (basal value). The values obtained at each time after surgery were compared to basal values unique to each animal. The mechanical allodynia of all groups was assessed 0.5, 1, 2, 4, 6, 24, 48, 72 and 96 h after incision, when necessary.

2.4. Treatments

A selective H_1 receptor antagonist (promethazine, 100 μg/paw, i.pl.), a selective 5-HT_3 receptor antagonist (ondansetron, 10 μg/paw, i.pl.), a selective 5-HT_{2A} receptor antagonist (ketanserin, 5 μg/paw, i.pl.) or vehicle (PBS, 20 μl/paw, i.pl.) was administered 0.5 h before the incision procedure. Sodium cromoglycate (200 μg/paw, i.pl.), a mast cell membrane stabilizer, was administered 15 min before the incision procedure. The mast cell degranulator compound 48/80 was administered daily at increasing doses (1, 3, 10 and 10 μg/paw, i.pl.). The dosages and timing of the drugs were obtained from previous studies (Bileviciute et al., 1998; Jaffery et al., 1994; Parada et al., 2001; Piovezan et al., 2004; Wei et al., 2005; Zeitz et al., 2002). The effects of the treatments on nociception were assessed just until the end of the antinociceptive effect to avoid unnecessary discomfort for the animals.

Another group of animals, which did not undergo the incisional procedure, received intraplantar administration of sodium cromoglycate, promethazine, ondansetron or ketanserin as described above to determine whether these compounds alone altered the mechanical threshold of the animals. Lidocaine, a local anesthetic, was used as a positive control (Wang et al., 2010).

2.5. Measurement of histamine and serotonin levels

To confirm the mast cell depletion produced by repeated compound 48/80 treatment, separate groups of mice were killed by cervical dislocation 24 h after the last injection of compound 48/80. Histamine and serotonin levels were measured in homogenates of the paw skin of animals, as described in detail below.

Histamine levels were evaluated as previously described by Anton and Sayre (1968). Paw skin samples were homogenized in PBS (50 mM, pH 7.4) with 1 mM metronidazole (a histamine methyl transferase inhibitor used to reduce histamine degradation), centrifuged at 12,000 ×*g*, 4 °C for 10 min, and the resulting supernatants were used to evaluate histamine content. Then, 150 μl of NaOH (1 M) was added to 400 μl of supernatant and incubated with 40 μl of 1% *o*-phthaldialdehyde (OPT). Next, 75 μl of HCl (3 M, pH 10.4) was added to stop the reaction and to allow for the development of fluorescence. Samples were read with an excitation wavelength of 360 nm and an emission wavelength of 450 nm using a fluorescence photometer.

Serotonin levels were evaluated as previously described by Vanable (1963). Paw tissue samples were homogenized in 500 μl perchloric acid (0.4 M) and centrifuged at 1000 ×*g* for 10 min. Then, 300 μl of the supernatants was combined with 125 μl of borate buffer (0.5 mM, pH 10, NaCl saturated) and 3 ml of 1-butanol. This solution was mixed for 10 min. The organic phase was then separated, incubated with 350 μl of phosphate buffer (0.05 M, pH 7.4) and 3 ml of *n*-heptane and mixed for 2 min. The aqueous phase (500 μl) was incubated with 500 μl of ninhidrin (0.24%) and 500 μl of phosphate buffer (100 mM, pH 7.0). The reaction was incubated at 100 °C for 10 min and allowed to rest at room temperature in the dark for 5 h. The levels of fluorescence were

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read using an excitation wavelength of 380 nm and an emission wavelength of 500 nm with a fluorescence photometer. To confirm the serotonin levels determined by the fluorimetric method, we also detected its level by a reverse-phase HPLC (High Performance Liquid Chromatography) with electrochemical detection (ED). The system consisted of a Synergi Fusion-RP C-18 reverse-phase column (150×4.6 mm i.d., 4-um particle size, Phenomenex, Torrance, CA, USA), a dual coulometric electrochemical detector (Coulochem III, ESA, Chelmsford, USA), and an LC-20AT pump (Shimadzu, Kyoto, Japan). This detector consisted of two cells successively connected, both containing a porous graphite working electrode together with associated reference and counter electrodes. The detector was equipped with a guard cell (ESA 5020) electrode set at +350 mV and the working electrodes (5011 analytical cell, ESA) set at E1 = +100 and E2 = +450 mV versus a solid state palladium reference electrode. The column was maintained inside a temperature-controlled oven (25 °C). The tissue samples were homogenized with an ultrasonic cell disrupter (Sonics, Newtown, CT, USA) in 0.1 M perchloric acid. After centrifugation at 15,000 ×g for 30 min, 20 µl of the supernatant was injected into the chromatograph. The mobile phase, used at a flow rate of 1 ml/min, had the following composition: 20 g citric acid monohydrated, 200 mg octane-1-sulfonic acid sodium salt, 40 mg ethylenediaminetetraacetic acid (EDTA), 900 ml HPLC-grade water, 10% methanol and pH 4. The peak areas of the external standards were used to quantify the sample peaks.

Experiments were performed to verify whether the surgical procedure promotes degranulation of mast cells and whether sodium cromoglycate is capable of protecting mast cell membranes by hindering its degranulation after incision. Separate groups of mice received an intraplantar administration of sodium cromoglycate or PBS, and after 15 min, they were subjected to a surgical or sham procedure. After 1 or 48 h of surgery, the mice were sacrificed by cervical dislocation, and the operated or sham-operated paws were perfused as previously described (Ferreira et al., 2004). A double polyethylene tube was inserted into the subcutaneous space of the paw, and 100 µl of PBS (or PBS plus metronidazole for histamine) was perfused at a rate of 200 µl min^{-1} through the inner tube, after which the perfusate was collected through the outer tube. Histamine and serotonin levels in the perfusates were measured by fluorimetric methods as described above.

2.6. Histology

To confirm the degranulation of mast cells in the tissues of the right hind paws of the animals that received or did not receive a plantar incision, we carried out histological analyses. Samples were collected 1 h after the incision or sham procedure. Mice were sacrificed, and their paws were removed and fixed in alfac solution (16:2:1 mixture of ethanol 80%, formaldehyde 40% and acetic acid) and then decalcified. Each sample was embedded in paraffin wax, sectioned at 5 µm and stained with toluidine blue. A representative area was selected for qualitative light microscopic analysis of the inflammatory cellular response with a 10× and 100× objective (Recio et al., 2000). To minimize any source of bias, the investigator analyzing the samples did not know the identity of the group that he was analyzing.

2.7. Statistical analyses

The results are expressed as means + S.E.M. Data were analyzed by a Student's t-test or one- or two-way analysis of variance (ANOVA). P values less than 0.05 ($P<0.05$) were considered significant, and F values presented in the text are demonstrated by treatment versus time interactions only when $P<0.05$.

3. Results

The depletion of mast cell mediators produced by the repeated treatment of animals with compound 48/80 (1, 3, 10 and 10 µg/paw, i.pl.) markedly decreased mechanical allodynia (inhibition of 98 ± 23%) in mice after plantar incision [$F(1, 112) = 0.92$, $P<0.0001$; Fig. 1A]. This anti-allodynic effect occurred 1 to 96 h after surgery. Confirming that mast cell degranulation occurred, the repeated treatment of animals with compound 48/80 also significantly decreased the levels of histamine and serotonin (88 ± 4% and 68 ± 10%, respectively) present in the skin of the injected paw, when evaluated by fluorimetric methods (Fig. 1B and C). Likewise, there was a decrease of the same magnitude of serotonin levels in the skin paw of animals injected with compound 48/80 (decrease of 65 ± 6%), when evaluated by HPLC method (the levels of serotonin in the paw injected with PBS or 48/80 were 0.26 ± 0.08 or 0.096 ± 0.01 ng/mg of tissue, $P<0.05$, Student's t test). Histological analyses revealed that the surgical procedure clearly promoted mast cell degranulation in paw tissue 1 h after surgery has been made (Fig. 2C). In animals that were submitted to sham procedure, we only verified the presence of intact mast cells (Fig. 2A). We confirmed that mast cell degranulation resulted in increases in the levels of serotonin (threefold) and histamine (fifteen-fold) in the tissue perfusate of operated mice, 1 h but not 48 h after surgery, when compared to the levels of sham-operated animals (Fig. 3B and C).

Treatment with the mast cell stabilizer cromoglycate (200 µg/paw, i.pl.) significantly reduced mechanical allodynia (inhibition of 96 ± 21%) when compared to the control [$F(1, 98) = 11.92$, $P<0.0001$; Fig. 3A]. This effect occurred from 0.5 to 24 h after plantar incision. Likewise, local cromoglycate treatment (200 µg/paw, i.pl.) also significantly prevented the increase in the levels of histamine (44 ± 10% of inhibition) and serotonin (73 ± 5% of inhibition) in the perfused tissue of operated, but not sham-operated, animals at 1 h after surgery (Fig. 3D and E). However, cromoglycate (200 µg/paw, i.pl.) administered 7 h after surgery did not cause antinociceptive effect from 8 to 96 h after surgery (data not shown).

Similarly to treatment with cromoglycate, treatment with the H_1 receptor antagonist promethazine (100 µg/paw, i.pl.) partially but significantly reduced mechanical allodynia in the animals after plantar incision when compared to controls [$F(1,84) = 9.527$, $P<0.0001$; Fig. 4A]. This effect was observed from 0.5 to 6 h after plantar incision. Pre-treatment with the selective 5-HT_3 receptor antagonist ondansetron (10 µg/paw, i.pl.) or with the selective 5-HT_{2A} receptor antagonist ketanserin (5 µg/paw, i.pl.) also partially decreased the allodynia after plantar surgery [$F(1, 56) = 6.4$, $P<0.0001$; Fig. 4B], [$F(1, 44) = 6.9$, $P<0.0001$; Fig. 4C], respectively. This reduction in the mechanical allodynia was significant 0.5, 1 and 2 h after incision. When the three antagonists were co-administered together they completely reversed mechanical allodynia with maximum effect in 2 h (100% of inhibition; $F(1, 54) = 5.7$, $P<0.0001$; Fig. 4D). This effect was observed from 0.5 to 6 h after plantar incision.

Finally, we verified that local administration of cromoglycate, promethazine, ondansetron or ketanserin did not alter the mechanical threshold of sham-operated animals (Supplemental Fig. 1). Conversely, lidocaine, which was used as a positive control, increased the mechanical threshold [$F(1,40) = 7$, $P<0.05$] of sham-operated animals 1 to 6 h after the procedure (Supplemental Fig. 1).

