PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA E CIÊNCIAS DA SAÚDE
FARMACOLOGIA BIOQUÍMICA E MOLECULAR

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PARTICIPAÇÃO DOS RECEPTORES CXCR2 PARA QUIMIOCINAS NA TOXICIDADE INDUZIDA PELO PARAQUAT EM ROEDORES

Porto Alegre
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Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-Graduação em Medicina e Ciências da Saúde, Área de Concentração em Farmacologia Bioquímica e Molecular, da Pontifícia Universidade Católica do Rio Grande do Sul.

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2014

Dados Internacionais de Catalogação na Publicação (CIP)

C837p Costa, Kesiane Mayra da

Participação dos receptores CXCR2 para quimiocinas na toxicidade induzida pelo paraquat em roedores / Kesiane Mayra da Costa. – Porto Alegre, 2014.
60 f. : il.

Diss. (Mestrado em Medicina e Ciências da Saúde) – Área de Concentração em Farmacologia Bioquímica e Molecular.
Orientador: Dr. Maurício Reis Bogo.
Co-orientador: Drª. Maria Martha Campos.

1. Medicina. 2. Farmacologia. 3. Paraquat/toxicidade. 4. Experimentação Animal. 5. Quimiocinas. 6. Receptores de Interleucina-8B. 7. Ratos Wistar. I. Bogo, Maurício Reis. II. Campos, Maria Martha. III. Título.

CDD 615.1
NLM QV 601

Ficha Catalográfica elaborada por
Vanessa Pinent
CRB 10/1297

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Aprovada em: 02 de Janeiro de 2014.

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Porto Alegre

2014

Aos meus pais, pelo exemplo de vida e apoio incondicional.

AGRADECIMENTOS

À Deus, pela força e coragem de cada dia!

Aos meus pais, Rudemar e Lisete Ana da Costa, meus exemplos de vida e caráter. Por acreditarem em mim e acima de tudo, me dar o mais incondicional dos sentimentos, o amor imenso.

Ao meu irmão, Cristian Eduardo da Costa, por lembrar a todo o momento de que eu era capaz!

Ao meu noivo, Alexandre Telles, por pacientemente compreender a minha ausência e escolhas, mas mesmo assim me dedicar tanto amor.

À minha segunda família, Jorge, Mari e Lucas Grün, por me deixarem fazer parte de suas vidas e me acolherem com tanto carinho.

Ao meu orientador, Prof. Dr. Maurício Reis Bogo, por acreditar em mim e possibilitar a realização deste trabalho; pelo incentivo e apoio, mas principalmente pelos ensinamentos, paciência e amizade.

À Profa. Dra. Maria Martha Campos, por ter estendido a mão quando mais precisei e por ter acreditado (sempre) que este trabalho seria possível. Agradeço muito pela co-orientação e acima de tudo pelos ensinamentos, apoio, paciência e amizade.

Ao colega Izaque S. Maciel, por ter me ensinado todo o necessário para que este trabalho pudesse acontecer e sobretudo, pela amizade.

À colega Luiza W. Kist, pelo excelente apoio técnico.

Ao colega Rodrigo B. M. da Silva, pela ajuda e apoio técnico.

Aos colegas do Laboratório de Farmacologia Aplicada 1, pelo carinho, amizade e companheirismo.

Aos colegas do Laboratório de Habilidades Médicas e Pesquisa Cirúrgica, pela acolhida e presteza.

Aos colegas do Instituto de Toxicologia e Farmacologia, pela parceria e dedicação.

Aos colegas do Laboratório de Pesquisa Biologia Genômica e Molecular, Laboratório de Cultura de Células e Laboratório de Análises Hematolócias, pelo apoio técnico.

Minhas queridas: Ana Letícia V. Barcellos, Joice N. Ott, Priscilla B. Pail e Raquel Dal Sasso Freitas pelo apoio, incentivo e principalmente pelo “ombro” amigo.

“A Força não vem da capacidade física, ela vem de uma vontade inabalável.”

Mahatma Gandhi

RESUMO

O paraquat (PQ) é um composto químico bastante utilizado no mundo, o qual é aliado a potenciais riscos de intoxicação. Este herbicida induz a formação de espécies reativas de oxigênio (ROS) que podem comprometer vários órgãos, especialmente os pulmões (através da recaptação de poliaminas), e o cérebro (pela perda de neurônios dopaminérgicos). Este trabalho avaliou os efeitos deletérios do paraquat frente ao Sistema Nervoso Central (SNC) (como mudanças nos parâmetros fisiológicos, respostas nociceptivas, atividade locomotora e coordenação motora, e o perfil de expressão de alguns marcadores moleculares de inflamação), e periférico (como células inflamatórias no sangue e pulmões), com o objetivo de avaliar o possível papel protetor do antagonista de receptores de quimiocinas CXCR2, o SB225002, nestes parâmetros. A toxicidade ao paraquat foi induzida em ratos machos Wistar, em uma dose total de 50 mg/kg, administrada intraperitonealmente (i.p.) (10 mg/kg a cada três dias). Animais do grupo controle receberam solução salina (10 ml/kg) no mesmo protocolo de administração. Diferentes grupos de animais foram tratados com o antagonista SB225002 (1 ou 3 mg/kg, i.p.), administrado 30 minutos antes de cada aplicação do paraquat. As prinicpais mudanças encontradas nos animais tratados com paraquat foram: diminuição do peso corporal e hipotermia, aumento da resposta nociceptiva, diminuição da capacidade de marcha e locomoção, aumento da expressão de *TNF-α* e *IL-1β* no estriado, e a migração de células inflamatórias no sangue e pulmões. Alguns destes parâmetros foram revertidos quando administrado o antagonista SB225002, como recuperação dos parâmetros fisiológicos, diminuição da nocicepção, melhora na atividade de marcha, modulação da expressão estriatal de *TNF-α* e *IL-1β*, e diminuição da migração neutrofílica para o sangue e pulmões. Em conjunto, nossos resultados demonstram que os danos causados pelo paraquat ao sistema central e periférico podem ser prevenidos através da inibição farmacológica dos receptores de quimiocinas CXCR2. A evidência experimental aqui apresentada extende a compreensão dos efeitos toxicodinâmicos do paraquat, e proporciona novas possibilidades para tratar a intoxicação causada por este herbicida.

Palavras-chave: Paraquat; inflamação; quimiocinas; CXCR2; SB225002.

ABSTRACT

Paraquat (PQ) is an agrochemical agent commonly used worldwide, which is allied to potential risks of intoxication. This herbicide induces the formation of reactive oxygen species (ROS) that ends up compromising various organs, particularly the lungs (by the polyamine uptake system), and the brain (by dopaminergic neuronal cell loss). This study evaluated the deleterious effects of paraquat on the central nervous system (CNS) (changes in physiologic parameters, nociceptive responses, locomotor activity and motor coordination, and expression profile of some inflammatory markers), or peripherally (inflammatory cells in the blood and lungs), with special attempts to assess the putative protective effects of the selective CXCR2 receptor antagonist SB225002 on these parameters. PQ-toxicity was induced in male Wistar rats, in a total dose of 50 mg/kg, given by the intraperitoneal (i.p.) route (10 mg/kg each three days). Control animals received saline solution (10 ml/kg) at the same schedule of administration. Separate groups of animals were treated with the selective CXCR2 antagonist SB225002 (1 or 3 mg/kg, i.p.), administered 30 min before each paraquat injection. The major changes found in paraquat-treated animals were: decreased body weight and hypothermia, nociception behavior, impairment of locomotor and gait capabilities, enhanced *TNF-α* and *IL-1β* expression in the striatum, and cell migration to the lungs and blood. Some of these parameters were reversed when the antagonist SB225002 was administered, including recovery of physiological parameters, decreased nociception, improvement of gait abnormalities, modulation of striatal *TNF-α* and *IL-1β* expression, and decrease of neutrophil migration to the lungs and blood. Taken together, our results demonstrate that damage to the central and peripheral systems elicited by paraquat can be prevented by the pharmacological inhibition of CXCR2 chemokine receptors. The experimental evidence presented herein extends the comprehension on the toxicodynamic aspects of paraquat, and opens new avenues to treat intoxication induced by this herbicide.

Keywords: Paraquat; inflammation; chemokines; CXCR2; SB225002.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIAÇÕES

PQ – Paraquat

i.v. – Intravenoso

SNC – Sistema Nervoso Central

BHE – Barreira Hematoencefálica

EROs – Espécies Reativas de Oxigênio

SOD – Superóxido Dismutase

PMNs – Polimorfonucleares

ELR – Glutamic acid-leucine-arginine

IL-8/CXCL8 – Interleucina-8

MPO – Mieloperoxidase

SB225002 – N-(-2-hidroxi-4-nitrofenil) – N*(2-bromofenil)ureia

TNBS – Ácido 2,4,6-trinitrobenzeno sulfônico

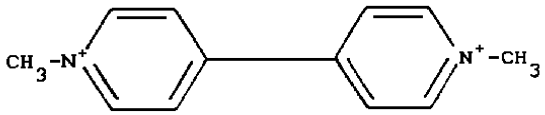
i.p. – Intraperitoneal

SUMÁRIO

1 INTRODUÇÃO

1.1 Paraquat

O paraquat (1,1´-dimetil-4,4´-bipiridílio dicloro; PQ) (Figura 1) pertence ao grupo dos herbicidas bipiridílios quarternários de amônia, apresentando como característica a sua falta de seletividade, mas alta eficiência. Em vista a estes atrativos e por apresentar baixo custo, é amplamente utilizado na agricultura. Além disso, não possui efeito poluente ambiental considerável e, por esta razão, os cuidados em relação ao uso são negligenciados[1; 2].



Neurobiology of Disease, 2002.

Figura 1. Estrutura química do herbicida paraquat.

O abuso deste herbicida também pode ser intencional nas tentativas de suicídio. Porém, muitas vezes, os casos de intoxicações são acidentais[1]. O paraquat é encontrado em vários órgãos incluindo o coração, fígado, rins, timo e pulmões[3]. Estudos demonstraram que este herbicida também já foi encontrado no sistema imune e hematopoiético, sendo localizado em vários tipos de células sanguíneas (granulócitos, eritrócitos e megacariócitos) e seus precursores entre 24 h e sete dias após administração intravenosa (i.v.)[4]. No entanto, é nos pulmões que ocorre o maior acúmulo, devido à grande afinidade pelos pneumócitos tipo I e II, além das células de Clara[5]. Este órgão é severamente afetado, o que pode resultar em inflamação alveolar endotelial e fibrose, que pode culminar na falência respiratória e morte[1]. A lesão pulmonar aguda seguida de fibrose, e a massiva inflitração celular nos espaços alveolares podem ser observadas após a intoxicação com uma única aplicação de paraquat[3].

