Universidade Estadual de Londrina

RENATO DANIEL RAMALHO CARDOSO

EFEITO PROTETOR DA MARESINA 2 (MaR2) E DA RESOLVINA D5 (RvD5) EM MODELO DE ENDOTOXEMIA INDUZIDO POR LIPOPOLISSACARÍDEO (LPS) EM CAMUNDONGOS

Londrina
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Trabalho de Conclusão de Curso apresentado à Universidade Estadual de Londrina - UEL, como requisito para a obtenção do título de Doutor em Patologia Experimental.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior

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“Outros podem caminhar COM você, mas
ninguém pode caminhar POR você.”

(Autor desconhecido)

RESUMO

CARDOSO, Renato Daniel Ramalho Cardoso. **Efeito protetor da Maresina 2 (MaR2) e da Resolvina D5 (RvD5) em modelo de endotoxemia induzido pelo Lipopolissacarídeo (LPS) em camundongos.** 2022. 92 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2022.

Os mediadores lipídicos pró-resolução (SPMs) têm a capacidade de regular e reduzir a resposta inflamatória através de diferentes vias, modulando a produção de citocinas pró-inflamatórias, inibindo o recrutamento de células inflamatórias e diminuindo a lesão mediada por espécies reativas de oxigênio (EROs). Portanto, considerando o potencial protetor, anti-inflamatório e antioxidante desses mediadores lipídicos, e que a endotoxemia via LPS pode induzir lesões inflamatórias e oxidantes, entende-se que esses mediadores possuem potencial para serem utilizados terapeuticamente em casos de endotoxemia. O objetivo geral deste trabalho foi avaliar o efeito terapêutico dos mediadores lipídicos pró-resolução Maresina 2 (MaR2) e Resolvina D5 (RvD5) em um modelo de endotoxemia induzida por LPS. Os animais foram tratados com MaR2 (1ng ou 10 ng/animal) ou RvD5 (0,1ng, 1ng ou 10 ng/animal) 60 minutos antes de receber LPS (10mg/kg) por via intravenosa. O sangue foi coletado por punção cardíaca 6 horas após o tratamento e os níveis séricos da ureia, creatinina, ALT e AST foram quantificados. A atividade da MPO e da NAG foram observadas no fígado, pulmões e rins, bem como os níveis de citocinas pró-inflamatórias. Os testes de FRAP, ABTS, GSH, TBARS e NBT foram realizados nos mesmos órgãos para avaliar a capacidade antioxidante total dessas amostras e para observar a atividade e lesão oxidativa. Além disso, fígado, pulmões e rins foram avaliados histologicamente e o resultado foi quantificado por meio de um escore histopatológico. Demonstramos que a MaR2 diminuiu os níveis séricos de ureia e creatinina, atuando de forma protetora, principalmente nos rins, moderando a atividade inflamatória, aumentando a capacidade antioxidante total dessas amostras, diminuindo os níveis de peroxidação lipídica e evitando alterações histológicas severas. Portanto, a MaR2 demonstrou um papel protetor nos rins, reduzindo a inflamação e o estresse oxidativo. Em paralelo, a RvD5 exerceu atividade protetora renal, pulmonar e hepática com melhora dos parâmetros histológicos, diminuição dos níveis de citocinas pró-inflamatórias, redução da peroxidação lipídica e dos da produção do ânion superóxido. Além disso, a RvD5 também reduziu a atividade de NAG e MPO, restaurou os níveis de antioxidantes totais, assim como os níveis de GSH. Portanto, nossos dados sugerem que MaR2 e RvD5 seriam uma possibilidade terapêutica para endotoxemia/choque endotoxêmico.

Palavras-chave: mediadores lipídicos pró-resolução; inflamação; estresse oxidativo.

ABSTRACT

CARDOSO, Renato Daniel Ramalho Cardoso. **Maresin 2 (MaR2) and Resolvin D5 (RvD5) reduce LPS-induced endotoxemia in mice.** 2022. 92 p. Thesis (Doctorade in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2022.