4. Discussion

Postoperative pain is a common form of acute pain. Several studies have indicated that effective postoperative analgesia reduces morbidity following surgery, thereby improving patient outcome and reducing clinical expenses. Adequate knowledge regarding treatment of postoperative pain is important to reduce the morbidity and mortality of patients after surgery (Kehlet et al., 2006; Pogatzki-Zahn et al.,

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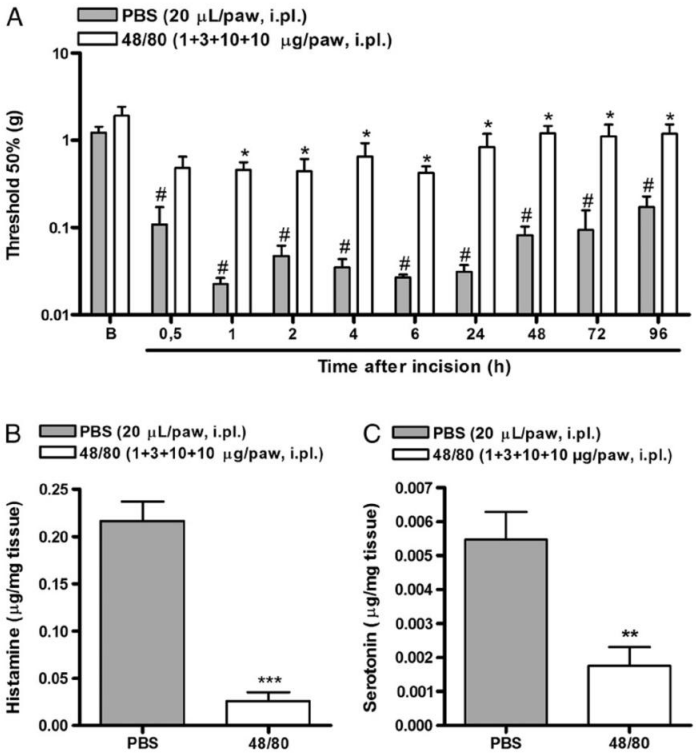

Fig. 1. Effect of pre-treatment for 4 days (1 + 3 + 10 + 10 µg/paw) of compound 48/80 on mechanical allodynia (A) or on histamine (B) or serotonin (C) levels after plantar incision in mice. The vertical bars represent the means of 8 animals + standard errors of the mean for (A) and the means of 5–6 animals + standard errors of the mean for (B) and (C). *P<0.05 when compared to the PBS-treated group; Student's t-test. #P<0.01 when compared to baseline; one-way ANOVA followed by a Student–Newman–Keuls test.

2007). However, recent surveys have demonstrated that about 50–70% of patients experience moderate to severe pain after surgery, indicating that despite the development of new drugs and improved analgesic techniques, postoperative pain remains under-evaluated and poorly treated (Pogatzki-Zahn et al., 2007). It is important to obtain a better understanding of the cellular events that control the peripheral mechanisms of postoperative pain (Wilder-Smith and Arendt-Nielsen, 2006). In the present study, we demonstrated that

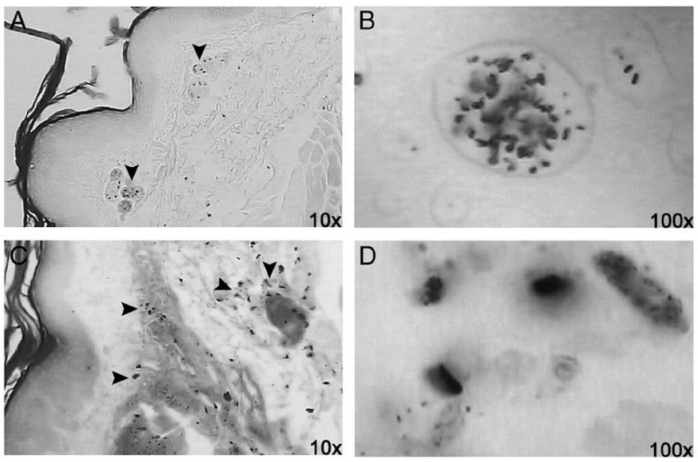

Fig. 2. Representative light microphotograph showing the presence of mast cells after sham (A, B) or surgical (C, D) procedures in the paw tissue of mice. A section was obtained from the paw tissue 1 h after the surgical or sham procedures. (A, B) Granulated mast cells; (C, D) degranulated mast cells.

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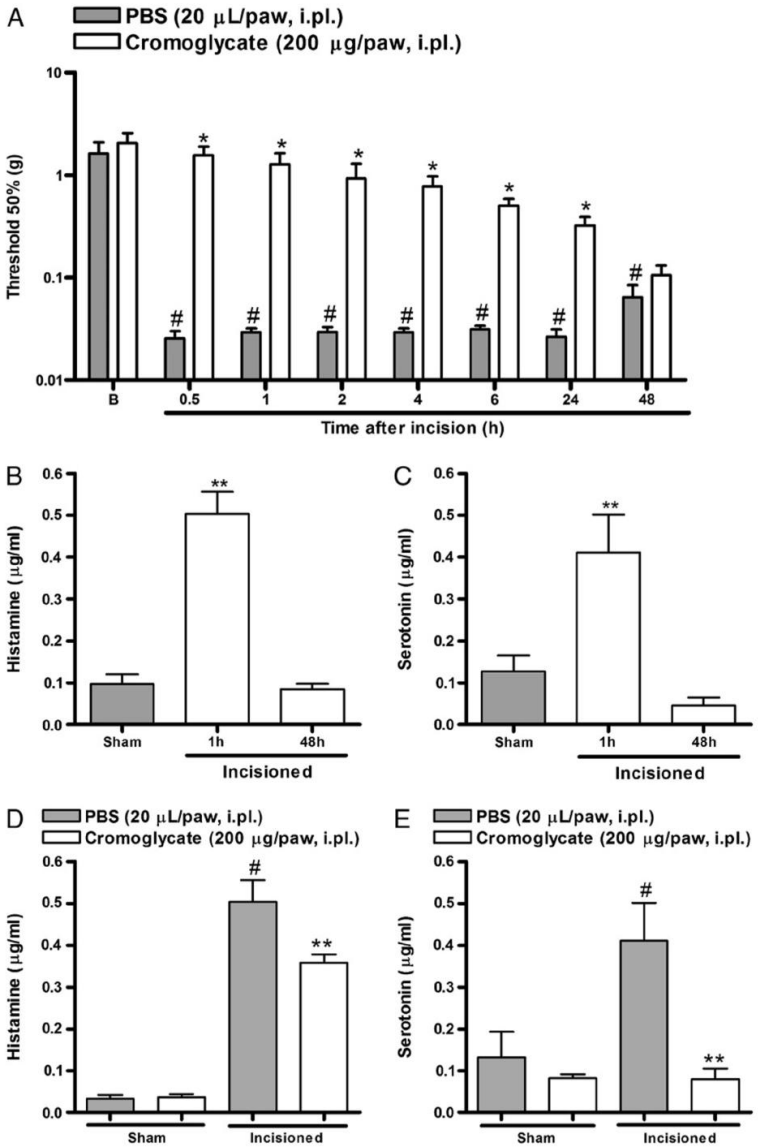

Fig. 3. Effect of cromoglycate (200 µg/paw, i.pl.) on mechanical allodynia (A) or on the increase of histamine (D) and serotonin (E) levels 1 h after plantar incision in mice. The vertical bars represent the means of 8 animals + standard errors of the mean for (A) and the means of 5 animals + standard errors of the mean for (D) and (E). (A) *P<0.05 when compared to the PBS-treated group; Student's t-test. #P<0.01 when compared to baseline; one-way ANOVA followed by a Student–Newman–Keuls test. (D, E) #P<0.01 as compared to sham-PBS-treated mice; **P<0.01 compared to the respective control group (one-way ANOVA followed by Student–Newman–Keuls test). (B, C) Effect of surgery on histamine (B) and serotonin (C) levels 1 or 48 h after plantar incision in mice. The vertical bars represent the means of 5–8 animals + standard errors of the mean for (B) and (C); **P<0.01 compared to sham group (one-way ANOVA followed by Student–Newman–Keuls test).

mast cell activation is essential to the development of postoperative nociception.

First, we demonstrated that the depletion of mast cell mediators by degranulation after pre-treatment with compound 48/80 reduced postoperative nociception. A previous study has described that compound 48/80 may lead to non-cytotoxic degranulation of mast cells by retaining the integrity of their plasma membranes and by promoting the release of their mediators (Bundoc and Myers, 2007). In accordance with previous findings (Di Rosa et al., 1971), we demonstrated that pre-operative mast cell degranulation induced by repeated compound 48/80 treatment reduced the levels of mast cells mediators, such as histamine and serotonin, in the injected tissue. Moreover, we found that surgery in the mast cell mediator-depleted tissue efficiently and extensively reduced postoperative nociception in mice, indicating that mast cell activation is important for the development of postoperative pain.

We also observed histologically that 1 h after surgery, a time when we detected a high-intensity allodynia, there was immense mast cell

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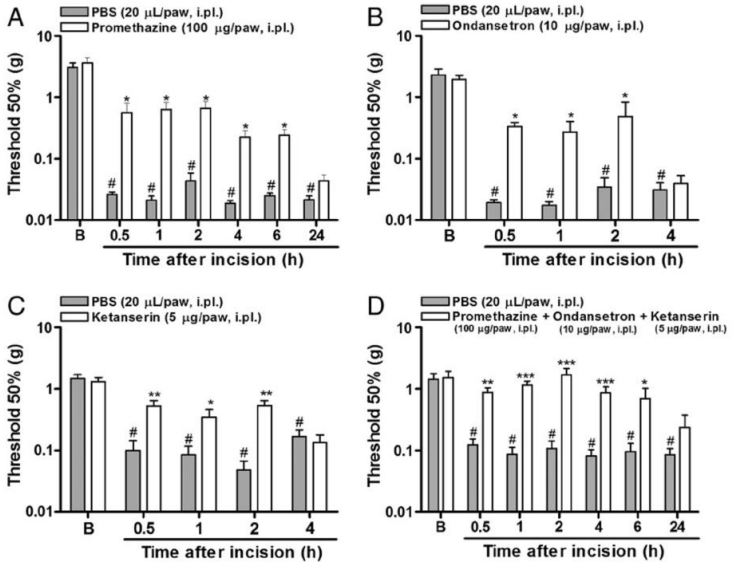

Fig. 4. Effect of pre-treatment with promethazine (100 µg/paw, i.p.l.) (A), ondansetron (10 µg/paw, i.p.l.) (B), ketanserin (5 µg/paw, i.p.l.) (C) or of the combination of promethazine (100 µg/paw, i.p.l.), ondansetron (10 µg/paw, i.p.l.) plus ketanserin (5 µg/paw, i.p.l.) (D) on mechanical allodynia after plantar incision in mice. The verticals bars represent the means of 8 animals + standard errors of the mean. *P<0.05 when compared to the PBS-treated group; Student's t-test. #P<0.01 when compared to baseline; one-way ANOVA followed by a Student–Newman–Keuls test.

degranulation in the incised skin. Similar findings were found by Weller et al. (2006), who demonstrated that the majority of skin mast cells directly adjacent to a skin wound in mice exhibit extensive degranulation. Moreover, at 1 but not 48 h, after the surgical procedure, we also observed that there was an increase in the levels of serotonin and histamine in the tissue perfusate, indicating that the incision produced an early release of mast cell mediators. To support the idea that this cell type is involved in nociception induced by an incision, we treated the animals with sodium cromoglycate. Cromoglycate is capable of protecting mast cell membranes, thereby hindering their degranulation (Parada et al., 2001). According to the results obtained from mast cell depletion, the cromoglycate pre-treatment was capable to reduce nociception and prevent an increase in the levels of released histamine and serotonin induced by surgical injury after 1 h of surgery. However, when cromoglycate was administered few hours after surgical procedure, it was unable to prevent the nociception indicating that mast cell degranulation had already occurred. Thus, the degranulation of mast cells and the release of their pro-nociceptive mediators appear to mediate the development of postoperative nociception in mice.