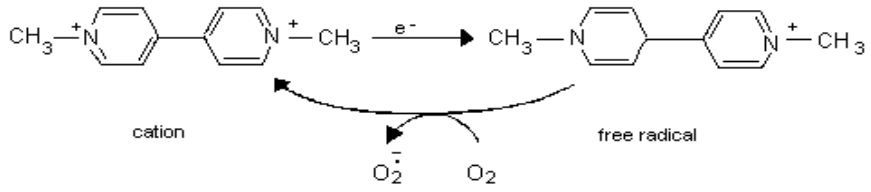
O Sistema Nervoso Central (SNC) também é drasticamente lesado pelo herbicida, o qual induz alterações preferenciais no sistema nigrostriatal, acúmulo no córtex cerebral e hipocampo[6; 7; 8]. Após 30 minutos da administração do paraquat, este alcança seu pico máximo de concentração no cérebro e plasma[9], onde estudos realizados em roedores indicam

que este herbicida atravessa a barreira hematoencefálica (BHE) através do sistema de transporte ativo envolvendo aminoácidos neutros grandes[10]. Após esta exposição, pode haver lesão de neurônios ligados à aprendizagem e memória[11], bem como, indução de alterações no sistema motor e disfunções mitocondriais estriatais [8; 12; 13].

Vários estudos *in vivo* têm demonstrado que repetidas administrações de paraquat – que variam de cinco dias a seis semanas – em camundongos ou ratos, produz diminuição do número de neurônios dopaminérgicos da substância nigra, diminuição de dopamina no estriado e seus metabólitos, diminuição da expressão e atividade da tirosina hidroxilase, e imunorreatividade do transporte de dopamina[14; 15]. Velez-Pardo e colaboradores (2010) demonstraram, em estudos prévios *in vivo*, que os efeitos diretos deste herbicida no córtex cerebral são associados com o aumento da produção de superóxido e despolarização das organelas mitocondriais, apresentando uma clara disfunção mtocondrial[16].

As propriedades tóxicas do paraquat podem estar relacionadas à possibilidade de dano neuronal, através de mecanismos de estresse oxidativo[17], onde as reações de oxirredução levam à formação de radicais livres. Os radicais livres são moléculas instáveis e quimicamente reativas que podem ser geradas no citoplasma celular, mitocôndrias e membrana das células[1]. Segundo a literatura, as mitocôndrias são as maiores fontes de produção de espécies reativas de oxigênio (EROs) quando expostas ao herbicida [13; 18].

Os radicais livres e as espécies reativas de oxigênio – superóxido, peróxido de hidrogênio e radicais hidroxila – reagem com lipídios, ácidos graxos e DNA das células-alvo, causando danos graves. Existem diferentes sistemas antioxidantes endógenos, incluindo superóxido dismutase (SOD), catalase e glutationa. As substâncias antioxidantes interceptam os radicais livres gerados evitando lesões e a perda da integridade da célula. Entretanto, em situações de intoxicações graves, como no caso do paraquat, as moléculas anti-oxidantes não são capazes de impedir os danos causados ao organismo pelos radicais livres, gerando o aumento do estresse oxidativo[1] (Figura 2).



Bus and Gibson, 1984.

Figura 2. Esquema de oxirredução do paraquat.

1.2 Receptores CXCR2

O paraquat pode causar danos em vários tecidos[19]. No tecido lesado, através da ação de quimiocinas, ocorre o recrutamento e a migração de leucócitos polimorfonucleares (PMNs) da corrente sanguínea para o local da inflamação por quimiotaxia, um processo regulado por diversos mediadores de diferentes classes [20].

Quimiocinas são um subconjunto das citocinas (8-10 kDa) que regulam seletivamente o recrutamento de leucócitos para tecidos inflamados, pricipalmente através da ativação de receptores acoplados à proteína G[21; 22]. Estes receptores são classificados em diferentes famílias, de acordo com o tipo de quimiocinas as quais se ligam (CC, CXC, C ou CX_3C)[23]. As quimiocinas CXC podem ser divididas em um grupo de quimiocinas que contém um domínio ácido glutâmico-leucina-arginina (ELR) modificado e o grupo de quimiocinas sem esta modificação. Sete dos quinze ligantes CXC (CXCL1-3 e CXCL5-8) contém o terminal ELR modificado, com alta afinidade para os receptores de quimiocina CXCR2[24]. Quimiocinas CXC são especialmente envolvidas na migração de leucócitos polimorfonucleares, onde o protótipo deste grupo é a interleucina-8 (IL-8/CXCL8), que se liga aos receptores CXCR1/2[25], e recruta leucócitos da corrente sanguínea para o tecido inflamado[20]. A comparação entre as sequências CXCR1 e CXCR2, mostrou aproximadamente 70% de identidade entre os genes humanos e de ratos (*Rattus norvegicus*) e camundongos (*Mus musculus*)[24].

Quimiocinas e receptores de quimiocinas são potenciais alvos terapêuticos para uma variedade de doenças[26; 27]. Além de células hematopoiéticas, outros tipos celulares como células endoteliais, musculares, e epiteliais expressam os receptores para quimiocinas[28]. Embora haja 77% de similaridade entre estes receptores[21], em 95% dos casos ocorre

internalização do CXCR2 em cerca de 2-5 minutos após sua ativação, em comparação com apenas 10% para o receptor CXCR1. Esta diferença entre o tempo de ativação dos receptores é um importante fator que distingue a habilidade do CXCR1 e CXCR2 em mediar ativação e regulação de leucócitos em resposta ao mediador CXCL8[29].

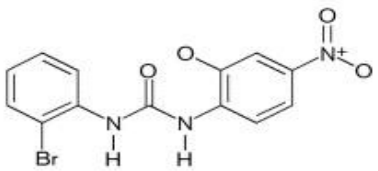
O receptor para quimiocinas CXCR2 e seus ligantes têm sido associados a uma variedade de patologias neuroinflamatórias, bem como, no recrutamento de alguns tipos de leucócitos especializados e no desenvolvimento de células neurais[24]. A ativação de neutrófilos por estímulos endógenos (como citocinas) é seguida pela ativação do consumo de oxigênio para a produção de ânion superóxido – precursor de uma série de espécies reativas de oxigênio. A partir desse processo, um grande número de oxidantes altamente microbicidas são formados, tais como HOCl, HO•, ONOO[30].

Tais oxidantes são produzidos com a finalidade de combater microrganismos invasores, embora também provoquem danos, estando envolvidos em um grande número de alterações patológicas. A formação de espécies reativas é equilibrada naturalmente pela existência de compostos e sistemas antioxidantes. Os grânulos azurófilos dos neutrófilos apresentam níveis elevados de mieloperoxidase (MPO), a qual, juntamente com a NADPH oxidase de membrana, está envolvida na geração de espécies reativas de oxigênio e oxidação de biomoléculas[30].

1.3 Antagonista SB225002

Após 20 anos de pesquisas, foi identificado no SNC e em doenças relacionadas o papel do CXCR2 na resposta imune[31]. Assim, diferentes antagonistas dos receptores de quimiocinas estão sendo desenvolvidos com o objetivo de modular a inflamação e a resposta imune[32].

Em meados da década de 1990, foram iniciados alguns estudos a fim de identificar antagonistas potentes e seletivos dos receptores CXCR2[33]. O SB225002 (N-(-2-hidroxi-4-nitrofenil) – N*(2-bromofenil)ureia) (Figura 3) representa o primeiro antagonista seletivo e não-peptídico dos receptores CXCR2, que foi desenvolvido em 1998 por White e colaboradores[20].



White et al., 1998.

Figura 3. Estrutura química do antagonista SB225002.

Estudos realizados por Bento e colaboradores (2008)[34], empregando um modelo *in vivo* de colite em camundongos induzido por ácido 2,4,6-trinitrobenzeno sulfônico (TNBS), demonstrou que a administração intraperitoneal (i.p.) de SB225002, em baixas doses como 1 mg/kg, foi capaz de prevenir vários quadros inflamatórios, como a migração neutrofílica no cólon[34]. Este antagonista, quando testado em protocolos experimentais *in vitro*, produziu inibição da quimiotaxia para neutrófilos humanos e de coelhos. Os resultados obtidos *in vitro* foram confirmados por estudos *in vivo*, sendo que o SB225002 inibiu seletivamente a marginação de neutrófilos induzida por IL-8/CXCL8 em coelhos. Esse antagonista também foi capaz de inibir as respostas nociceptivas induzidas por vários agentes em camundongos[20].

Manjavachi e colaboradores (2010), demonstraram que o tratamento sistêmico em camundongos com este antagonista não provocou efeitos tóxicos significativos quando em protocolos de tratamentos crônicos, reforçando a segurança desta droga. Além disso, mostrou ser eficaz em diminuir a atividade da mieloperoxidase induzida por aplicações de carragenina nas patas bem como a diminuição das respostas nociceptivas abdominais induzida por ácido acético[20]. O SB225002 também se mostrou eficaz em reduzir a proliferação de células tumorais de carcinoma de boca de linhagens humanas e de roedores através de mecanismos que parecem estar relacionados com a modulação do processo inflamatório[35].

Finalmente, em estudos recentes Zhang e colaboradores (2013), demonstraram que aplicações na região espinhal com o antagonista SB225002 (5 e 20 µg/sítio) inibiu a alodínia mecânica causada por CXCL1/KC em camundongos no modelo de dor neuropática[22]. Dornelles e colaboradores (2013), demonstraram que este antagonista sozinho ou em combinação ao antagonista de TRPV1, o SB266791, foi capaz de de prevenir em ratos alterações funcionais, inflamatórias e nociceptivas relacionadas à cistite hemorrágica induzida através de aplicações i.p. de ciclofosfamida[36].

Uma vez que, a indução da inflamação tem sido associada à regulação de quimiocinas e seus receptores no SNC[37], o uso do antagonista SB225002 pode tornar-se uma valiosa ferramenta farmacológica no bloqueio e manejo de mediadores inflamatórios através da inibição da ativação de receptores CXCR2.

2 OBJETIVOS

2.1. Objetivos Gerais

O presente trabalho teve como objetivo avaliar a toxicidadde central e periférica causada pelo herbicida paraquat, bem como, os efeitos do tratamento com o SB225002, um antagonista dos receptores CXCR2, sobre estes efeitos em ratos machos Wistar.

2.2. Objetivos Específicos

- Avaliar, por meio de parâmetros fisiológicos, como peso e temperatura, o impacto causado no organismo antes e após cada exposição ao paraquat.
- Determinar, por meio de testes comportamentais/motores, como Campo Aberto; Teste de Equilíbrio e Coordenação Motora; e Teste de Marcha, o grau de comprometimento do SNC após toxicidade ao herbicida.
- Analisar, por meio de técnica de coloração e contagem diferencial hematológica, a quantificação de células leucocitárias no sangue periférico.
- Analisar, por meio de técnica de mieloperoxidade, a migração de células inflamatórias para os tecidos pulmonares.
- Analisar, por meio de técnica de RT-qPCR, a expressão gênica de marcadores inflamatórios como *COX-2*; *IL-1β*; *TNF-α* e *NF-κB1* no estriado cerebral de ratos.