Specialized pro-resolving mediators (SPMs) modulate and reduce the inflammatory response through different pathways, modulating the production of pro-inflammatory cytokines, inhibiting the recruitment of inflammatory cells, and decreasing reactive oxygen species (ROS)-induced injury. Considering the protective, anti-inflammatory and antioxidant potential of SPMs and that LPS-induced endotoxemia triggers inflammatory and oxidant lesions, the main objective of this work was to evaluate the therapeutic effect of the SPM Maresin 2 (MaR2) and Resolvin D5 (RvD5) in a model of LPS-induced endotoxemia. Mice were treated with MaR2 (1ng or 10 ng/animal) or RvD5 (0.1ng, 1ng, or 10 ng/animal) 60 minutes before receiving LPS (10mg/kg) intravenously. Blood was collected by cardiac puncture 6 hours after the treatment. Urea, creatinine, ALT, and AST were quantitated in the plasma. MPO and NAG activity were analyzed in the liver, lungs and kidney, as well as the levels of pro-inflammatory cytokines. FRAP, ABTS, GSH, TBARS, and NBT colorimetric tests were performed in samples from the same organs, to assess the total antioxidant capacity and oxidative activity. In addition, liver, lungs, and kidneys were histologically evaluated, and the result was quantified using a histopathological score. MaR2 decreased serum urea and creatinine levels exert protective effect in the kidneys. MaR2 modulated inflammatory activity, increased total antioxidant capacity, decreased lipid peroxidation levels, and moderated histological changes. Therefore, our data supports that MaR2 exert a protective role in the kidneys by reducing inflammation and oxidative stress. In addition, we demonstrate that RvD5 have renal and hepatic protective activity. Moreover, RvD5 improved histological parameters, decreased expression of pro-inflammatory cytokines, reduced levels of lipid peroxidation and the production of superoxide anion. Further, RvD5 decreased NAG and MPO activity, restored total antioxidant levels, including GSH. In summary, our data suggest that MaR2 and RvD5 are suitable therapy candidates for endotoxemia/endotoxemic shock.

Key words: specialized pro-resolving mediators; inflammation; oxidative stress, endotoxemic.

LISTA DE ABREVIATURAS E SIGLAS

5-LOX	5-Lipoxigenase
12-LOX	12-Lipoxigenase
15-LOX	15-Lipoxigenase
AA	Ácido araquidônico
ALT	Alanina aminotransferase
AST	Aspartato aminotransferase
CLP	Ligação e Perfuração Cecal
DHA	Ácido docosahexaenóico
DPA	Ácido docosapentaenóico
EPA	Ácido eicosapentaenóico
EROs	Espécies Reativas de Oxigênio
GSH	Glutationa forma reduzida
ICAM-1	Molécula de Adesão Intercelular - 1
INF-γ	Interferon gama
IL1- β	Interleucina 1 beta
IL1-ra	Antagonista de receptor da IL-1
IL-4	Interleucina 4
IL-6	Interleucina 6
IL-10	Interleucina 10
LPS	Lipopolissacarídeo
MCTR	Maresina conjugada na regeneração tecidual
MPO	Mieloperoxidase
NF-kB	Fator nuclear kappa B
RvD	Resolvina - D
RvE	Resolvina - E
SMPs	Mediadores lipídicos pró-resolução
TLR4	Receptor toll-like 4
TNF-α	Fator de necrose tumoral alfa
VCAM-1	Proteína de adesão celular vascular – 1

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1 INTRODUÇÃO

A Sepse pode ser definida como uma disfunção orgânica, com potencial letalidade, causada pela resposta exacerbada do hospedeiro frente à infecção, enquanto o choque séptico é ocasionado pela corrente disfunção homeostática do hospedeiro, no qual o metabolismo celular e circulatório é profundamente afetado, aumentando significativamente a mortalidade (SINGER et al., 2016; FERNANDO et al*.,* 2018).

Conforme dados da Fundação Oswaldo Cruz, a sepse causa 11 milhões de mortes no mundo a cada ano, principalmente em crianças, idosos e doentes crônicos. Além disso, no Brasil, aproximadamente 240.000 pessoas morrem a cada ano por complicações da sepse e do choque séptico, superando números relacionados com infarto agudo do miocárdio e câncer. O Instituto Latino-Americano de Sepse ressalta a necessidade de uma melhor vigilância nos leitos das Unidades Intensivas no Brasil, visto que a mortalidade chega a 65% dos casos, superando a média global que não ultrapassa 40% (FIOCRUZ, 2021).

Alguns modelos para estudo da sepse/choque séptico e das potenciais moléculas atenuadoras deste processo vêm sendo utilizados nas últimas décadas (BORGES, 2018), principalmente em relação ao agente indutor, como nos modelos em que há administração exógena de endotoxina (RUIZ-MIYAZAWA et al., 2015; ZUCOLOTO et al., 2017; FATTORI et al., 2020); nos modelos em que há administração exógena de um patógeno vivo (STAURENGO-FERRARI et al., 2017, STAURENGO-FERRARI et al., 2018); e os modelos que alteram alguma barreira de proteção endógena do animal, como o de ligação e perfuração cecal (CLP) (ALVES-FILHO et al., 2010).