It is known that pre-formed and neo-formed factors of mast cells are involved in situations where they are activated. Histamine is an important inflammatory mediator that is present in mast cell granules (Metcalfe et al., 1997). The administration of histamine in human skin is generally associated with the generation of intense pruritus, erythema and edema (Carstens, 1997). However, some studies have demonstrated that during a peripheral inflammatory response as well as during an incision procedure, mast cells are the main source of histamine and various other mediators (e.g., serotonin) that sensitize nociceptors and produce nociceptive response (Basbaum et al., 2009; Leon et al., 1994; Parada et al., 2001; Ren and Dubner, 2010; Zuo et al., 2003). Furthermore, Ferreira (1972) showed that when histamine is given intradermically in humans, it causes moderate to strong pain. Notably, nociceptors possess a great number of histaminergic H_1 receptors (Sawynok 2003). Confirming the participation of histamine liberated from mast cells in nociception induced for incision, we verified that promethazine, a selective antagonist of H_1 receptors (Bileviciute, et al., 1998), can reduce incision-induced nociception. Our results are in agreement with data from the literature demonstrating that preoperative administration of H_1 receptor antagonists reduces postoperative morphine consumption and the need for pain treatment in patients (Chia et al., 2004; Tarkkila et al., 1995). Thus, administration of H_1 receptor antagonists may partly prevent the establishment of peripheral sensitization, thereby reducing postoperative pain.

Serotonin [5-hydroxytryptamine (5-HT)], a major neurotransmitter component of the inflammatory chemical milieu, is released by mast cells (Dray, 1995; Kushnir-Sukhov et al., 2007). Once released, serotonin is free to interact with a number of molecularly distinct receptor subtypes (Hamon and Bourgoin, 1999; Martin et al., 1998). The 5-HT_3 and 5-HT_{2A} receptors are the main serotoninergic receptors present in nociceptors (Millan, 2002; Wei et al., 2005; Zeitz et al., 2002). Similar to H_1 receptor antagonists, we also verified that pre-treatment with peripheral administration of the 5-HT_3 receptor antagonist ondansetron or of the 5-HT_{2A} receptor antagonist ketanserin also partially prevented mechanical allodynia induced by incision in the mouse paw, indicating a role for serotonin in postoperative pain. In contrast to our pre-clinical study, clinical studies have shown that ondansetron administered systemically or a few hours after surgery does not have an analgesic effect itself and does not alter the consumption of analgesics (Jokela et al., 2010; Rauers et al., 2010). The discrepancies between our pre-clinical and the clinical studies might be explained by the fact that 5-HT_3 receptor activation has been described to induce nociception in the periphery, but not in the central nervous system (Alhaider et al., 1991; Bardin et al., 1997; Parada et al., 2001; Tambeli et al., 2006; Walstab et al., 2010). The same has been described to 5-HT_{2A} receptor activation (Abbott et al., 1997; Nakajima et al., 2009; Obata et al., 2001; Sasaki et al., 2001; Wei et al., 2005). Because we injected ondansetron or ketanserin locally at the site of incision, the antinociceptive action produced by the peripheral 5-HT_3 or 5-HT_{2A} blockade was favored. Another difference between our pre-clinical and the clinical studies is the time of administration.

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Typically, the administration of ondansetron in a clinical setting is carried out post-surgically to treat nausea and vomiting (Fujii, 2011; Kim et al., 2009). Conversely, we administered ondansetron or ketanserin before the incision to prevent the development of nociception. Moreover, our histological analyses demonstrated a massive mast cell degranulation as early as 1 h after incision. Thus, our treatment prevented the 5-HT_3 and 5-HT_{2A} receptor activation and the peripheral sensitization that may occur quickly after the incision, whereas the post-surgical treatment with ondansetron or ketanserin may be ineffective to produce analgesia in patients because mast cell degranulation and 5-HT_3 and 5-HT_{2A} receptor activation would have already occurred. Since the previous local administration of the antagonists H_1, 5-HT_3 or 5-HT_{2A} alone does not fully prevent the mechanical allodynia, we conducted the administration of the three antagonists in combination. When administered together, they were able to completely prevent the nociception induced by surgical procedure. These results indicate that histamine and serotonin acting in different receptors are important algogens in the early hours of the postoperative painful process.

Different from the antagonism of the histaminergic and serotoninergic receptors that produced short-lasting effect the stabilization or the preoperative degranulation of mast cells produced a more effective and long-lasting anti-nociceptive effect. Thus, other mediators liberated from mast cells also appear to mediate the development and the maintenance of postoperative pain. However, more studies must be undertaken to elucidate such mediators.

It has been suggested that certain drugs administered locally, such as histamine and serotonin antagonists could exert non-specific anti-nociceptive effects by acting as local anesthetics (Reeh, 2008). Discarding this possibility, we have shown that cromoglycate, promethazine, ondansetron or ketanserin at the doses that result in prevention of postoperative nociception did not alter the mechanical threshold of the sham-operated animals, which occurred after treatment with the positive control lidocaine.

Taken together, our findings indicate the great importance of mast cell activation as a peripheral mechanism that is involved in the development and maintenance of postoperative nociception. Thus, mast cell activation mechanisms could be interesting targets for the development of novel therapies to treat postoperative pain.

Supplementary materials related to this article can be found online at doi:10.1016/j.ejphar.2011.10.001.

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Supplementary Figure

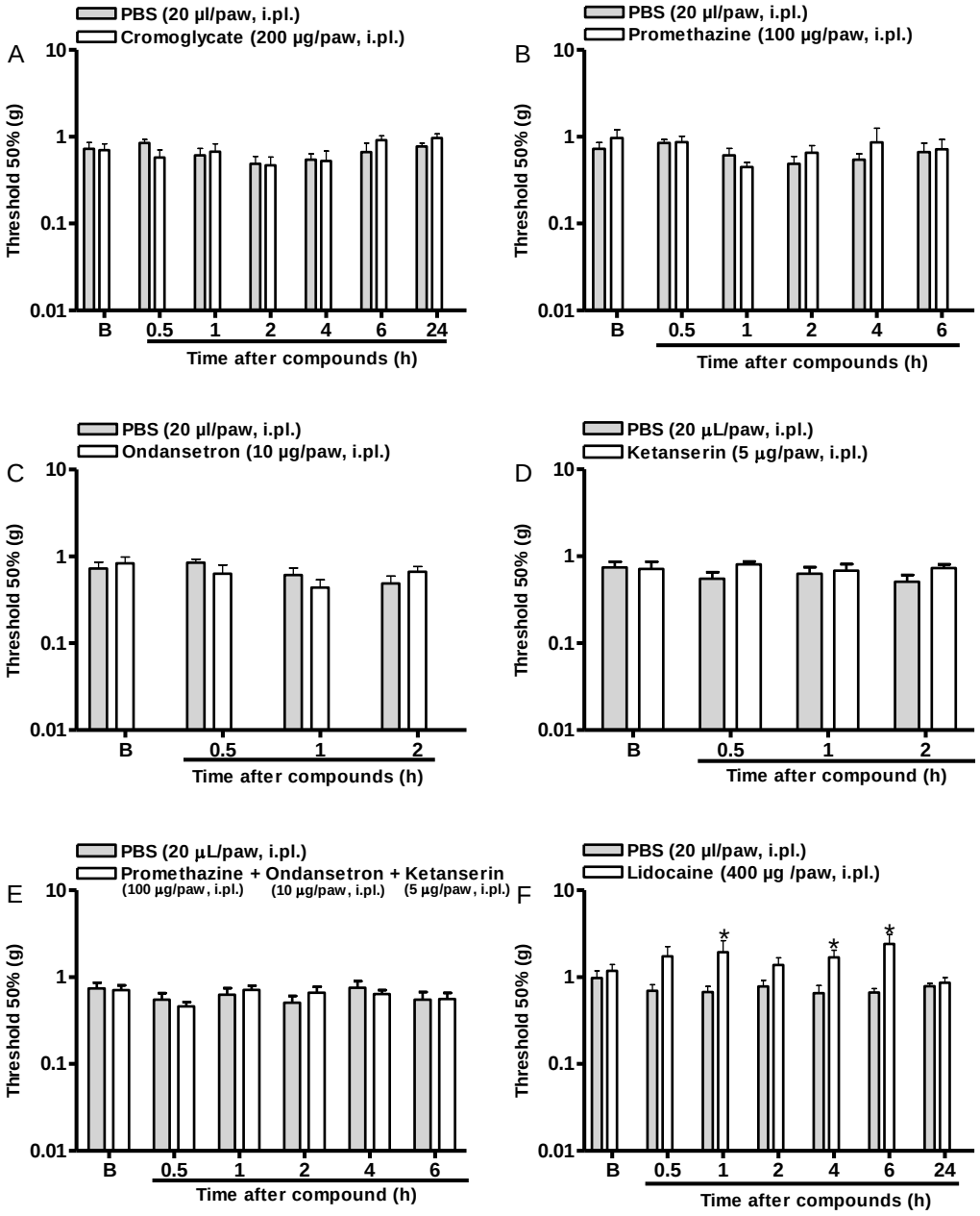

Figure 1- Effect of pre-treatment with cromoglycate (200 µg/paw, i.pl.) (A), promethazine (100 µg/paw, i.pl.) (B), ondansetron (10 µg/paw, i.pl.) (C), ketanserin (5 µg/paw, i.pl.) (D), promethazine (100 µg/paw, i.pl.), ondansetron (10 µg/paw, i.pl.) and ketanserin (5 µg/paw, i.pl.) (E) or lidocaine (400 µg/paw, i.pl.) (F), on the mechanical thresholds of sham-operated mice. The verticals bars represent the means of 6-7 animals + standard errors of the mean. *P<0.05 when compared to the PBS-treated group; Student's t-test.

4.1.2 MANUSCRITO

Critical role of PAR-2 activation by mast cell tryptase on the development of postoperative pain

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ABSTRACT

Background: Recently, studies have indicated that nearly half of all surgical patients still have inadequate pain relief what become important to understand the mechanisms involved postoperative pain to be better treat it. Previous studies have shown that incision caused mast cell degranulation which is related to postoperative pain. Thus, the aim of this study was to investigate the involvement of mast cell tryptase and its substrate, the protease-activated receptor 2 (PAR-2), in a model of postoperative pain in mice.