3 CONSIDERAÇÕES FINAIS

Considerando a gravidade do quadro de intoxicação causado pelo paraquat associado ao seu uso, observa-se limitadas opções terapêuticas para reverter os quadros de intoxicações, minimizando os efeitos causados após o contato. Até o presente, não há um antídoto específico para a intoxicação com o paraquat tanto a nível central quanto a nível periférico. A conduta terapêutica consiste em tratar os danos causados ao organismo com antioxidantes e anti-inflamatórios[1].

Segundo estudos de Manjavachi e colaboradores (2010), o uso do antagonista SB225002 parece ser uma alternativa promissora para o controle de alterações inflamatórias relacionadas ao SNC, visto que este conseguiu inibir a marginação de neutrófilos para locais de inflamação, diminuindo significativamente as reações de estresse oxidativo[20]. Portanto, este antagonista poderia ser uma alternativa a ser utilizada para tentar diminuir as alterações causadas pelo estresse oxidativo no SNC.

De acordo com Czerniczyniec e colaboradores (2011), em experimento realizado com exposições agudas de paraquat, roedores desenvolveram – no período de um mês – alterações comportamentais e comprometimento das regiões mitocondrial estriatal e cortical[12]. O uso deste antagonista mostrou-se também de extrema importância nos quadros de dor causado por processos inflamatórios mediado pelo receptor CXCR2[22].

O presente trabalho investigou a participação dos receptores CXCR2 para quimiocinas nas respostas inflamatórias mediadas pela toxicidade do herbicida paraquat em modelo *in vivo* em roedores, utilizando para isto o emprego de técnicas farmacológicas, comportamentais, bioquímicas e de biologia molecular. Os nossos resultados demonstraram, pela primeira vez, que o bloqueio dos receptores CXCR2, pela administração de inibidor seletivo, o antagonista SB225002, por via i.p., conseguiu diminuir de forma significativa as respostas inflamatórias no protocolo de toxicidade crônica induzido pelo herbicida.

Observou-se que os animais apenas tratados com paraquat apresentaram ao longo do protocolo, e sendo mais evidente a medida em que houve a sobreposição das doses, uma significante mudança nos parâmetros fisiológicos e comportamentais. Houve também, a gradual perda tanto da atividade quanto da habilidade locomotora destes animais, o que nos leva a sugerir e também com base em estudos prévios, que o paraquat seria um potencial mediador de lesão a nível de SNC. Contudo, verificamos também que houve o aumento da

expressão gênica de marcadores moleculares inflamatórios a nível estriatal, bem como a migração de PMNs a nível de sistema periférico e no tecido pulmonar.

No presente estudo, os animais tratados somente com o antagonista em doses de 1 a 3 mg/kg não demonstraram nenhum efeito *per se*, verificando que os achados encontrados nas análises ao longo do protocolo permaneceram iguais ao grupo controle, o que nos mostra que este composto não acarreta toxicidade ao modelo.

Interessantemente, observou-se que os ratos tratados previamente com o antagonista SB225002 apresentaram uma significativa melhora nos parâmetros fisiológicos e comportamentais, mostrando também que a parte locomotora destes animais foi mais preservada do que o grupo que recebeu somente as aplicações do herbicida. Para nossa constatação, este antagonista se mostrou bastante eficaz em diminuir a quimiotaxia de neutrófilos para o sistema periférico, bem como para os tecidos pulmonares, reduzindo consideravelmente a atividade da mieloperoxidase, contribuindo na diminuição do agravamento do quadro inflamatório em ratos quando expostos a aplicações de paraquat. Em relação aos marcadores inflamatórios cerebrais, observou-se que o SB225002 modulou a expressão estriatal de *TNF-α* e *IL-1β.*

Os resultados deste estudo reforçam achados prévios[38], sugerindo que a exposição ao herbicida paraquat causa uma condição inflamatória sistêmica que é evidenciada pela participação dos receptores de quimiocinas, principalmente no que diz respeito a recrutação de leucócitos específicos para os tecidos lesados, onde o bloqueio seletivo pelo antagonista SB225002 pode representar uma importante alternativa nos processos inflamatórios e dolorosos. Os dados aqui apresentados são consistentes com a ideia de que este potente e seletivo antagonista dos receptores CXCR2, pode representar uma alternativa considerável nos casos de respostas inflamatórias e nociceptivas causadas por este xenobiótico, especialmente onde a quimiotaxia de neutrófilos está diretamente relacionada.

Torna-se imprescindível a realização de mais estudos, a fim de determinar os mecanismos que levam ao estresse oxidativo, bem como a análise fisiopatológica que este herbicida causa, tanto a nível basal quanto comportamental.

Formatado: Inglês (EUA)

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ANEXOS

ANEXO A – CARTA DE APROVAÇÃO CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 097/12 – CEUA

Porto Alegre, 02 de agosto de 2012.

Senhor Pesquisador:

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 12/00295, **"Participação dos receptores CXCR2 para quimiocinas na toxicidade induzida pelo paraquat em roedores".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora da CEUA/PUCRS

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ANEXO B – MANUSCRITO DO TRABALHO EXPERIMENTAL

Os resultados do presente trabalho foram submetidos à revista PlosOne®, fator de impacto 4.09 (JCR 2013).

Formatado: Inglês (EUA)

PHARMACOLOGICAL INHIBITION OF CXCR2 CHEMOKINE RECEPTORS MODULATES PARAQUAT-INDUCED INTOXICATION IN RATS

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Código de campo alterado

Abstract

Paraquat (PQ) is an agrochemical agent commonly used worldwide, which is allied to potential risks of intoxication. This herbicide induces the formation of reactive oxygen species (ROS) that ends up compromising various organs, particularly the lungs (by the polyamine uptake system), and the brain (by dopaminergic neuronal cell loss). This study evaluated the deleterious effects of paraquat on the central nervous system (CNS) (changes in physiologic parameters, nociceptive responses, locomotor activity and motor coordination, and expression profile of some inflammatory markers), or peripherally (inflammatory cells in the blood and lungs), with special attempts to assess the putative protective effects of the selective CXCR2 receptor antagonist SB225002 on these parameters. PQ-toxicity was induced in male Wistar rats, in a total dose of 50 mg/kg, given by the intraperitoneal (i.p.) route (10 mg/kg each three days). Control animals received saline solution (10 ml/kg) at the same schedule of administration. Separate groups of animals were treated with the selective CXCR2 antagonist SB225002 (1 or 3 mg/kg, i.p.), administered 30 min before each paraquat injection. The major changes found in paraquat-treated animals were: decreased body weight and hypothermia, nociception behavior, impairment of locomotor and gait capabilities, enhanced *TNF-α* and *IL-1β* expression in the striatum, and cell migration to the lungs and blood. Some of these parameters were reversed when the antagonist SB225002 was administered, including recovery of physiological parameters, decreased nociception, improvement of gait abnormalities, modulation of striatal *TNF-α* and *IL-1β* expression, and decrease of neutrophil migration to the lungs and blood. Taken together, our results demonstrate that damage to the central and peripheral systems elicited by paraquat can be prevented by the pharmacological inhibition of CXCR2 chemokine receptors. The experimental evidence presented herein extends the comprehension on the toxicodynamic aspects of paraquat, and opens new avenues to treat intoxication induced by this herbicide.

Keywords: Paraquat; inflammation; chemokines; CXCR2; SB225002.

Introduction

Paraquat (PQ) dichloride is a fast-acting, non-selective bipyridylium herbicide, widely used in many countries worldwide due to the low costs and effectiveness against a range of weeds. The accidental or intentional poisoning with this agent has been associated with fatal cases since there is a lack of effective treatments to intoxication (for review see: [1]). In fact, the mechanisms implicated in the toxicity caused by paraquat exposure are not yet completely understood [2]. Regardless, it has been demonstrated that paraquat effects are primarily allied with oxidative stress through the production of reactive oxygen species (ROS) [3,4]. After exposure, the herbicide can be detected in several organs including heart, kidneys, liver, thymus, and lungs [5]. The concentration in lungs is commonly highly elevated, as a consequence of its affinity for type 2 pneumocytes. Of note, acute lung injury with fibrosis and massive cell infiltration in the alveolar spaces can be observed after intoxication with a single dose of paraquat [6].

Chemokines are a subset of cytokines that selectively regulate the recruitment of leukocytes to inflammatory sites, mainly through the activation of G-protein-coupled receptors [7,8]. These receptors are classified into different families according to the type of chemokines they bind (CC, CXC, C, or CX_3C) (for review see: [9]). CXC chemokines are especially involved in migration of polymorphonuclear leukocytes (PMNs), and the prototype of this group is interleukin-8 (IL-8/CXCL8), which binds to the CXCR1/2 receptors [10]. Of note, it has been demonstrated that paraquat is able to activate a set of genes related to inflammatory pathways (IL-10, CXL10, and CXL11), according to evaluation of the human keratinocyte cell line HaCaT [11].

In 1998, White et al. [12] described the first non-peptide, selective, and competitive CXCR2 receptor antagonist, named SB225002, which was found effective in preventing a series of IL-8-mediated inflammatory responses, both *in vitro* and *in vivo*. By using an *in vivo* model of mouse colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS), Bento et al. [13] demonstrated that intraperitoneal (i.p.) administration of SB225002, in doses as low as 1 mg/kg, was able to prevent several inflammatory parameters, such as neutrophil migration to the colon tissue. Furthermore, i.p. treatment with SB225002 resulted in a marked and long-lasting inhibition of acute or chronic nociception in mice [14]. More recently, SB225002 was demonstrated to be effective in preventing the functional, inflammatory, and nociceptive alterations related to cyclophosphamide-induced cystitis in rats when dosed i.p., alone or in combination with the transient receptor potential vanilloid 1 (TRPV1) antagonist SB266791

[15]. Another recent study demonstrated that spinal injection of SB225002 (5 and 20 µg/site) significantly inhibited the mechanical allodynia caused by CXL1/KC in mice [8].

The present study examined, for the first time, the possible beneficial effects of SB225002 in a rat model of paraquat intoxication, induced by repeated exposure to low doses of this herbicide. Interestingly, our results revealed that pharmacological inhibition of CXCR2 receptors was allied to improvement of both central and peripheral complications induced by paraquat, thus contributing to clarify its mechanisms of toxicity.

Materials and Methods

Ethics Statement

Experiments were conducted in accordance with current guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals [16]. All the experimental procedures were approved by the Animal Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul (RS) (Protocol Number: CEUA 12/00295).