Destes modelos descritos, o mais amplamente utilizado está relacionado com a administração intravenosa ou intraperitoneal de Lipopolissacarídeos (LPS), uma endotoxina advinda da membrana externa de bactérias gram-negativas, com potencial endotoxêmico capaz de ativar resposta imunológica e metabólica no hospedeiro (BORGES, 2018) e que caso estejam exacerbadas, poderão levar ao desenvolvimento de choque endotoxêmico, caracterizado por um quadro clínico de hipotensão, perfusão tecidual inadequada e falência sistêmica de órgãos (STABILE, 2005).

O LPS se liga e ativa receptores do tipo *toll-like* (TLRs), preferencialmente o receptor TLR4, presente na superfície de macrófagos, linfócitos, monócitos e granulócitos (RUIZ-MIYAZAWA et al., 2015; MOLTENI; GEMA; ROSSETTI, 2016; MOHAMMAD; THIEMERMANN, 2021). Ao reconhecer a molécula de LPS, o TLR4 irá ativar vias intracelulares de fatores de transcrição como o NF-κB, favorecendo a produção de citocinas pró-inflamatórias como o TNF-α, IL-6, INF-γ, IL-1β, IL-33 e quimiocinas (LU et al., 2008; ZUCOLOTO et al., 2017). Essas citocinas, por sua vez, funcionam como mediadores para amplificar a resposta inflamatória, incluindo o recrutamento de células do sistema imune com capacidade de produzir espécies reativas de oxigênio (EROs) (RUIZ-MIYAZAWA et al., 2015) e produção de óxido nítrico (STABILE, 2005).

Porém, caso ocorra descontrole ou excessiva ativação dessas vias anteriormente desc

ritas, poderá ocorrer um desbalanço inflamatório sistêmico, promovendo danos teciduais, alterações metabólicas e piora dos sinais clínicos, proveniente de uma possível resposta séptica exacerbada do hospedeiro, com grande potencial de morbidade e letalidade (BORGES, 2018; MOHAMMAD; THIEMERMANN, 2021).

A resposta inflamatória aguda inicial pode ser considerada protetora, principalmente no início do processo, no qual a ativação decorre por consequência de estímulos nocivos advindos de agentes químicos, físicos ou biológicos (FLOWER, 2006). As fases iniciais do processo inflamatório orquestram e mobilizam as células e moléculas inflamatórias com intuito de promover a resolução do estímulo nocivo, ao passo que na fase final do processo se objetiva destruir o agente agressor e reparar o dano tecidual (FLOWER, 2006; SERHAN, 2014).

Os mediadores lipídicos pró-resolução (SPMs) são moléculas especializadas em promover a resolução do processo inflamatório por diferente vias e mecanismos, sendo classificadas de acordo com suas características estruturais em quatro principais famílias: **lipoxinas, resolvinas, protectinas e maresinas** (SERHAN; SAVILL, 2005; SERHAN, 2014). Os SPMs são sintetizados endogenamente por mamíferos, principalmente os humanos e podem ser encontrados em diferentes tecidos, órgãos e líquidos corporais (CHIANG; SERHAN, 2020).

A síntese biológica dos SPMs ocorre através da ação de algumas fosfolipases em moléculas de ácidos graxos poli-insaturados (ômega 3 e 6), para que assim aconteça a formação de lipoxinas oriundas do ácido araquidônico (AA), para

que ocorra a formação das resolvinas – E a partir do ácido eicosapentaenóico (EPA) e a produção das protectinas, maresinas e resolvinas – D advindas do ácido docosahexaenóico (DHA) e do ácido docosapentaenóico (DPA) (JORDAN; WERZ, 2021).

Estes mediadores lipídicos promovem a resolução do processo inflamatório através da inibição da expressão das moléculas de adesão ICAM-1, VCAM-1 e P-Selectina (CHEN et al., 2014; GONG et al., 2014), diminuindo diretamente a migração de neutrófilos nos sítios inflamatórios, limitando a lesão tecidual, sem afetar a eliminação efetiva de bactérias na ocorrência de modelos infecciosos (WU et al., 2014).