Results: The previous treatment (30 min before) with the tryptase inhibitor (gabexate, 0.01-1 nmol/paw) or the PAR-2 antagonist (ENMD-1068, 10-100 nmol/paw) in the local of the incision prevented postoperative nociception as well as the nociception induced by the PAR-2 activator tryptase (5 ng/paw). Neither gabexate nor ENMD-1068 was capable of reversing nociception when administered 30 min after incision. Indicating an early tryptase release by mast cells postoperatively, plantar surgery increased activity of tryptase (two–fold higher) in perfusates and produced mast cell degranulation on the incised tissue 10 min after surgery. The local pre-treatment with the mast cell membrane stabilizer cromoglycate (200 µg/paw) fully prevented the increase in tryptase release and the nociception induced by surgery. Accordingly, the depletion of mast cell mediators produced by repeated pre-treatment with compound 48/80 (1, 3, 10 and 10 µg/paw) largely reduced tryptase activity in the paw tissue and postoperative nociception.

Conclusion: Thus, the mast cell release of tryptase and the further PAR-2 activation are interesting targets for the development of novel therapies to treat postoperative pain.

Keywords: hyperalgesia; nociception; surgery; protease.

1. INTRODUCTION

During the past two decades, the under treatment of acute pain in surgical patients has been widely recognized as an important issue in health care. Patients who have well-controlled pain have an improved health-related quality of life and overall greater satisfaction with their experience. Adequate knowledge regarding treatment of postoperative pain is important to reduce the morbidity and mortality of patients after surgery (Kehlet et al., 2006; Gandhi et al., 2011). Unfortunatly, despite the introduction of new standards, guidelines, and educational efforts, data from around the world suggest that postoperative pain continues to be managed inadequately (Weiser, 2008; Wu and Raja, 2011). Of note, the treatment to produce postoperative analgesia is frequently complicated by the limited efficacy and undesirable side effects of currently available analgesic drugs (Dahl and Kehlet, 2006; Alkaitis et al., 2010). So, study the mechanisms involved in postoperative pain would be an interesting target to better treat it.

The mechanisms involved are probably multiple, including tissue injury related to the incision itself, secondary inflammation, and damage to nerves caused by tissue retraction during the surgery (Flatters, 2008). The place of incision presents signs of inflammation including local edema, hyperthermia, hyperemia and pain (Clark et al., 2007; Swarm et al., 2001) indicating that surgery causes cellular and vascular release of pro-inflammatory substances that mediate postoperative pain. It was previously demonstrated that mast cells degranulate and are drastically decreased in number within hours following tissue incision (Egozi et al., 2003). Furthermore, we previously demonstrated that the prevention of mast cell degranulation largely reduced nociception in a model of postoperative pain (Oliveira et al., 2011). However, the antagonism of histamine or serotonin, two important mast cell mediators, just partially reduce postoperative nociception, indicating that other mast cell components must be involved.

In addition of histamine and serotonin, mast cell degranulation releases tryptase which has been demonstrated to be an important pro-nociceptive proteinase related with some painful diseases, such as irritable bowel syndrome

(Barbara et al., 2004; Barbara et al., 2007; Cenac et al., 2007). Tryptase is known to be a potent activator of protease-activated receptor 2 (PAR-2) (Nystedt et al., 1994; Molino et al., 1997; Fox et al., 1997). PAR-2 is a G-protein coupled receptor which is expressed in peripheral terminals of sensory neurons and seems to play an important role in inflammatory pain (Macfarlane et al., 2001; Ossovskaya and Bunnett, 2004; Steinhoff et al., 2005).

The close association between mast cells and nerves in peripheral tissues, and the fact that large amounts of tryptase are released upon mast-cell degranulation, makes tryptase an ideal candidate to activate PAR-2 on peripheral neurons (Vergnolle et al., 2003). However, it is not known the putative role of tryptase or PAR-2 in postoperative pain. Thus, in the present study we assess the ability of mast cell tryptase mediates nociceptive responses via PAR-2 receptor in a model postoperative pain in mice.

2. MATERIALS AND METHODS

2.1. Animals

The experiments were conducted using male Swiss mice (25-35 g) that had free access to food and water and were maintained in a temperature-controlled room (22 ± 2 °C) under a 12-h light-dark cycle before and after surgery. Animals were acclimatized to the laboratory for at least 2 h before experiments and were used only once. The experiments were performed with the approval of Ethics Committee of the Universidade Federal de Santa Maria (process number 45/2010) and were carried out in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.2. Drugs

Gabexate mesilate, N-p-Tosyl-Gly-Pro-Arg p-nitroanilide acetate salt (pNA), tryptase from human lung, compound 48/80, sodium cromoglycate and lidocaine were obtained from Sigma Chemical Company (St. Louis, USA). N3-methylbutyryl-N-6-aminohexanoyl-piperazine (ENMD-1068) was obtained from Enzo Life Sciences (Farmingdale, USA). All compounds were diluted in a phosphate-buffered solution (PBS; 137 mM NaCl and 10 mM phosphate buffer; pH 7.4). We have used PBS to avoid that pH alterations in solution could interfere with the nocicepive test (Meotti et al., 2010). For the histological procedures, we used paraffin, formaldehyde, ethanol, acetic acid, xylol and toluidine blue obtained from Merck (Rio de Janeiro, Brazil).

2.3. Postoperative pain model

Mice were anesthetized with 2% isoflurane via a nose cone. After anti-septic preparation of the right hind paw with 10% povidone-iodine solution (PVPI), a 5 mm longitudinal incision was made with a number 11 blade through the skin and fascia of the plantar foot. The incision was started 2 mm from the proximal edge of the heel and extended toward the toes. The underlying muscle

was elevated with a curved forceps, leaving the muscle origin and insertion intact. The skin was apposed with a single mattress suture of 6.0 nylon (Pogatzki and Raja, 2003). The animals sham-operated were only anesthetized.

For the behavioral assessment of mechanical hyperalgesia, mice were placed individually in clear plexiglass boxes (7 x 9 x 11 cm) on elevated wire mesh platforms to allow access to the ventral surface of the hind paws. Before the test procedures, the animals remained in the box for approximately 1.5 hours for behavioral accommodation. The mechanical stimulus was applied in the plantar surface. The mechanical threshold was determined before and after incision with flexible nylon von Frey filaments using the Up-and-Down method, which consisted of six applications of a particular filament applied once every 3-4 s. Testing was initiated with the 0.4 g filament. The response was defined as a withdrawal of the stimulated paw. In the absence of a response to a particular filament, the next strongest filament was utilized, and in the case of a response, the next weakest filament was presented (Chaplan et al., 1994). Mechanical hyperalgesia was considered as a decrease in the threshold when compared with the same paw before surgery (basal value). The values obtained at each time after surgery were compared to basal values unique to each animal. The mechanical hyperalgesia of all groups was assessed 0.5, 1, 2 and 4 h after incision, when necessary.

For spontaneous nociception measurement, mice were placed individually in the same boxes used for the measurement of mechanical hyperalgesia and guarding behavior was observed conform Xu and Brennan (2010), with few modifications. Incised hind paw was closely observed during a 1 min period repeated every 30 min for 1 h. According to the hind paw position at the time of observation, a score of 0, 1, or 2 was given. Zero was scored when the incised area was touching the mesh, and the area was blanched or distorted by the mesh; 1 was scored when the incised area touched the mesh without blanching or distortion; 2 for the position when the incised area was completely off of the mesh. For each hind paw, a sum score was obtained by subtracting the score of the incised hind paw from its score before surgery.

2.4. Treatments

A selective PAR-2 antagonist (N3-methylbutyryl-N-6-aminohexanoyl-piperazine: ENMD-1068, 1-100 nmol/paw, i.pl.), an inhibitor of tryptase (gabexate mesylate, 0.1-1 nmol/paw, i.pl.) or vehicle (PBS, 20 µL/paw, i.pl.) was administered 0.5 h before of the incisional or sham procedure. Tryptase, an activator PAR-2 (5 ng/paw, i.pl.) was administered 0.5 h before of ENMD-1068, gabexate or vehicle in sham-operated mice. Sodium cromoglycate (200 µg/paw, i.pl.), a mast cell membrane stabilizer, was administered 15 minutes before of the incisional procedure. The mast cell degranulator compound 48/80 was administered daily at increasing doses (1, 3, 10 and 10 µg/paw, i.pl.) and the animals were used 24 h after the last injection of compound 48/80. The dosages and timing of the drugs were obtained from previous studies (Vergnolle et al., 2001; Hoffmeister et al., 2011; Oliveira et al., 2011; Kelso et al., 2007). The effects of the treatments on nociception were assessed just until the end of the antinociceptive effect to avoid unnecessary discomfort for the animals. Another group of animals, which did not undergo the incisional procedure, received intraplantar administration of sodium cromoglycate, gebexate or ENMD-1068 as described above to determine whether these compounds alone altered the mechanical threshold of the animals. Lidocaine, a local anesthetic, was used as a positive control (Wang et al., 2010).

2.5. Measurement of tryptase activity

To confirm the mast cell depletion produced by repeated compound 48/80 treatment, separate groups of mice were euthanized by cervical dislocation 24 h after the last injection of compound 48/80. Tryptase activity was measured in homogenates of the paw skin of animals, as described in detail below.

Tryptase activity was evaluated as previously described by Hoffmeister et al. (2011). Paw skin samples were homogenized in 10 mM tris hydroxymethyl aminomethane (Tris; pH 6.1) containing 2 M NaCl and centrifuged at 700 x g, 4 °C for 10 min, and the resulting supernatants were used to evaluate tryptase activity. Then, 50 µl of supernatant was combined with 50 µl of 500 µg/ml N-p-Tosyl-Gly-Pro-Arg-p-nitroanilide in Tris buffer (60 mM, pH 7.8) containing 0.4%

DMSO and 30 µg/ml heparin at 37 °C for 1 h. The enzymatic activity of tryptase was determined measuring the hydrolysis of the substrate N-p-Tosyl-Gly-Pro-Arg-p-nitroanilide to its product p-nitroanilide in a spectrophotometer at 405 nm.

Separated groups of animals were submitted to a surgical or sham procedure and 10, 30 or 60 min after surgery the animals were euthanized by cervical dislocation, and the operated or the sham-operated paws were perfused as previously described (Ferreira et al., 2004). A double polyethylene tube was inserted into the subcutaneous space of the paw, and 100 µl of PBS was perfused at a rate of 200 µl/min through the inner tube, after which the perfusate was collected through the outer tube. Tryptase activity in the perfusates was measured as described above.

2.6. Histology

To confirm the degranulation of mast cells in the tissues of the right hind paws of the animals, that suffered or did not suffered plantar surgery, we carried out histological analyses. Samples were collected 10 min after the surgical or sham procedure. Mice were sacrificed, and their paws were removed and fixed in alfac solution (16:2:1 mixture of ethanol 80%, formaldehyde 40% and acetic acid) and then decalcified. Each sample was embedded in paraffin wax, sectioned at 5 µm and stained with toluidine blue. A representative area was selected for qualitative light microscopic analysis of the inflammatory cellular response with a 10x and 100x objective (Oliveira et al., 2011). To minimize any source of bias, the investigator analyzing the samples did not know the identity of the group that he was analyzing.