Animals

Male Wistar rats (weighing 180 g at the beginning of experiments) used in this study were obtained from the Central Animal House of Universidade Federal de Pelotas (UFPEL, Brazil). The animals were housed in groups of four and maintained in controlled temperature (22 ± 1ºC) and humidity (60–70%), under a 12 h light-dark cycle (lights on 07:00 AM). Food and water were available *ad libitum*. Animals were acclimatized to the laboratory for at least 1 h before testing and all the tests were performed between 9:00 AM and 5:00 PM. The number of animals and intensity of noxious stimuli used were the minimum necessary to provide consistent effects of the treatments. Analgesics were not administered to avoid any possible interference with the obtained results. In all experimental protocols, the animals were monitored twice daily for signals of severe distress. The criteria adopted to decide on euthanasia included the occurrence of tremors, convulsions, and/or chromodacryorrhea. The euthanasia was performed by deep anesthesia with isoflurane.

General protocols of treatment

Initially, a total of 28 rats received i.p. applications of paraquat at different doses [5 (n=4), 10 (n=14), 15 (n=4), and 20 (n=6) mg/kg] [17], twice a week for 28 days, in order to determine the appropriate dose for the next experiments. A separate group of animals received saline solution (n=8) as control (Figure 1A). The number of animals used to determine the experimental design was 36 rats. Next, on the basis of the previous set of experiments and based on the literature [18], animals were provided with paraquat (10 mg/kg), twice a week for 14 days, at a total dose of 50 mg/kg. The animals used in the behavioral analysis were further used in molecular and biochemical analysis. Briefly, rats were randomly divided into six groups comprising: (A) vehicle/saline (1% Tween-80 in 0.9% NaCl solution; 10 ml/kg), with a total number of 15 animals; (B) SB225002 (1 mg/kg)/saline, with a total number of six

animals; (C) SB225002 (3 mg/kg)/saline, with a total number of four animals; (D) vehicle/paraquat, with a total number of 20 animals; (E) SB225002 (1 mg/kg)/paraquat, with a total number of nine animals and (F) SB225002 (3 mg/kg)/paraquat, with a total number of 13 animals. The number of animals used in the behavioral, molecular, and biochemical analysis was 67 rats. Therefore, the total number of animals used in this study was 103 rats. The doses of SB225002 were selected on the basis of previous publications [14]. The antagonist (or the vehicle) was administered 30 min before each paraquat or saline application. The protocol of the treatments is outlined in Figure 1B.

Body weight measurement

Rat body weight was recorded (in g) using a digital balance (Bioprecisa®). All the animals were weighed once a day at the basal timepoint, and before the 2nd, 3rd, 4th, and 5th applications. The values are expressed as the difference between the initial weights minus the weight values on the day of measurement.

Body temperature assessment

The rectal temperature was recorded (in ^{o}C) using a commercially available thermometer (Pro-check®). After recording the initial rectal temperature (basal measurement; in ^{o}C), the body temperature was evaluated on days corresponding to the 2nd, 3rd, 4th, and 5th applications at two different timepoints: immediately before, and 1 h after the treatments. The values are expressed as the difference between the temperatures before and after application.

Nociceptive responses

The method adopted in the present study was similar to that described previously [19,20,21,22], with minor modifications. The animals were analyzed in their cages in order to minimize the potential variations in the behavioral responses. After the treatments, the animals were assessed in two different approaches: (i) the spontaneous behavior was measured 25 min after treatment for 5 min; (ii) the spontaneous behavior was measured after 55 min for 5 min, over a total period of a 1 h. The behavioral alterations were scored according to the following scale: 0 = absence of any signal, with normal eyes; 1 – piloerection; 2 – stretching; 1 – hump-backed position; 1 – half-closed eyes; 2 – completely closed eyes. All the experiments were performed in a blinded manner, each experimenter

scoring four rats in parallel. The scores were registered over each 5-min period, and summed for an overall pain score (maximum score= 6).

Open-field test

To analyze the locomotor activity, the animals were evaluated in the open-field test, according to the methodology described before [23], with minor modifications. The experiments were conducted in a sound-attenuated room, under low-intensity light. Rats were individually placed in the center of a plywood box (34.5 cm height x 249 cm circumference x 77.5 cm radius), with the floor divided into 17 squares. Duration of grooming (s) was cumulatively registered over 5 min. The number of rearing behaviors and fecal boluses was also recorded. Additionally, the number of squares crossed with the four paws was registered. All the parameters were measured at the basal timepoint and after the 3rd and 5th applications.

Beam-walking test

To assess the locomotor skills, the animals were evaluated in the beam-walking test. The methodology used was the same described previously [24]. Each rat was positioned in the center of a wooden narrow bridge (3.5 cm wide and 85 cm long), suspended between two platforms at an angle of 8º [25]. The time taken to cross the beam (in seconds, s), and the number of paw slips and falls was registered during 3 min. Each animal was acclimatized on the platform on three consecutive days before the experiments. The measurements were made at the basal timepoint and after the 3rd and the 5th applications.

Footprint test

To determine the gait abnormalities induced by paraquat, the animals were evaluated in the footprint test according to the methodology described by Richter et al. [26] with minor modifications, at the baseline and after the 3rd and the 5th applications of the herbicide. The rats were allowed to walk through an 85-cm-long, 5-cm-wide runway corridor, with a darkened cage at the end of the corridor. The floor of this corridor was covered with a new white absorbing paper for each run. The animals were first trained to pass straight forward through the corridor on three consecutive days before the experiments. After the training, the paws were colored with different colors (red for the forepaws and black for the hindpaws), and the rats were placed into the corridor. For footprint analysis, both the initial and the final 10 cm of the stamped paper was excluded to avoid false-positive results [27]. Two distinct

parameters were considered for analysis: (i) the stride length was measured as the average distance between the extremity of third toes of the forepaws and hindpaws, for both left and right paws; (ii) the step frequency was evaluated according to the total number of footprints. The averages obtained by two blinded researchers were considered as a duplicate.

Gene expression analysis by quantitative real-time RT-PCR (RT-qPCR)

Following the behavioral tests assessments, the animals were euthanized by deep inhalation of isoflurane and all the right and left striatum was collected. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry (A260/280 nm) and after treated with deoxyribonuclease I (Invitrogen) to eliminate genomic DNA contamination. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 µg total RNA, following the manufacturer´s instruction. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-stranded cDNA synthesis. Reactions were done in a volume of 25 µL using 12.5 µL of diluted cDNA, containing a final concentration of 0.2x SYBR® Green I (Invitrogen), 100 µM dNTP, 1x PCR Buffer, 3 mM $MgCl_2$ 0.25 U Platinum® Taq DNA Polymerase (Invitrogen), 0.5 M betaine, and 200 nM of *COX-2* [28], *IL-1β*, *TNF-α* [29], *NF-κB1* [30], or *Rpl13α* [31] primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95°C, 40 cycles of 15 s at 95°C for denaturation, 35 s at 60°C for annealing, and 15 s at 72°C for elongation. At the end of the cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99°C and showed in all cases one single peak. *Rpl13α* was used as an endogenous control. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 2012.3 Software (http://LinRegPCR.nl). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method [32].

Código de campo alterado

Hematologic parameters

After the behavioral assessments, the animals were euthanized by deep inhalation of isoflurane and the blood was collected. Immediately after, a small drop of blood was taken for smear evaluation using May-Grunewald-Giemsa staining [33]. Differential counts (neutrophils, eosinophils, basophils, lymphocytes, monocytes, and immature cells) were

estimated under a ×40 objective (Olympus® CH30 model), by counting 100 cells. Representative pictures were captured by Zeiss® Axio Imager A1.

Myeloperoxidase (MPO) activity

Neutrophil recruitment to the rat lung was measured by means of tissue MPO activity, according to the method described by Souza et al. [34], with minor modifications. After euthanasia by deep inhalation of isoflurane, the lungs of animals were removed and immediately stored at -80°C. The tissues were homogenized in 5% (w/v) EDTA/NaCl buffer (pH 4.7) and centrifuged at 4000 rpm for 25 min, at 4°C. The pellet was resuspended in 0.5% hexadecyltrimethyl ammonium bromide buffer (pH 5.4), and the samples were re-centrifuged (4000 rpm, 25 min, 4°C). Twenty-five microliters of the supernatant were used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM $NaPO_4$, and 0.3 mM hydrogen peroxide. The absorbance was measured at 595 nm, and the results are expressed in optical density (OD) per milligram of tissue.

Statistical analysis

Results are presented as mean ± standard error mean (SEM). The statistical comparison of the data was performed by one-way analysis of variance (ANOVA), followed by Bonferroni´s post-hoc test. Comparison of the survival curves was performed using the Log-rank test. *P* values less than 0.05 ($p<0.05$) were considered as indicative of significance (GraphPad Prism 5.0, La Jolla, CA, USA).

Drugs and Reagents

The following drugs and reagents were used: paraquat was purchased from Syngenta Limited (Huddersfield, West Yorkshire, UK). N-(2-hydroxy-4-nitrophenyl)-N9-(2-bromophenyl) Urea (SB225002) was synthesized as described before [39] and provided by Universidade Federal de Santa Catarina (UFSC). SB225002 was identified by comparing the 1H NMR data with those published beforehand (Yield 70%, m.p. = 193–194 _C. 1H NMR (400 MHz) (ppm) (DMSO-d6) 11.05 (br. s, 1H), 9.48 (s, 1H), 9.12 (s, 1H), 8.36 (d, 1H), 7.93 (d, 1H), 7.75 (dd, 1H), 7.69 (s, 1H), 7.63 (d, 1H), 7.36 (t, 1H), 7.03 (t, 1H); IR (cm_1) 3364, 3212, 1692, 1588, 1536, 1507, 1430, 1306, 1267, 1212, 1086, 746, 649. LC–MS-ESI (m/z) 350 (M-H)-. SB225002 was dissolved in 1% Tween-80 (Merck, Haar, Germany) in 0.9% NaCl solution.

Results

Dose-related toxicity of paraquat and experimental design

This experimental set was designed to characterize the behavioral changes and mortality after treatment with different doses of paraquat in order to define the ideal scheme of treatment for the next experiments. Thus, different doses of paraquat [5 (n=4), 10 (n=14), 15 (n=4), and 20 (n=6) mg/kg] were administered to rats, twice a week over 28 days, and the percentage of survival (Figure 1A) and general behavioral changes (open-field test and beam-walking test) were registered (results not shown).

The animals treated with 5 mg/kg per dose of paraquat did not show significant changes in behavioral tests, displaying low toxicity, as was observed in the negative control group (treated with saline). In the 10 mg/kg paraquat group, significant time-related changes were observed in the behavioral tests, and 7/14 of animals died within the first 14 days. With paraquat doses of 15 mg/kg and 20 mg/kg, all the animals died within the first 14 days of treatment. Therefore, further experiments were performed using the dose of 10 mg/kg (sub-lethal dose), twice a week over 14 days, with a total cumulative dose of 50 mg/kg (Figure 1B).