Além disso, SPMs conseguem modular a via de sinalização do NF-κB realizando a regulação da expressão de citocinas pró-inflamatórias e quimiocinas, amplificando a diminuição da expressão das moléculas de adesão e a migração de células inflamatórias (BUCKLEY; GILROY; SERHAN, 2014; ROMANO et al., 2015; SERHAN et al., 2015; JORDAN; WERZ, 2021).

O potencial anti-inflamatório desses lipídios também é possível devido ao aumento da expressão da enzima heme-oxigenase 1, ao aumento da produção de moléculas anti-inflamatórias IL-4, IL-10 e IL-1ra (antagonista de receptor da IL-1) e pela capacidade de eliminação bacteriana via macrófagos (BUCKLEY; GILROY; SERHAN, 2014; SERHAN et al., 2015). Além disso, os mediadores lipídicos pró-resolução possuem a capacidade de diminuir os níveis séricos de marcadores do estresse oxidativo (WANG et al., 2020) e de aumentar os níveis da superóxido dismutase (SOD), catalase e da glutationa GSH em camundongos tratados com LPS (LI et al., 2021).

Ademais, em outro modelo de sepse em camundongos, foi observado que os SPMs atuaram na proteção de órgãos vitais durante a resposta séptica sistêmica, diminuindo os níveis séricos de alanina aminotransferase (ALT), aspartato aminotransferase (AST), creatinina e ureia (LI et al., 2016).

A resolução do processo inflamatório é um evento coordenado pelos SPMs que possuem a capacidade de diminuir a infiltração excessiva de neutrófilos, promovem também a apoptose acelerada de neutrófilos já ativados e a eferocitose dessas células, além de realizar regeneração tecidual, depuração bacteriana e influenciam no aumento da sobrevida global (SERHAN, 2014; JORDAN; WERZ, 2021).

As maresinas, derivam do DHA, sua biossíntese ocorre principalmente em macrófagos do tipo M2 com envolvimento e participação da enzima 12-lipoxigenase (12-LOX), transformando o DHA em 14S-HpDHA e posteriormente em 13S, 14S-epóxido maresina que poderá ser convertida em maresina 1, maresina 2 e maresina conjugada na regeneração tecidual (MCTR) (SASAKI et al., 2011; LI et al., 2020).

Já as resolvinas podem ser subdividas em Resolvinas – E (RvE) e em Resolvinas – D (RvD), sendo as RvDs derivadas do DHA e sintetizadas por diferentes células (polimorfonucleares e macrófagos) através da ação da lipoxigenase 15 (LOX-15) que transforma o DHA em 17S-hidroperoxi-DHA, este intermediário, por sua vez, sofre ação de mais uma lipoxigenase a LOX-5 gerando seis subtipos de RvD (DEMARQUOY; BORGNE, 2014; DARTT et al., 2020; MOLAEI et al., 2021).

Sendo assim, considerando o potencial protetor, anti-inflamatório e antioxidante apresentado pelos mediadores lipídicos (BUCKLEY; GILROY; SERHAN, 2014; ROMANO et al., 2015; SERHAN et al., 2015; WANG et al., 2020; LI et al., 2021) e que a endotoxemia via LPS pode induzir lesões inflamatórias e oxidantes por diferentes mecanismos (BORGES, 2018), entende-se que estes mediadores possuem importante potencial para serem utilizados terapeuticamente em casos de endotoxemia e choque endotoxêmico.

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Avaliar o efeito dos mediadores lipídicos pró-resolução MaR2 e RvD5 em modelo de choque endotoxêmico induzido pelo LPS.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito da MaR2 e da RvD5 nas modificações histopatológicas do fígado, rim e pulmão;

- Avaliar o efeito da MaR2 e da RvD5 sobre a atividade da mieloperoxidase (MPO) e N-acetilglucosamina (NAG);

- Avaliar o efeito da MaR2 e da RvD5 sob os marcadores de lesão hepática (AST e ALT) e lesão renal (Ureia e Creatinina);

- Avaliar o efeito da MaR2 e da RvD5 sobre a produção das citocinas IL-1β, TNF-α, IL-6 e IL-10 por ELISA;

-Investigar a ação da MaR2 e da RvD5 em relação aos parâmetros de estresse oxidativo através das técnicas de redução do NBT, ABTS, FRAP, TBARS e GSH;

3 ARTIGO MARESINA 2 (MaR2)

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Protective effect of Maresin 2 in a mouse model of LPS-induced systemic inflammation and oxidative stress: kidney protection.