2.7. Statistical Analysis

The results are expressed as means ± standard error of the mean (S.E.M.), except for the ID_{50} values (i.e. gabexate or ENMD-1068 dose that reduces nociceptive responses to the order of 50% related to the control value), which were expressed as geometric means accompanied by their respective 95% confidence limits and spontaneous nociception scores, which are reported as medians followed by their 25ht and 75ht percentiles. Spontaneous nociception scores were analyzed by Mann-Whitney test. All other data were

analyzed by a Student's t-test or one- or two-way analysis of variance (ANOVA). P values less than 0.05 ($P<0.05$) were considered significant, and F values presented in the text are demonstrated by treatment versus time interactions only when $P<0.05$.

3. RESULTS

3.1. The inhibition of the tryptase activity or the PAR-2 antagonism prevents the development of postoperative nociception

The previous treatment (30 min before surgery) gabexate (1 nmol/paw, i.pl.), a selective tryptase inhibitor, reduced mechanical hyperalgesia in mice after plantar surgery [F (1, 45) = 3.56, P<0.001; Fig. 1A]. Its anti-hyperalgesic effect occurred from 0.15 to 2 h after surgery and at doses of 0.1 and 1 nmol/paw [F (3, 18) = 15.6, P<0.001; Fig. 1B]. The calculated ID_{50} value was 0.30 (0.072-1.22) nmol/paw and the maximum inhibition (I_{max}) was 76 ± 15%.

Similarly, the previous treatment with the selective PAR-2 antagonist ENMD-1068 (100 nmol/paw) was also able to prevent postoperative hyperalgesia in mice [F (1, 50) = 5.03, P<0.0001; Fig. 1C]. The anti-hyperalgesic effect of ENMD-1068 occurred from 0.15 to 2 hours after surgery, with an ID_{50} value of 59 (27-130) nmol/paw and an I_{max} of 80 ± 13% (Fig. 1D).

Demonstrating that the effects of gabaxate and ENMD-1068 were specific to postoperative hyperalgesia, the local administration of either compounds did alter the mechanical threshold of sham-operated animals. Conversely, lidocaine (2 µmol/paw; used as a positive control), increased the mechanical threshold of sham-operated animals 0.5 to 4 hours after its administration (Fig. 2).

Besides preventing the mechanical hyperalgesia, previous administration of either gabexate (1 nmol/paw, i.pl.) or ENMD-1068 (100 nmol/paw, i.pl.) was capable of inhibiting spontaneous nociception in animals operated (Fig. 3).

3.2. The injection of tryptase in hinpaw mimics surgery inducing hyperalgesia

Similar to plantar surgery, the intraplantar injection of tryptase (5 ng/paw, i.pl.) was able to produce mechanical hyperalgesia in mice [F (1, 63) = 6.01, P<0.0001; Fig. 4A]. This hyperalgesic effect occurred from 10 minutes to 6 hours after its administration. The pre-treatment with gabexate (1 nmol/paw, F (1, 60) = 5.40, P<0.0001; Fig. 4B) or ENMD-1068 (100 nmol/paw, F (1, 65) = 5.79, P<0.0001; Fig. 4C) was able to largely prevent the reduction in the

mechanical threshold induced by tryptase from as early as 10 minutes and lasting up to 2 h, with inhibitions at the peak of 100% and 88 ± 14%, respectively.

3.3. The inhibition of tryptase or the PAR-2 antagonism does not reverse the established postoperative nociception

Different from the results obtained with previous treatment, gabexate (1 nmol/paw) and ENMD-1068 (100 nmol/paw) were not able to reverse the established postoperative hyperalgesia when they were administered 30 minutes after incision (Fig. 5).

3.4. Incision induces an early mast cell degranulation and tryptase release

Indicating an early tryptase release by mast cells postoperatively, plantar surgery increased the activity of tryptase (two–fold higher) in paw tissue perfusate of operated mice when compared with sham-operated animals in 10 min, but not in 30 or 60 min after surgery (Fig. 6; $P<0.001$, Student's t-test). Accordingly, we also detected mast cell degranulation as early as 10 min after surgery by histological analyses (Fig. 7).

3.5. The reduction of trypase in tissue or of its release prevented postoperative hyperalgesia

We next used two different approaches to confirm the critical role of tryptase in postoperative pain. Firstly, we detected that the depletion of mast cells mediators by repeated treatment with compound 48/80 was able to largely reduce the tryptase activity in paw skin of mice (inhibition of 81 ± 14%; Fig. 8A) as well as the postoperative hyperalgesia (inhibition 74 ± 3%) (Fig. 8B; $P<0.001$, Student's t-test).

Secondly, we also observed that the previous treatment of mice with the mast cell membrane stabilizer cromoglycate (200 µg/paw) prevented both the increase of tryptase activity (100% of inhibition, Fig. 8C) and the mechanical hyperalgesia (inhibition 76 ± 18%) after surgical procedure (Fig. 8D; $P<0.001$, Student's t-test).

4. Discussion

Since postoperative pain remains poorly treated, it is important to understand the mechanisms involved in postoperative pain to better treat it. Previously, we showed that the surgery caused mast cell degranulation and increased histamine and serotonin local levels (Oliveira et al., 2011). Apart the prevetion of mast cell degranulation largely reduce nociception, the antagonism of histamine or serotonin receptors only partially decreased postoperative nociception in mice, suggesting that other mast cell-derived mediators are involved. Here, we found that mast cells contribute to the development of postoperative pain through early tryptase release and further PAR-2 activation.

Histochemical and immunohistochemical techniques, demonstrated that the serine protease tryptase is localized exclusively in mast cells and may be used as an indicator of mast cell activation (Chen et al., 1993; The et al., 2007). However, the role of tryptase in painful processes are largely unknown. Clinical studies demonstrated that tryptase are released in irritable bowel syndrome and that they can directly stimulate sensory neurons and generate hypersensitivity symptoms (Klooker et al., 2010; Cenac et al., 2007). In the present study we have observed that the selective tryptase inhibitor gabexate was capable of preventing postoperative nociception. Of note, a recent study demonstrated that manipulation of the operated site promotes mast cell degranulation with subsequent local release of tryptase (conventional laparotomy), while minimally invasive technique (laparoscopy) did not present increased levels of tryptase in tissue operated (The et al., 2007). This indicates mast cell may be involved in postoperative pain, especially where surgical manipulation is more extensive.

Tryptase is a selective agonist of the protease-activated receptor 2 (PAR-2). Studies have shown that PAR-2 activation is implicated in mast cell degranulation-induced hyperalgesia, as well as in formalin-induced hyperalgesia, while PAR-2-deficient mice showed no signs of thermal hyperalgesia after the intraplantar injection of the mast cell degranulator compound 48/80 (Vergnolle et al., 2001). Likewise, intraplantar injections of PAR-2 agonists promote mechanical and thermal hyperalgesia in rats and mice (Vergnolle et al., 2001; Kawabata et al., 2001). Moreover, PAR-2 blockade

appears as a reasonable therapeutic option for a number of chronic inflammatory diseases: arthritis, skin inflammation, inflammatory bowel diseases (Vergnolle, 2009). Recently, a novel PAR-2 selective antagonist ENMD-1068 has been developed. Systemic administration of this antagonist produced anti-inflammatory actions in a murine model of arthritis (Kelso et al., 2006), and reduced osteoarthritis progression and joint degradation in vivo (Ferrel et al., 2010). Preclinical trials with PAR-2 antagonists, such as ENMD-1068, are important to reveal whether PAR-2 is a potential therapeutic target for the treatment of postoperative pain. We observe that intraplantar pre-administration of ENMD-1068 was able to prevent both mechanical hyperalgesia and spontaneous nociception after surgery. Our results are in according with literature data cited above, involving agonists and antagonists PAR-2 in inflammatory pain conditions.

Tryptase administered into the mice hind paw mimics the postoperative nociception and promotes a reduction in mechanical threshold of mice. However, this threshold reduction occurs within the first 6 hours after its administration, whereas pain initiated by surgical procedure lasts about 4 to 5 days. This indicates that tryptase is involved early in the process painful. Moreover, gabexate or ENMD-1068 was able to prevent the reduction of mechanical threshold induced by tryptase. This is consistent with our results in postoperative pain, where gabexate or ENMD-1068 was able to reduce postoperative nociception preventing the activation the PAR-2 receptor.

The partial prevention of postoperative mechanical hyperalgesia by the previous administration of ENMD-1068 or gabexate is consistent with previous data where local treatment with H_1, 5-HT_3 or 5-HT_{2A} receptor antagonists partially decreased postoperative nociception in mice, indicating other mast cell mediators besides histamine and serotonin are involved in postoperative nociception (Oliveira et al., 2011).

When ENMD-1068 or gabexate were administered after plantar surgery found that they were unable to prevent the postoperative nociception, indicating that mast cell degranulation had already occurred. Consequently after mast cell degranulation there was an early release of inflammatory mediators, such as tryptase, with nociceptor activation and installation of the painful process, which

was not reversed by treatment with ENMD-1068 or gabexate. In acordance with this idea, we observed an increase in the activity of tryptase in the tissue perfusate few minutes after surgery, when compared with sham-operated animals indicating that the surgery produced an early release of mast cell mediators. This early increase in the activity of tryptase demonstrates its importance in the development of postoperative pain, since in 30 min after the surgery it is no longer released. Confirming the data of tryptase release, we demonstrated by histological procedure that as early as 10 min after surgery, at the same time when we detected increased activity of tryptase, that mast cell degranulation had already occurred. Thus, therapeutic strategies to treat postoperative pain with mast cell mediators must be preventive.

To bring more evidences regarding the role of tryptase in postoperative pain, we used different approaches to confirm the critical role of tryptase in postoperative pain. Compound 48/80 lead to degranulation of mast cells promoting the release of their mediators such as histamine, serotonin and tryptase (Cavalcante et al., 2000; Kivinen et al., 2001; Bundoc and Keane-Myers, 2007; Crivellato and Ribatti, 2010; Oliveira et al., 2011). However, its repeated administration promote depletion of these mediators (Di Rosa et al., 1971; Oliveira et al., 2011). We demonstrated that the depletion of mast cell mediators by degranulation after pre-treatment with compound 48/80 was able to reduce the activity of tryptase in the paw skin as well as to prevent postoperative nociception in the animals. We also verified that mast cell stabilizer, cromoglycate, was able to prevent the tryptase release induced by surgical injury after 10 min of surgery, such as its previous administration prevent postoperative nociception in the animals. These is according with previous results where was demonstrated that cromoglycate is capable of protecting mast cell membranes, thereby hindering their degranulation and is able to prevent nociception induced by surgical injury (Oliveira et al., 2011). Thus, the early degranulation of mast cells and the release of their pro-nociceptive mediators appear to mediate the development of postoperative nociception in mice.