Physiological changes evoked by paraquat: effects of SB225002

The treatment with 10 mg/kg per dose of paraquat, with a total dose of 50 mg/kg within 14 days, resulted in a significant and time-related reduction of body weight ($p<0.01$) in comparison to the control vehicle/saline group. This effect was partially, although not significantly, prevented by pre-treating animals with the selective CXCR2 antagonist SB225002 (3 mg/kg), as observed at the 5th application (Figure 2A).

The rectal temperature was checked at two distinct timepoints, immediately before and 1 h after treatments. The administration of paraquat resulted in a time-dependent and significant reduction of body temperature in both cases ($p<0.01$). This effect was significant from the 3rd application, when the temperature was checked before each administration of paraquat (Figure 2B), and at all the experimental times following paraquat dosage (Figure 2C). Of note, the treatment with SB225002 (3 mg/kg) was able to significantly reverse the body temperature decrease, according to assessment after the last application of paraquat ($p<0.05$; Figure 2C). SB225002 (either 1 or 3 mg/kg) did not elicit any significant effect (Figures 2A and 2B), except by a significant decrease of rectal temperature after 1 h of

antagonist application (4th application, 3 mg/kg SB225002), an action that was reversed in the subsequent measurement (5th application, 3 mg/kg SB225002) (Figure 2C).

Effects of treatment with SB225002 on paraquat-elicited nociception

We also investigated whether nociceptive changes induced by paraquat might be prevented by SB225002. Intoxication with paraquat was associated with time-dependent nociceptive behavior, which was significantly different from vehicle/saline control animals, as recorded at 25 (Figure 3A) or 55 min (Figure 3B) following the herbicide application. In either protocol, the maximal effects of paraquat was observed at the 5th application ($p<0.01$). Of note, the administration of SB225002 (3 mg/kg) was able to significantly reduce paraquat-evoked nociception, at either 25 ($p<0.05$) or 55 min ($p<0.01$) timepoints (Figure 3A and 3B). However, 1 mg/kg SB225002 displayed a significant effect on paraquat-induced nociception only at 55 min of evaluation ($p<0.01$).

Effects of paraquat administration in the open-field paradigm

In these experiments, we analyzed to what extent the treatment with paraquat might affect locomotor and exploration tasks in the open-field apparatus. Paraquat-intoxicated animals displayed significant reductions of crossing (Figure 4A) and rearing counts (Figure 4B) at both the 3rd and 5th applications ($p<0.01$). Although grooming behavior was not significantly affected (Figure 4C; $p>0.05$), paraquat administration caused a significant decrease in the number of fecal boluses (Figure 4D; $p<0.01$). SB225002 (1 or 3 mg/kg) did not display any unspecific effect, and this antagonist failed to significantly modify the effects of paraquat in the open-field model (Figure 4A-4D; $p>0.05$).

Assessment of motor coordination

The motor coordination was firstly evaluated in the beam-walking test. The traverse time was not significantly affected in either experimental group (Figure 5A). During the analysis of falls, one animal (1/18) fell after the 3rd application and another one (1/18) fell after the 5th application (Figure 5B), both belonging to the vehicle/paraquat group. Regarding paw slips, two animals (2/18) from the vehicle/paraquat group presented paw slips after the 3rd application, and five animals (5/18) of this group presented paw slips after the 5th application. One animal from the 1 mg/kg SB225002 plus PQ group (1/9) presented a paw fault at the 3rd application (Figure 5C).

Next, the animals were evaluated in the footprint model by assessing the stride length mean and the average step frequency. Representative images of footprints after the 5th application are depicted in Figure 6A-D. The administration of paraquat caused a significant reduction of mean stride length (Figure 6E), associated with an increase of step frequency (Figure 6F), after both the 3rd and the 5th applications. Although not significant, effects were observed in the groups pretreated with SB225002 in which the effects of paraquat in motor coordination were virtually prevented by this antagonist, especially at the dose of 3 mg/kg.

The exposure of paraquat increases inflammatory marker expression in the striatum

Figure 7 shows the expression profile of some inflammatory markers in the striatum of rats. The relative gene expression of *TNF-α* was significantly increased in the vehicle/paraquat group ($p<0.05$) in comparison to the control (vehicle/saline) group (Figure 7B). The relative expression of *IL-1β* was increased in the vehicle/SB225002 (3 mg/kg) and vehicle/paraquat ($p<0.05$) groups when compared to the control (vehicle/saline) group (Figure 7C). Of note, the induction of *TNF-α* and *IL-1β* expression in the striatum was abolished by treatment with SB225002 (Figure 7B and C). *NF-κB1* and *COX-2* mRNA levels did not show any changes in the expression profile amongst different experimental groups (Figure 7A and D, respectively).

Hematologic changes and neutrophil migration

At the end of the behavioral experiments, we decided to assess the possible changes of leukocyte populations throughout the different groups. Representative images of different experimental groups are depicted in Figure 8A-F. Total blood cell counts (Figure 8G) revealed a predominance of lymphocytes in the vehicle/saline group and this feature was not significantly modified by SB225002 (1 and 3 mg/kg). Vehicle/paraquat-treated animals showed a predominance of neutrophils, accompanied by a slight increase in the number of immature cells and a reduction of lymphocyte numbers ($p<0.01$). Pretreatment with SB225002 (1 and 3 mg/kg) was able to significantly modify the effects of paraquat ($p<0.05$ and $p<0.01$, respectively), as these experimental groups presented the same percentages of lymphocytes and neutrophils.

Neutrophil migration to the rat lungs was assessed by means of MPO activity. The vehicle/paraquat group presented a significant increase of MPO levels ($p<0.05$) in comparison

to the control group. This parameter was reversed by the co-administration of SB225002 (3 mg/kg) ($p<0.05$), returning to the control levels (Figure 8H).

Discussion and Conclusions

The herbicide paraquat is widely employed worldwide, although its use has been prohibited in some countries due to the potential toxic effects [35]. Nevertheless, traces of paraquat can be detected in fruits and vegetables, and even in processed products, which might be related to intoxication events [36]. This agent promotes the formation of ROS and leads to toxicity of the central nervous system (CNS) linked to the loss of dopaminergic neurons, inducing Parkinson-like motor alterations in animal models [17,37,38]. The lungs are the major targets of paraquat intoxication, as the herbicide is transported by the polyamine uptake system, accumulating within the alveolar type II epithelial cells, which results in pulmonary fibrosis [39]. In the present study, we evaluated whether the administration of the selective chemokine CXCR2 receptor antagonist SB225002 might prevent either peripheral or central alterations related to paraquat intoxication in rats. We believe that our study contributes to the further understanding of the mechanisms involved in paraquat-induced toxicity, and might open new opportunities to develop potential therapeutic options to treat intoxications caused by this agent.

Initially, we performed a dose-response curve in order to select a sub-lethal scheme of treatment with paraquat using a series of behavioral evaluations. We employed an experimental design in which the rats received five applications of paraquat (10 mg/kg, i.p.), during a 14-day period, with a total dose of 50 mg/kg. Similarly, Czerniczyniec et al. [18] demonstrated that administration of paraquat (10 mg/kg, i.p.) in rats, dosed weekly for a month (40 mg/kg overall), resulted in behavioral changes associated with brain mitochondrial dysfunction, which corroborates our choice of protocol.

The schedule of treatment with paraquat adopted by us led to a time-related reduction of body weight and rectal temperature in rats, which is in accordance with previous literature [40,41,42,43,44]. Although the treatment with SB225002 (3 mg/kg, i.p.) was able to produce only a partial effect on body weight loss, the same dose of this antagonist largely prevented the hypothermia induced by paraquat. This series of results indicates that pharmacological inhibition of CXCR2 receptors by SB225002 can provide protective effects against some complications related to paraquat poisoning. Of high interest, the study conducted by Bento et al. [13] demonstrated that repeated administration of SB225002, at doses as low as 0.3 mg/kg, was effective in markedly preventing the body weight loss in the mouse model of TNBS-induced colitis. Nonetheless, to our knowledge, there is no previous report showing the ability of SB225002 to modulate hypothermia, thus we reveal a novel effect for this antagonist. In

spite of that, chemokines and their receptors have been detected in most cell types in CNS [45,46,47] (for review see: [48]), which might help to explain our data.

Among the symptoms of paraquat poisoning, it is relevant to cite the occurrence of intense abdominal pain in clinics [49,50]. In our study, we employed a scoring system previously used to assess visceral pain in rodents [19,20,21,22] to evaluate paraquat-related nociception behavior. The administration of paraquat resulted in a marked and time-related increase of nociception scores, and pretreatment with SB225002 significantly reduced paraquat-elicited nociception at all evaluated timepoints, displaying an apparent dose-related profile. It is well known that paraquat toxicity is likely related to oxidative stress [51], and generation of ROS by this herbicide is able to modulate neutrophil function [52]. Remarkably, prior experimental evidence showed that visceral pain elicited by colorectal distention relies on oxidative stress [21]. Thus, we hypothesize that inhibition of neutrophil migration by SB225002 might prevent the complications originating from paraquat-induced ROS generation, including the abdominal discomfort. Corroborating our data, it was previously demonstrated that treatment with SB225002, in doses ranging from 0.3 to 3 mg/kg, significantly inhibited the visceral nociception induced by acetic acid in mice [14] or by cyclophosphamide in rats [15].

We also aimed to investigate whether the treatment with SB225002 would prevent motor deficits induced by paraquat intoxication in rats. In fact, it has been largely demonstrated that paraquat exposure is associated with serious motor impairments that originate from oxidative stress and neurotransmission deficits in several brain regions (for review see: [37,53]). In this study, the exposure to paraquat led to decreased locomotor and exploratory activities (open-field test), which is in agreement with previous literature [38,44,54,55]. Furthermore, a reduction of fecal bolus number was also observed in paraquat-treated rats, as demonstrated before [36]. Furthermore, the administration of paraquat resulted in worsened performance in beam-walking and footprint paradigms. A series of alterations related to locomotor dysfunction were observed in previous publications dealing with the consequences of paraquat poisoning, mainly via mechanisms involving dopaminergic neurotoxicity [17,35,44,49,51,56]. Concerning the treatment with SB225002, this antagonist presented only partial effects on paraquat-induced motor deficits, although a general improvement of coordination skills was achieved, according to assessment in the beam-walking test or the footprint model. As mentioned beforehand, chemokine receptors are expressed throughout different brain regions [48], which might support the beneficial effects

of SB225002 in our experimental paradigm, although the ability of this antagonist to cross the blood-brain barrier remains to be further investigated. Furthermore, it is possible to surmise that prevention of peripheral inflammation by SB225002 might avoid paraquat-induced oxidative stress within the CNS, contributing to the positive effects of this antagonist on motor performance.

It has been previously shown that the striatum is considered the most important site of dopamine action and motor control [57]. For this reason, the striatum was used in the transcriptional analysis of known molecular markers of inflammation. The expression profile of nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NF-κB1*), tumor necrosis factor-α (*TNF-α*), interleukin-1β (*IL-1β*), and cyclooxygenase-2 (*COX-2*) was determined. *TNF-α* and *IL-1β* expression was induced in the striatum after paraquat administration, which, in turn, was abolished by treatment with SB225002. It is important to highlight that *TNF-α* expression is persistent in inflammatory response [29] and *IL-1β* is responsible for recruitment and activation of other immune cells at the inflammatory site (for review see: [58]).

Considering the evidence discussed above, we decided to examine to what extent the administration of SB225002 might modulate peripheral inflammation induced by paraquat exposure. Strikingly, the repeated application of paraquat (10 mg/kg, i.p.), in a scheme of five doses over the course of 14 days, resulted in a marked reduction of blood lymphocytes in comparison to saline-treated control animals, accompanied by an increase of neutrophil counts, and the scarce presence of immature cells. More interestingly, SB225002-treated groups (with either 1 or 3 mg/kg) presented the same percentages of lymphocytes and neutrophils, indicating the ability of the tested antagonist to reverse the peripheral inflammation elicited by paraquat. Similar to our data, Aires et al. [59] recently demonstrated that an acute single administration of paraquat (20 mg/kg, i.p.) resulted in marked neutrophilia, an effect that was prevented by the TNF-α inhibitor etanercept. Aligned to these experimental pieces of evidence, an attractive clinical study recently published by Fareed et al. [60] highlighted the gravity of hematological alterations observed in agricultural workers exposed to some pesticides. Furthermore, a recent publication by Kang et al. [61] demonstrated a correlation between the absolute lymphocyte counts and the 30-day mortality rates after paraquat poisoning in humans, suggesting an immunomodulatory action for this herbicide. Whether or not the therapy with anti-inflammatory agents (such as SB225002)

might prevent the clinical complications related to paraquat intoxication needs additional investigation.

Lungs are well recognized as the main targets of paraquat intoxication [62]. Paraquat-induced lung toxicity is likely dependent on development of pulmonary edema, inflammatory cell infiltration, and damage of alveolar cells, resulting in fibrosis [63]. A study conducted by Venkatesan [64] demonstrated that single administration of paraquat at a high dose (50 mg/kg, i.p.) led to a significant increase of lung neutrophil migration in rats, as indirectly measured by means of MPO activity. Furthermore, the acute exposure to paraquat (20 mg/kg, i.p.) produced a marked increase of neutrophil influx to the broncoalveolar lavage of mice, and this parameter was reduced by TNF-α inhibition [59]. In the present study, the repeated i.p. administration of paraquat at an overall dose of 50 mg/kg over 14 days caused a significant increase of lung MPO activity, which was prevented by pretreating animals with SB225002 at 3 mg/kg. Nevertheless, the increase of MPO activity was not modified by treatment with SB225002 at 1 mg/kg.

In summary, on the basis of results present herein, it is possible to suggest that pharmacological inhibition of CXCR2 receptors by the antagonist SB225002 is able to interfere, in a dose-dependent manner, with paraquat-related systemic toxicity. Further studies are necessary to reinforce our findings.

Acknowledgements

The authors thank Rodrigo B. M. da Silva and Priscilla B. Pail for their technical support. We also thank Dr. Paulo C. Leal for the kind donation of antagonist SB225002.

Author Contributions

Conceived and designed the experiments: KMC, ISM, MMC, and MRB. Performed the experiments: KMC, ISM, and LWK. Analyzed the data: KMC, ISM, LWK, MMC, and MRB. Contributed reagents/materials/analysis tools: MMC and MRB. Wrote the manuscript: KMC, LWK, MMC, and MRB.

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Table:

Table 1. Primer sequences for RT-qPCR experiments.

Gene	Forward primer	Reverse primer
COX-2[a]	5'-CCCCAAGGCACAAATATGATG-3'	5'-CCTCGCTTCTGATCTGTCTTGA-3'
IL-1β[b]	5′-GGACAGAACATAAGCCAACAA-3′	5′-CTTTCATCACACAGGACAGGT-3′
NF-κB[c]	5'-CAGCTCTTCTCAAAGCAGCA-3'	5'-TCCAGGTCATAGAGAGGCTCA-3'
Rpl13α[d]	5'-ACAAGAAAAAGCGGATGGTG-3'	5'-TTCCGGTAATGGATCTTTGC-3'
TNFα[b]	5′-AGCAGATGGGCTGTACCTTAT-3′	5′-GCTGACTTTCTCCTGGTATGA -3′

According to [a]Hanafy et al., 2012; [b]Singh et al., 2012; [c]Kireev et al., 2012; [d]Bonefeld et al., 2008.

Figures:

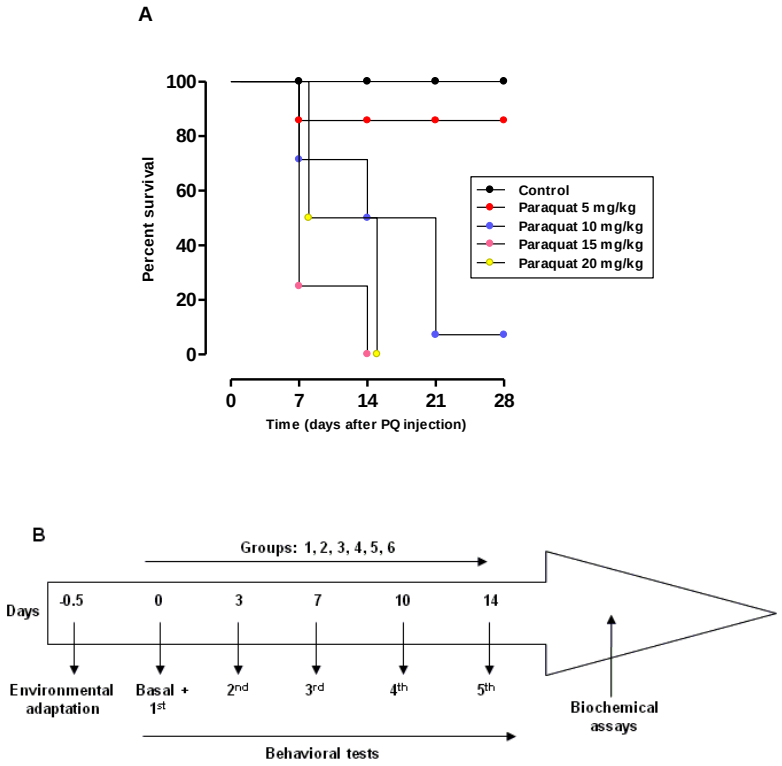

Figure 1. Paraquat survival curve and general schedule of treatment used in the study.
(A) Survival curve for cumulative paraquat administration through 28 days, given at different doses [5 (n=4), 10 (n=14), 15 (n=4), and 20 (n=6) mg/kg], twice a week. (B) The animals received five i.p. applications (at 0, 3, 7, 10, and 14 days) of vehicle/saline (10 ml/kg; group 1), SB225002 (1 and 3 mg/kg; groups 2 and 3)/saline, vehicle/paraquat (10 mg/kg; group 4), or SB225002 (1 and 3 mg/kg)/paraquat (10 mg/kg); groups 5 and 6, respectively. Behavioral tests were performed at days 0, 7, and 14, which corresponded to the basal timepoint, and the 3rd or the 5th application of drugs. In some cases, the behavioral measurements were also carried out under basal conditions (prior to any treatment).

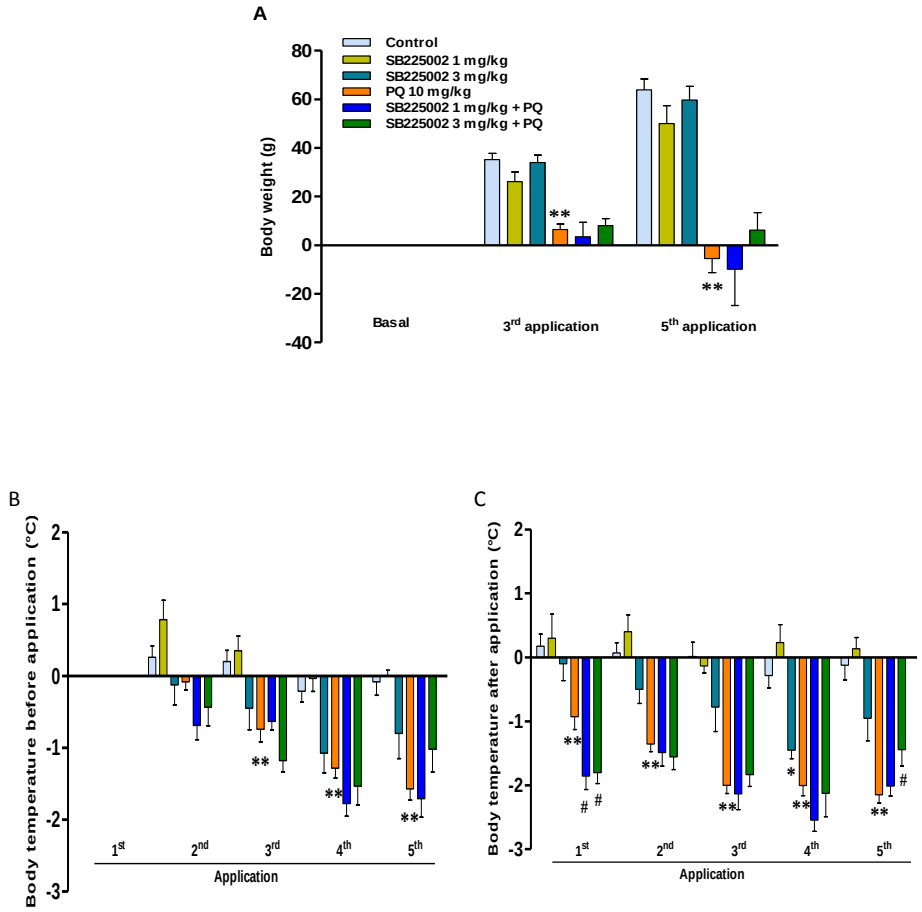

Figure 2. Physiological changes elicited by paraquat: effects of SB225002.

(A) Changes in body weight (Δ grams), before the 3rd and 5th application; (B) variation of body temperature (Δ ^{o}C), measured immediately before treatments; (C) variation of body temperature measured 1 h after each application. Each column represents the mean ± SEM of 4–18 animals per group. $^{*}p<0.05$ and $^{**}p<0.01$ significantly different from control (vehicle/saline) group; $^{\#}p<0.05$ significantly different from vehicle/paraquat group (PQ) (ANOVA followed by Bonferroni's post-hoc test).