Besides mechanical hyperalgesia, preclinical models of acute postoperative pain have been developed involving surgical incision of the skin,

muscle and fascia which leads to evoked and non-evoked pain-related behaviors that mirror the symptoms observed in patients undergoing surgery (Brennan et al., 1996; Buvanendran et al., 2004; Martin et al., 2004). It has also been shown that deep tissue (i.e., muscle) rather than skin incision is critical for the development of guarding pain, which is similar to pain at rest in postoperative patients, and for spontaneous activity (Xu and Brennan, 2010). Here, we demonstrated that tryptase inhibition or PAR-2 antagonism nor only reduced hyperalgesia, but also prevented the guarding pain induced by surgery. This finding reinforces the role of tryptase and PAR-2 in several painful symptoms that occurs in postoperative patients.

It has been suggested that certain drugs administered locally could exert non-specific antinociceptive effects by acting as local anesthetics (Reeh, 2008). Discarding this possibility, we have shown that gabexate or ENMD-1068 at the doses that result in prevention of postoperative nociception did not alter the mechanical threshold of the sham-operated animals, which occurred after treatment with the positive control lidocaine. Moreover, our results demonstrate clearly that the effect of tryptase inhibition or the PAR-2 antagonist is specific on postoperative hyperalgesia and not on normal mechanical stimuli detection.

Taken together, tryptase released from mast cells and its receptor PAR-2 appears to have an important role on the development of postoperative pain. Thus, mast cell activation mechanisms could be interesting targets for the development of novel therapies to treat pain that occurs after surgeries.

Acknowledgments

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Figure and Legends

Figure 1

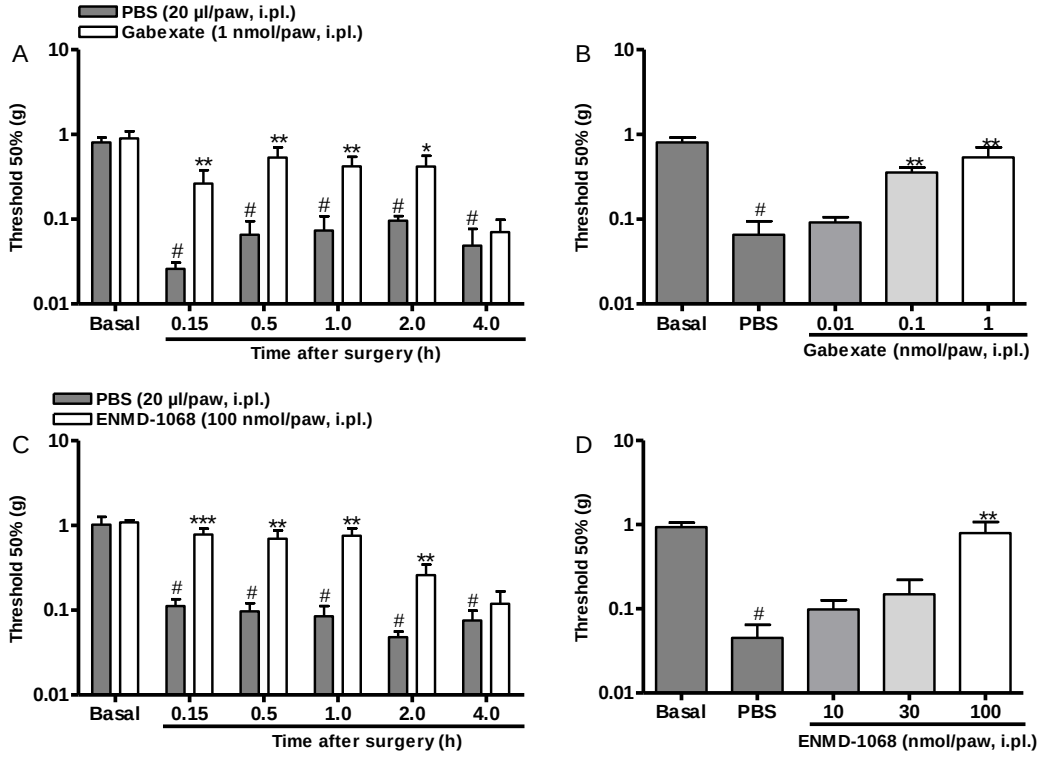

Fig. 1.

Effect of pre-treatment with gabexate or ENMD-1068 on mechanical hyperalgesia after plantar surgery in mice. Time-response curve of pre-administration of gabexate (1 nmol/paw, i.pl.) (A) or ENMD-1068 (100 nmol/paw, i.pl.) (C) or dose-response curve of pre-administration of gabexate (0.01-1 nmol/paw, i.pl.) (B) or ENMD-1068 (10-100 nmol/paw, i.pl.) (D) on mechanical hyperalgesia after plantar surgery in mice. The vertical bars represent the means of 5-7 animals + standard errors of the mean for (A, B) and the means of 6 animals + standard errors of the mean for (C, D). $^{\#}P<0.01$ when compared to baseline; one-way ANOVA followed by Dunnets' test. *P<0.05, **P<0.01, ***P<0.001 when compared to the PBS-treated group; one-way ANOVA followed by Dunnets' test (B, D) or Student's t-test (A, C).

Figure 2

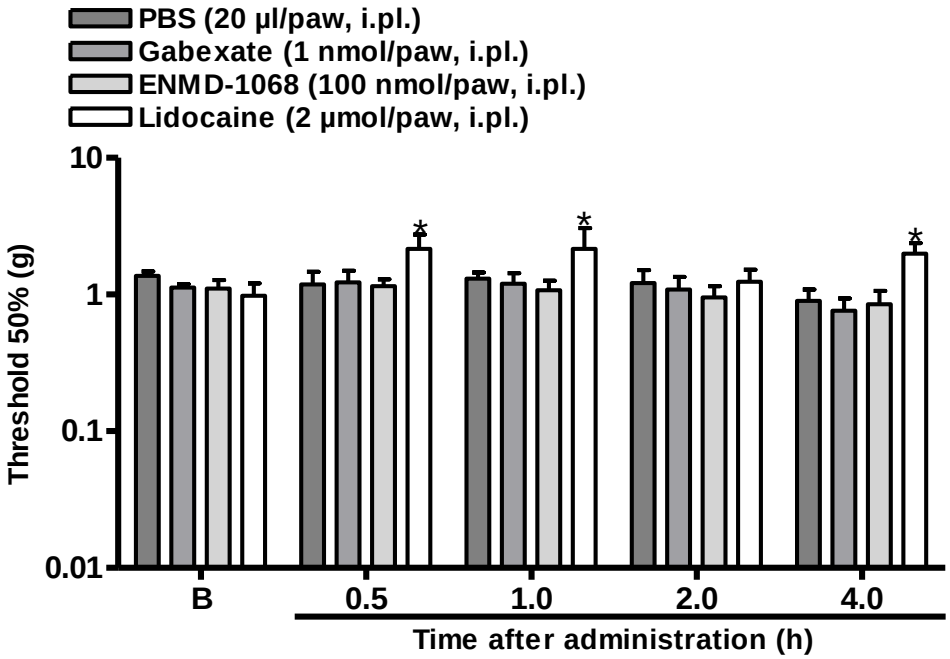

Fig. 2.

Effect of ENMD-1068 (100 nmol/paw, i.pl.), gabexate (1 nmol/paw, i.pl.) or lidocaine (2 µmol/paw, i.pl.) on the mechanical thresholds of sham-operated mice. The vertical bars represent the means of 5-6 animals + standard errors of the mean. *P<0.05 when compared to baseline; Student's t-test.

Figure 3

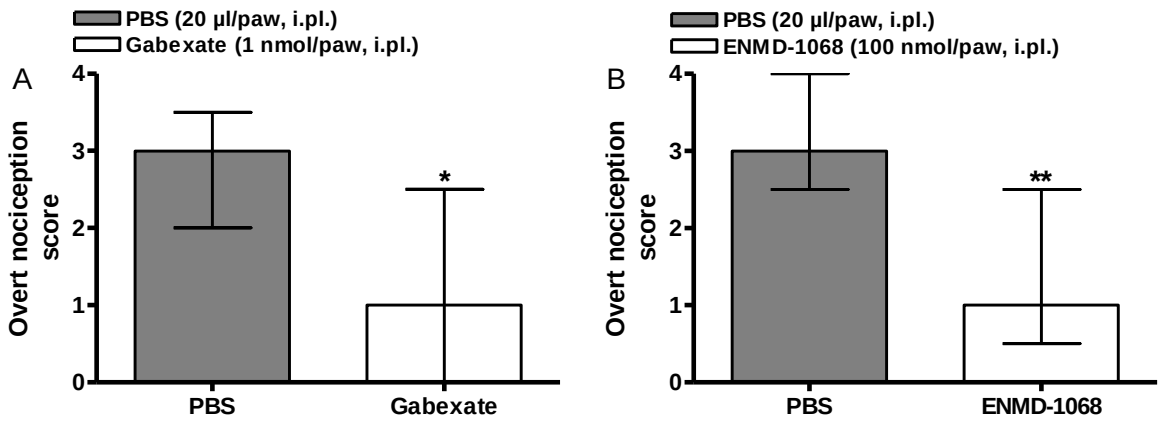

Fig. 3.

Effect of pre-treatment with gabexate or ENMD-1068 on spontaneous nociception after plantar surgery in mice. Effect of pre-administration of gabexate (1 nmol/paw, i.pl.) (A) or ENMD-1068 (100 nmol/paw, i.pl.) (B) on spontaneous nociception at 0.5 and 1 h after plantar surgery in mice. The vertical bars represent the means of 5-6 animals + standard errors of the mean for (A) and the means of 10 animals + standard errors of the mean for (B). *P<0.05 when compared to the PBS-treated group; Mann- Whitney test.