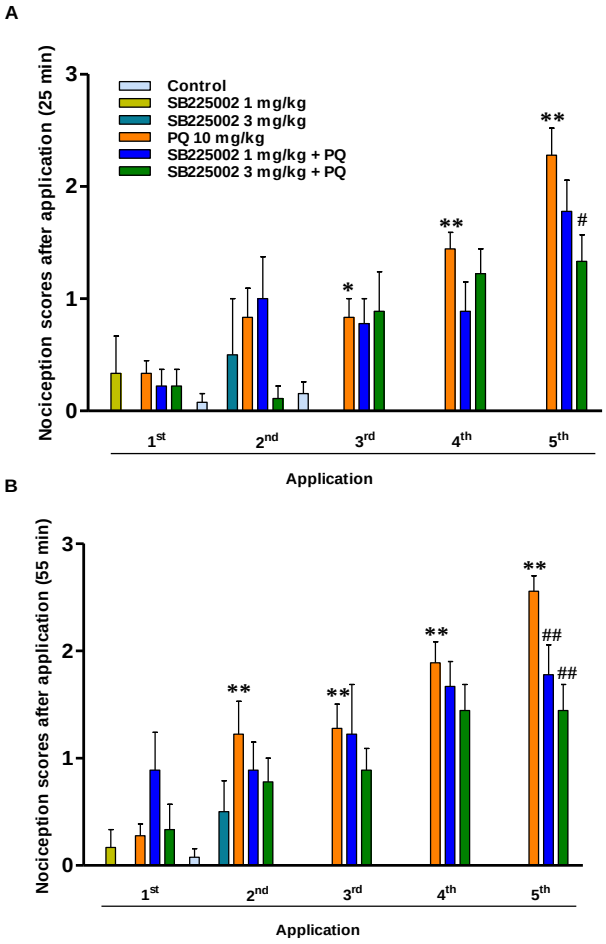

Figure 3. Behavioral scores of nociception.

Assessment of behavioral scores at 25 min (A) or 55 min (B) after each treatment. Data is plotted as the cumulative scores of nociception over 5 min. Each column represents the mean ± SEM of 4–18 animals per group. $^{*}p<0.05$ and $^{**}p<0.01$ significantly different from control (vehicle/saline) group; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ significantly different from vehicle/paraquat group (PQ) (ANOVA followed by Bonferroni's post-hoc test).

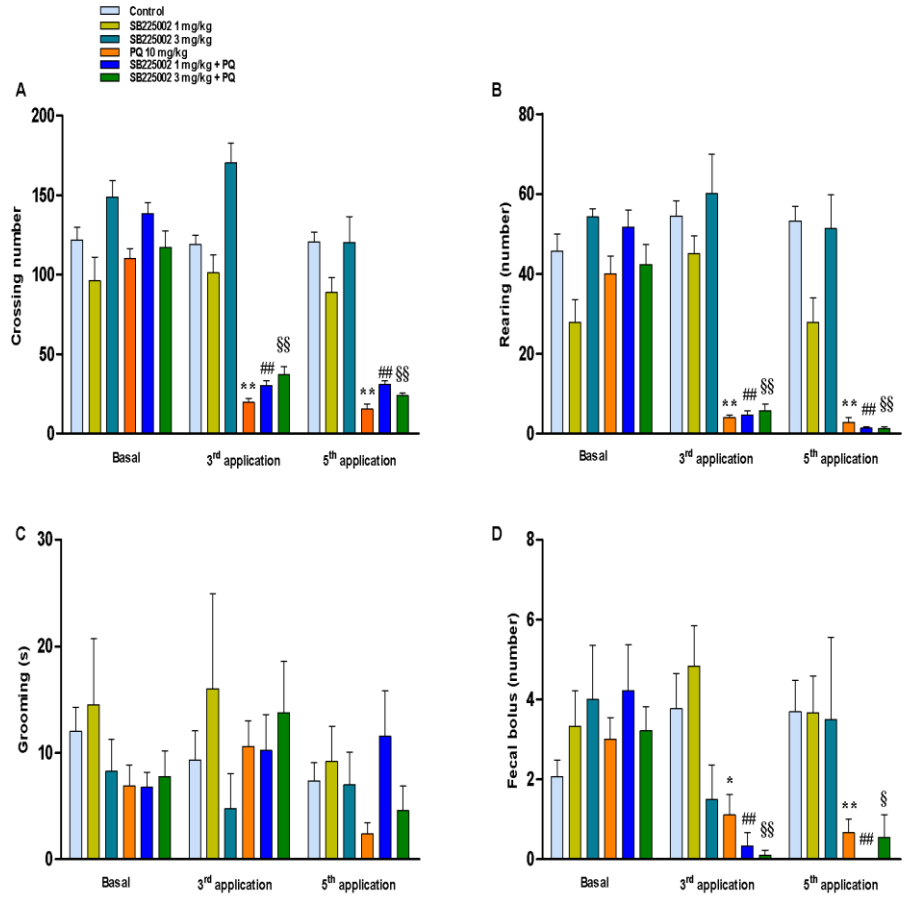

Figure 4. Evaluation of paraquat effects in the open-field test.

(A) Number of crossed squares; (B) number of rearings; (C) facial grooming in s; (D) number of fecal boluses. Each column represents the mean ± SEM of 4–18 animals per group. $^{*,\S}p<0.05$; $^{**,\#\#,\S\S}p<0.01$ compared with the basal measurements of the same experimental group (ANOVA followed by Bonferroni's post-hoc test).

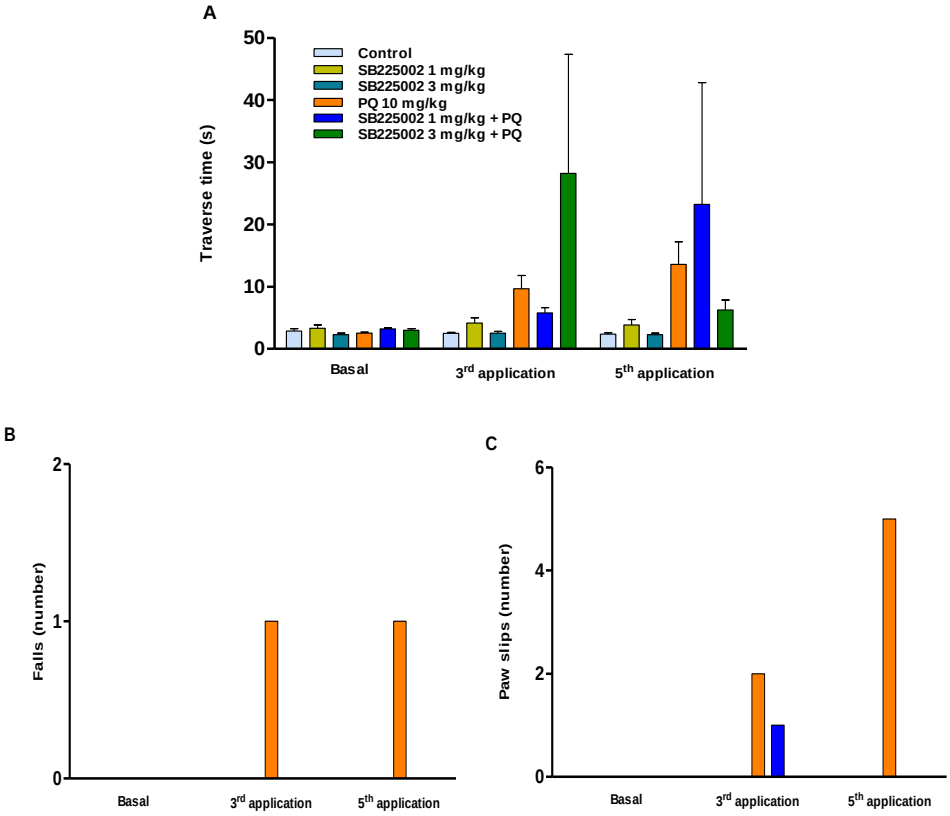

Figure 5. Locomotor skills in the beam-walking test.

(A) Time taken to cross the beam in s; (B) number of falls; (C) number of paw slips. Each column represents the mean ± SEM of 4–18 animals per group (ANOVA followed by Bonferroni's post-hoc test was used for analysis of data in Figure 5A).

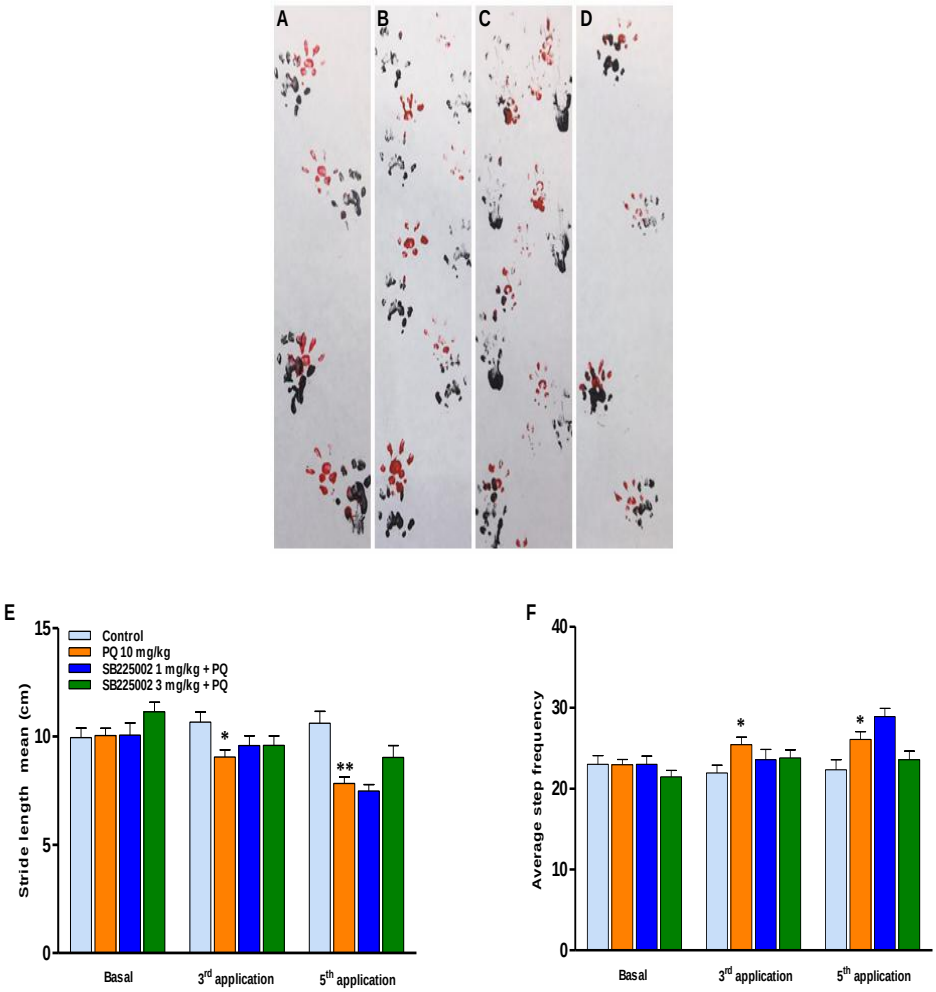

Figure 6. Symptoms of motor dysfunction.