Figure 4

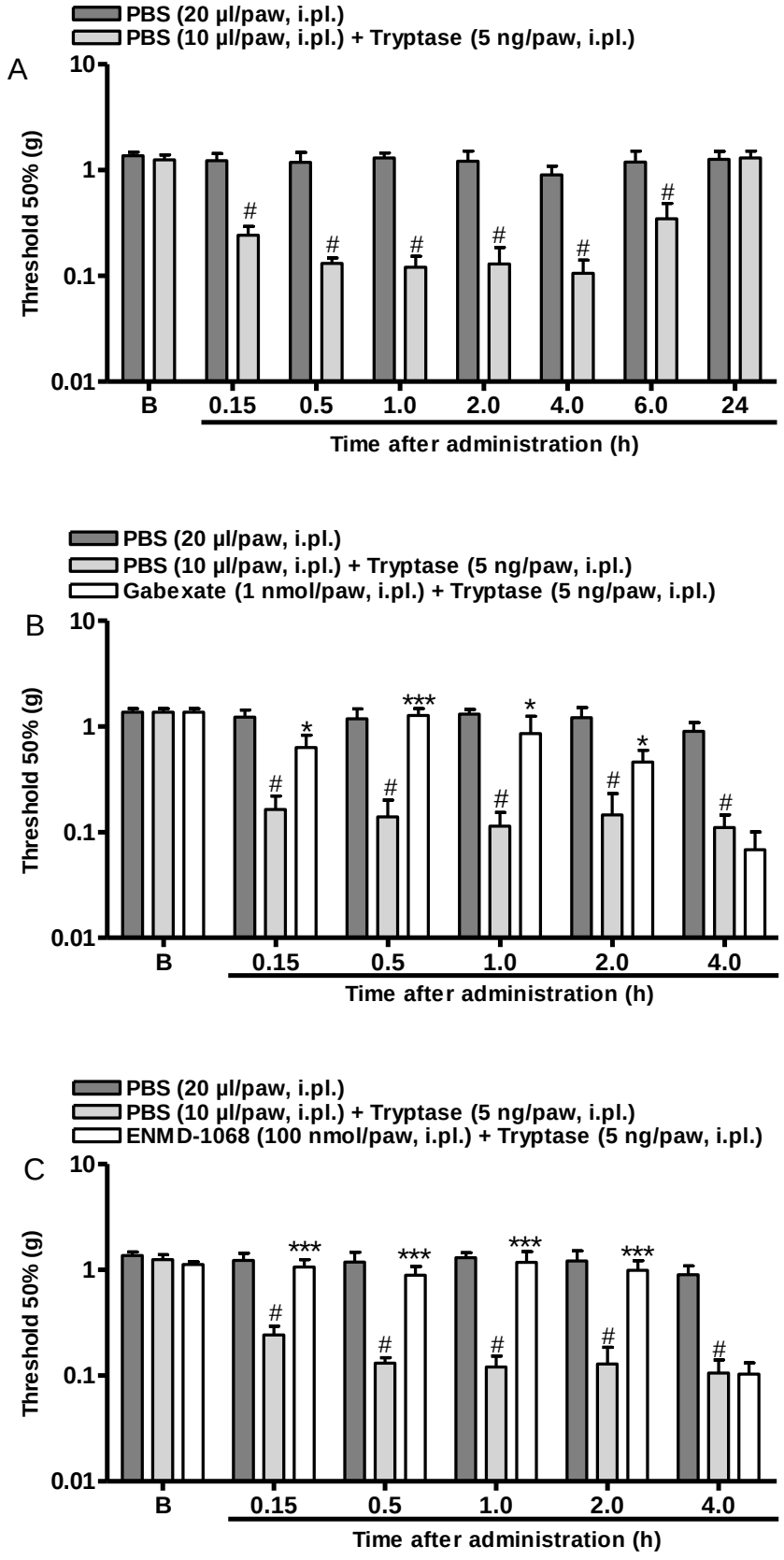
PBS (20 µl/paw, i.pl.)
PBS (10 µl/paw, i.pl.) + Tryptase (5 ng/paw, i.pl.)
A
Threshold 50% (g)
10
1
0.1
0.01
B
0.15
0.5
1.0
2.0
4.0
6.0
24
Time after administration (h)
PBS (20 µl/paw, i.pl.)
PBS (10 µl/paw, i.pl.) + Tryptase (5 ng/paw, i.pl.)
Gabexate (1 nmol/paw, i.pl.) + Tryptase (5 ng/paw, i.pl.)
B
Threshold 50% (g)
Time after administration (h)
PBS (20 µl/paw, i.pl.)
PBS (10 µl/paw, i.pl.) + Tryptase (5 ng/paw, i.pl.)
ENMD-1068 (100 nmol/paw, i.pl.) + Tryptase (5 ng/paw, i.pl.)
C
Threshold 50% (g)
Time after administration (h)

Fig. 4.

Effect of pre-treatment with gabexate or ENMD-1068 on reduction in mechanical threshold induced by intraplantar tryptase in mice. Effect of tryptase (5 ng/paw, i.pl.) on mechanical threshold in mice (A). Effect of pre-administration of gabexate (1 nmol/paw, i.pl.) (B) or ENMD-1068 (100 nmol/paw, i.pl.) (C) on mechanical hyperalgesia induced by intraplantar tryptase in mice. The vertical bars represent the means of 5-6 animals + standard errors of the mean (A, C) or the means of 5 animals + standard errors of the mean (B). $^{\#}P<0.01$ when compared to baseline and $*P<0.05$ and $***P<0.001$ when compared to the PBS-treated group; one-way ANOVA followed by Dunnets' test.

Figure 5

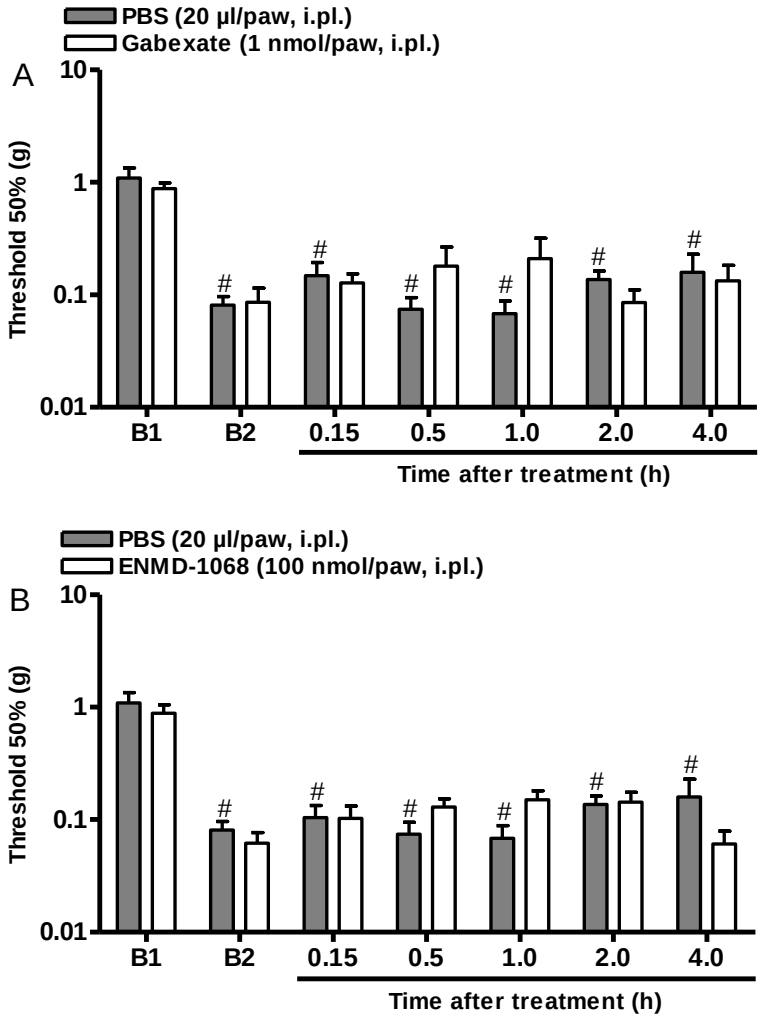

Fig. 5.

Effect of post-treatment with gabexate or ENMD-1068 on mechanical hyperalgesia after plantar surgery in mice. Effect of post-administration of gabexate (1 nmol/paw, i.pl.) (A) or ENMD-1068 (100 nmol/paw, i.pl.) (B) on mechanical hyperalgesia after plantar surgery in mice. The vertical bars represent the means of 7-8 animals + standard errors of the mean for (A) and the means of 6-7 animals + standard errors of the mean for (B). $^{\#}P<0.01$ when compared to baseline; one-way ANOVA followed by Dunnets' test.

Figure 6

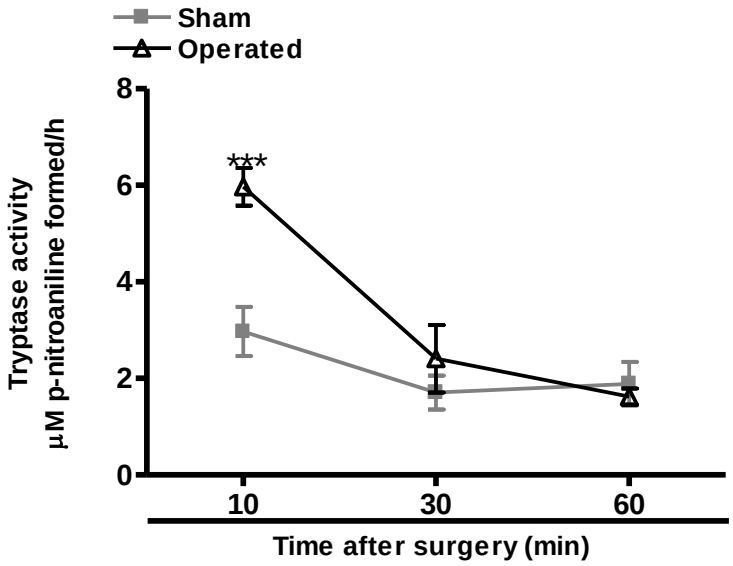

Fig. 6.

Effect of plantar surgery on tryptase activity in mice. Effect of surgery on tryptase activity at 10, 30 or 60 min after plantar surgery in mice. Each point represent the means of 6 animals + standard errors of the mean; ***P<0.001 compared to sham group; Student's t-test.

Figure 7

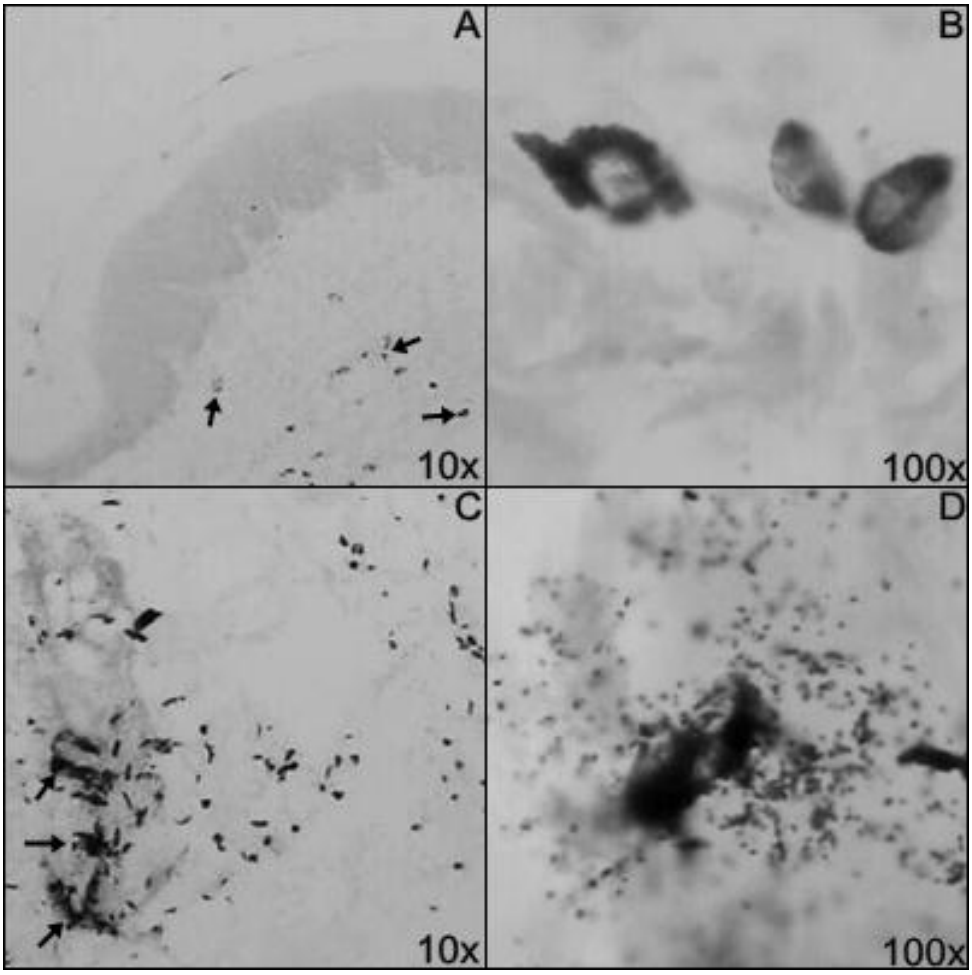

Fig.7.