Representative walking footprint patterns after the 5th treatment in (A) control (vehicle/saline) group; (B) vehicle/paraquat (PQ)- treated animals; (C) SB225002 (1 mg/kg)/plus PQ group; (D) SB225002 (3 mg/kg)/ plus PQ group. The bar graphs depict quantitative analysis of the footprint test regarding the stride length mean (E) and the average step frequency (F). Each column represents mean ± SEM of 9–19 animals per group. $^{*}p<0.05$ and $^{**}p<0.01$ compared with control (vehicle/saline) group (ANOVA followed by Bonferroni's post-hoc test).

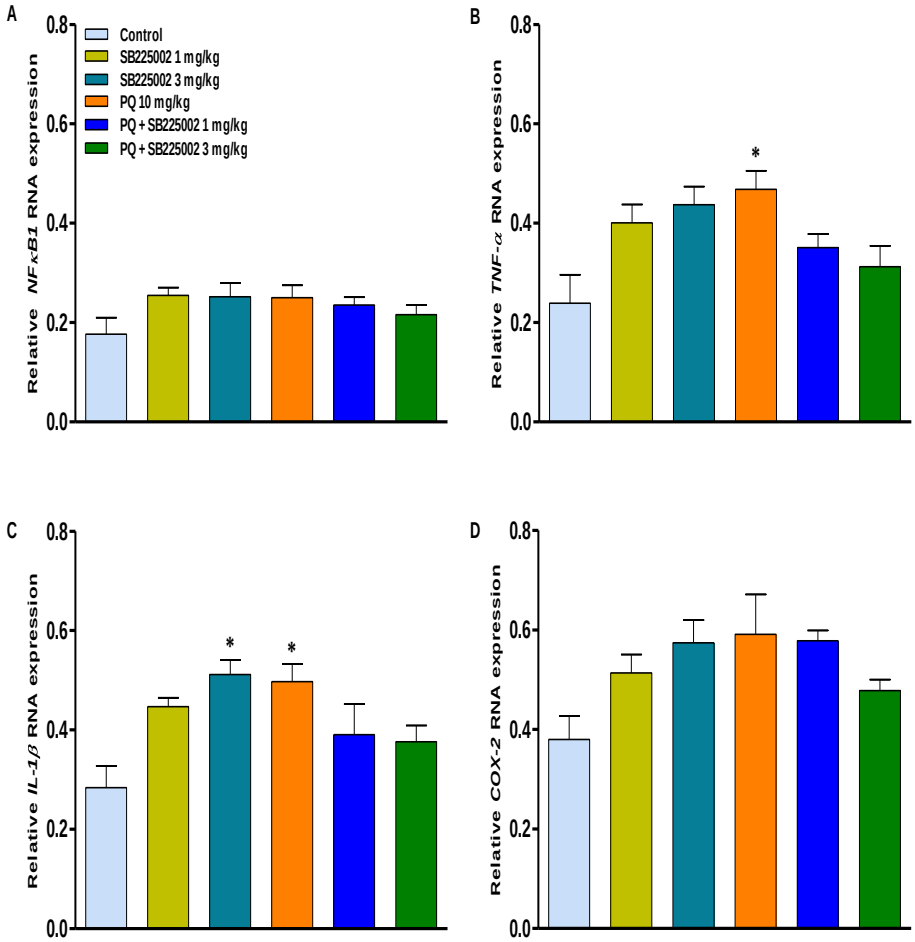

Figure 7. RT-qPCR analysis of inflammatory markers.

Relative expression profiles of (A) *NF-κB1*, (B) *TNF-α*, (C) *IL-1β*, and (D) *COX-2*, assessed in the striatum after the 5th paraquat treatment. Each column represents the mean ± SEM, 3–4 samples of striatum. $^{*}p<0.05$ compared with control (vehicle/saline) group (ANOVA followed by Bonferroni's post-hoc test).

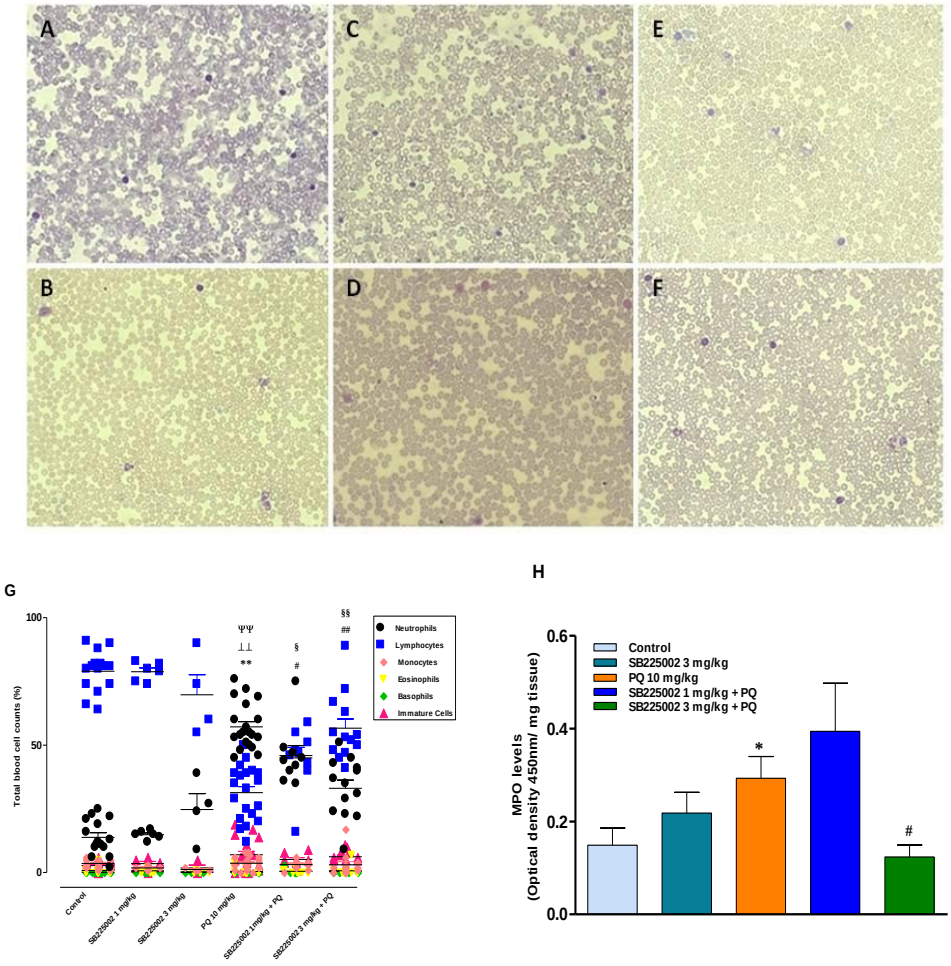

Figure 8. Hematological analysis and lung neutrophil migration.

Representative pictures showing the blood smear slides in (A) control (vehicle/saline) groups; (B) vehicle/paraquat (PQ)-treated animals; (C) SB225002 (1 mg/kg)/saline group; (D) SB225002 (3 mg/kg)/saline group; (E) SB225002 (1 mg/kg)/plus PQ group; (F) SB225002 (3 mg/kg)/plus PQ group. Effects of paraquat and SB225002 on the total blood cell counts (G) and neutrophil migration as assessed by MPO activity (H). Each column represents the mean ± SEM of 4–20 animals per group in blood cell counts and 3–12 animals per group in MPO activity. $^{**,\perp\perp,\Psi\Psi}p<0.01$ indicate significant differences of neutrophils, lymphocytes, and

immature cells in relation to control (vehicle/saline) group, respectively. $^{\#,\S}p<0.05$; $^{\#\#,\S\S}p<0.01$ indicate significant differences of neutrophils and lymphocytes in relation to vehicle/plus PQ group, respectively. $^{*}p<0.05$ indicates significant difference of MPO activity in relation to control (vehicle/saline) group; $^{\#}p<0.05$ indicates significant difference to vehicle/plus PQ group (ANOVA followed by Bonferroni's post-hoc test).

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ANEXO C – COMPROVANTE DE SUBMISSÃO DO ARTIGO

-----Mensagem original-----
De: em.pone.0.375fbd.d0e5d0d8@editorialmanager.com
[mailto:em.pone.0.375fbd.d0e5d0d8@editorialmanager.com] Em nome de PLOS ONE
Enviada em: segunda-feira, 25 de novembro de 2013 15:39
Para: Mauricio Reis Bogo
Assunto: Submission Confirmation for Pharmacological inhibition of CXCR2 chemokine receptors modulates paraquat-induced intoxication in rats - [EMID:6ff7ed8dc195f671]

Dear Dr. Bogo,

Your submission entitled "Pharmacological inhibition of CXCR2 chemokine receptors modulates paraquat-induced intoxication in rats" has been received by PLOS ONE.
You will be able to check on the progress of your paper by logging on to Editorial Manager as an author. The URL is http://pone.edmgr.com/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

PLOS ONE

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ANEXO D – ATA DE APRESENTAÇÃO DE DISSERTAÇÃO

Pontifícia Universidade Católica do Rio Grande do Sul
FACULDADE DE MEDICINA
PÓS-GRADUAÇÃO EM MEDICINA E CIÊNCIAS DA SAÚDE

ATA DE APRESENTAÇÃO DE DISSERTAÇÃO Nº 353

Aos dois dias do mês de janeiro do ano de dois mil e quatorze, no Curso de Mestrado em Medicina e Ciências da Saúde, área de concentração em Farmacologia Bioquímica e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul foi concluído o processo de avaliação da dissertação intitulada **"PARTICIPAÇÃO DOS RECEPTORES CXCR2 PARA QUIMIOCINAS NA TOXICIDADE INDUZIDA PELO PARAQUAT EM ROEDORES"** de autoria da pós-graduanda **Kesiane Mayra da Costa** sob orientação do **Prof. Dr. Maurício Reis Bogo** e co-orientação da **Profa. Dra. Maria Martha Campos.** A comissão examinadora foi constituída pelos professores: Dra. Vanessa Pinho da Silva (UFMG), Dr. Rafael Fernandes Zaninn (PUCRS), Dra. Fernanda Bueno Morrone (PUCRS) e Dr. Jarbas Rodrigues Oliveira, suplente (PUCRS). A aluna foi **APROVADA.** Para constar, lavrou-se esta ata que deverá ser anexada à documentação exigida para posterior expedição do diploma. A presente ata foi assinada pela Coordenadora do Programa de Pós-Graduação em Medicina e Ciências da Saúde. Porto Alegre, aos dois dias do mês de janeiro do ano de dois mil e quatorze.

Profa. Dra. Magda Lahorgue Nunes