Representative light microphotograph showing the presence of mast cells after sham or surgical procedures in the paw tissue of mice. A section was obtained from the paw tissue 10 min after the surgical (C, D) or sham (A, B) procedures.

Figure 8

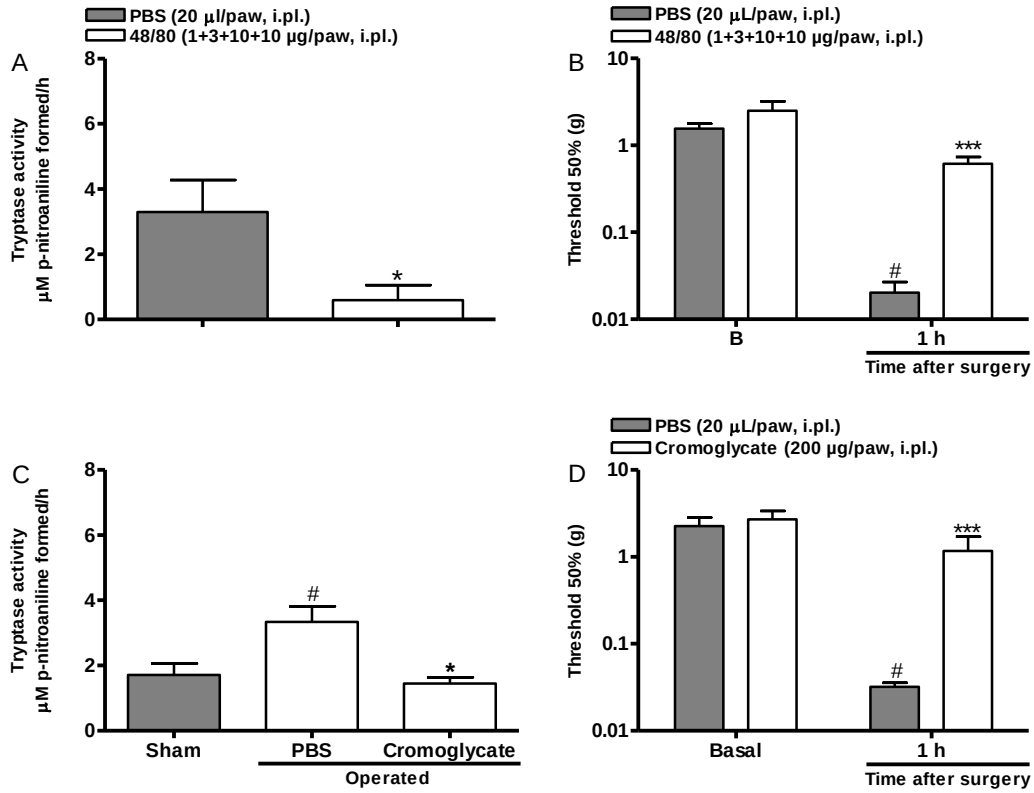

Fig. 8.

Effect of pre-treatment with compound 48/80 or cromoglycate on tryptase activity or on mechanical hyperalgesia in mice. Effect of pre-treatment for 4 days (1+ 3+ 10+ 10 µg/paw) with compound 48/80 on tryptase activity in the paw skin of mice (A) or on mechanical hyperalgesia induced by plantar surgery in mice (B). Effect of pre-treatment with cromoglycate (200 µg/paw) on tryptase activity in the paw perfusates of mice (C) or on mechanical hyperalgesia after plantar surgery in mice (D). The vertical bars represent the means of 5 animals + standard errors of the mean for (A, B, D) and means of 5-8 animals + standard errors of the mean for (C); $^{\#}P<0.01$ when compared to sham group or PBS; *P<0.05, ***P<0.001 when compared to PBS group; Student's t-test (A, B, D) or one-way ANOVA followed by Dunnets' test (C).

5. Discussão

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Atualmente, apesar dos inúmeros tratamentos farmacológicos e das medidas preventivas utilizadas no tratamento da dor pós-operatória, ela permanece pobremente tratada, levando à insatisfação dos pacientes, reabilitação prejudicada e hospitalização prolongada (Dolin et al., 2002; Joshi e Ogunnaike, 2005; Pogatzki-Zahn et al., 2007; Nossaman et al., 2010). Assim, é importante compreender melhor os eventos celulares que controlam os mecanismos periféricos da dor pós-operatória para melhor tratá-la (Wilder-Smith et al., 2006).

Di Rosa et al. (1971), demonstrou que a degranulação dos mastócitos induzida pelo tratamento repetido com o composto 48/80 reduz os níveis de mediadores inflamatórios dos mastócitos no tecido injetado. Nós demonstramos que a depleção dos mediadores dos mastócitos produzido pelo pré-tratamento repetido com o composto 48/80 preveniu a nocicepção pós-operatória e reduziu os níveis de histamina e serotonina e a atividade da triptase no tecido da pata, o que indica que a ativação do mastócitos é importante para o desenvolvimento da dor pós-operatória. Além disso, a cirurgia plantar produziu degranulação dos mastócitos, como avaliado por histologia e confirmado pelo aumento dos níveis de histamina e serotonina e pelo aumento da atividade da triptase no perfusato tecidual após a cirurgia. Nossos resultados estão de acordo com dados da literatura onde a maioria dos mastócitos da pele adjacente à pele ferida estão degranulados (Weller et al., 2006).

Para confirmar o envolvimento dos mastócitos na nocicepção pós-operatória, os animais receberam a administração de cromoglicato de sódio, que protege a membrana dos mastócitos impedindo a sua degranulação (Parada et al., 2001). O pré-tratamento com o estabilizador da membrana celular dos mastócitos, cromoglicato, preveniu a nocicepção mecânica e o aumento dos níveis de histamina e serotonina, bem como preveniu a liberação de triptase induzida pela cirurgia plantar. Assim, a degranulação de mastócitos e a liberação de seus mediadores pró-nociceptivos parecem mediar o desenvolvimento da nocicepção pós-operatória em camundongos. Os fatores

pré-formados e neo-formados dos mastócitos estão envolvidos em situações em que eles são ativados. A histamina, a serotonina e a triptase são mediadores inflamatórios que estão presentes nos grânulos dos mastócitos (Metcalfe et al., 1997). Alguns estudos têm demonstrado que durante uma resposta inflamatória periférica, os mastócitos são a principal fonte de histamina, serotonina e triptase que sensibilizam os nociceptores e produzem resposta nociceptiva (Fox et al., 1997; Parada et al., 2001; Vergnolle et al., 2001; Zeitz et al., 2002; Zuo et al., 2003; Ren e Dubner, 2010). Confirmando a participação desses mediadores dos mastócitos na nocicepção induzida pela cirurgia, nós verificamos que o tratamento local com os antagonistas dos receptores H_1 (prometazina), 5-HT_3 (ondansetrona), 5-HT_{2A} (cetanserina) ou PAR-2 (ENMD-1068) ou com o inibidor da triptase (gabexato) reduziram parcialmente a nocicepção pós-operatória em camundongos. Estudos da literatura demonstram que pacientes previamente tratados com antagonistas do H_1R requerem doses menores de morfina para promover analgesia após procedimentos cirúrgicos (Tarkkila et al., 1995; Chia et al., 2004). Por outro lado, antagonistas do receptor da serotonina 5-HT_3 são utilizados no pós-operatório, no tratamento de náuseas e vômitos e não apresentam efeitos analgésicos quando administrados sistemicamente, nem alteram o consumo de analgésicos (Jokela et al., 2010; Rauers et al., 2010). Assim como o receptor 5-HT_3, o receptor 5-HT_{2A} está presente nos nociceptores, estando envolvido em processos dolorosos (Wei et al., 2005; Sasaki et al., 2006) e não há dados na literatura demonstrando o uso clínico de antagonistas deste receptor. Devido ao fato de termos injetado ondansetrona ou cetanserina no local da incisão, a ação antinociceptiva produzida pelo bloqueio desses receptores pode ter sido favorecida. Antagonistas PAR-2 não são utilizados clinicamente para o tratamento de dor, porém, vários estudos sugerem o envolvimento deste receptor na amplificação de sinais dolorosos (Vergnolle et al., 2001b, 2003a,b; Cenac e Vergnolle, 2005; Dale e Vergnolle, 2008). Aqui, o antagonista PAR-2 e o inibidor da triptase foram capazes de prevenir a hiperalgesia e a nocicepção espontânea após a cirurgia, o que confirma o seu envolvimento na nocicepção pós-operatória. Assim, os nossos dados tornam-se interessantes, já que o gabexato é utilizado clinicamente na prevenção do dano pancreático que

ocorre em pacientes submetidos à endoscopia do sistema digestivo (Cavallini et al., 1996; Ueki et al., 2007). Estes resultados indicam que a histamina, a serotonina e a triptase agem em diferentes receptores e são agentes nociceptivos importantes nas primeiras horas do processo doloroso pós-operatório. Com isso, torna-se importante a realização de estudos clínicos para investigar o efeito de fármacos que controlem a ativação dos mastócitos com conseqüente prevenção da dor pós-operatória em humanos.

Assim, os mecanismos de ativação de mastócitos, bem como a liberação dos seus mediadores inflamatórios e conseqüente ativação dos seus respectivos receptores são alvos interessantes para o desenvolvimento de novas terapias para tratar a dor pós-operatória.

6. CONCLUSÕES

6. Conclusões

Tendo em vista os resultados obtidos no presente estudo, pode-se concluir que:

► Após o procedimento cirúrgico ocorre degranulação dos mastócitos;

► Histamina, serotonina e triptase são mediadores inflamatórios liberados pelos mastócitos após a cirurgia;

► A depleção dos mediadores dos mastócitos ou a estabilização da membrana dos mastócitos é capaz de prevenir a dor pós-operatória;

► O tratamento local com os antagonistas dos receptores H_1, 5-HT_3, 5-HT_{2A} ou PAR-2 confirma o envolvimento destes na dor pós-operatória.

Logo, a inativação dos mastócitos poderia ser um alvo interessante para o desenvolvimento de novas terapias para o tratamento da dor pós-operatória.

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