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Highlights

- MaR2 decreases serum levels of renal injury markers.

- MaR2 regulates the expression of pro-inflammatory cytokines and modulates the activity of NAG and MPO.

- MaR2 prevents lipid peroxidation and production of superoxide anion.

- MaR2 prevents endogenous antioxidant depletion.

- MaR2 decreases the renal lesion histopathological scores.

Abstract

In the present study we investigated the activity of Maresin 2 (MaR2) in a model of endotoxemia induced by systemic administration of lipopolysaccharide (LPS) in mice. LPS activate leukocytes triggering NF-κB phosphorylation, which culminates in the production of pro-inflammatory cytokines and reactive oxygen species. Combined inflammation and oxidative stress promote an intensive inflammatory response that can lead to endotoxemic chock. MaR2 is a specialized pro-resolving mediator (SPM) with anti-inflammatory and antioxidant property. Animals were treated with LPS (10mg/kg - iv.) 60 minutes before administration of MaR2 (1ng or 10ng/animal - i.p). Six hours after LPS injection, blood, kidneys, and lungs were collected. Serum levels of ALT, AST, Urea and Creatinine were evaluated. Activity of MPO, NAG, and pro-inflammatory cytokines in the liver, kidneys and lungs were observed. Moreover, total antioxidant capacity and the levels of lipid peroxidation were assessed in these samples, as well the histopathological score. MaR2 decreased serum levels of urea and creatinine exerting protective effects, mainly in the kidneys. Those effects were observed by moderating inflammatory activity, increasing total antioxidant capacity, decreasing the levels of lipid peroxidation and moderating histological changes. Therefore, we demonstrated that MaR2 has a protective role in the kidneys by reducing inflammation and oxidative stress during endotoxemia. Our data suggest that MaR2 might be a strong candidate for the treatment of endotoxemic shock.

Keywords: maresin 2; lipopolysaccharide; endotoxemia; inflammation; oxidative stress.

3.1 INTRODUCTION

Sepsis can be defined as an organic dysfunction with potential lethality, caused by the exacerbated response of the host to the infection. Septic shock, on the other hand, is caused by the homeostatic dysfunction of the host, which cellular and circulatory metabolism are affected, increasing mortality (Singer et al., 2016; Evans et al., 2021).

Several animal models for the study of sepsis/septic shock and the attenuating molecules of this process have been used to understand the mechanisms and pharmacological development for sepsis treatment. For instance, cecal ligation and perforation breaks the endogenous protective barrier exposing the peritoneal cavity to cecal bacteria. Therefore, it is an excellent model to study the balance between inflammation and anti-microbicidal activity against endogenous bacteria (Alves-Filho et al., 2010). Moreover, the exogenous administration of bacteria can also be used to study the specific contribution of the bacterium, or strains with higher virulence or antibiotic resistance (Bayer et al., 2016; Avci et al., 2018). Finnaly, the administration of endotoxins serves to focus on the inflammation mechanisms (Fullerton et al., 2016; Giordano et al., 2020).

The most widely used model of endotoxins administration is induced by the intravenous injection of lipopolysaccharide (LPS) (Seemann et al., 2017; Brinkhoff et al., 2018; Oliveira et al., 2019). LPS is an endotoxin from the outer membrane of gram-negative bacteria, with endotoxemic potential and able to triggers an immune and metabolic response in the host (Taniguchi; Yamamoto, 2005). Thus, LPS administration leads to the development of endotoxemic (septic) shock, which is characterized by hypotension, inadequate tissue perfusion and ultimately systemic

organ failure due to the entrapment of activated circulating leukocytes in the tissue (Stabile et al., 2007).

LPS is an agonist of the toll-like receptors 4 (TLR4) expressed by macrophages, lymphocytes, monocytes, and granulocytes. Thus, in the bloodstream, LPS activates blood leukocytes leading to the activation of intracellular signaling pathways such as the nuclear factor kappa B (NF-κB) (Molteni et al., 2016; Dickson; Lehmann, 2019). This pathway culminates in the production of pro-inflammatory cytokines, such as TNF-α, IL-6, IFN-γ, IL-1β, IL-33, and chemokines (Zucoloto et al., 2017).

Once activated, circulating leukocytes do not migrate to other foci, but rather get trapped in the microcirculation of organs such as kidneys, lungs, and liver (Maas et al., 2018). Activated leukocytes produce reactive oxygen (ROS) (Ruiz-Miyazawa et al., 2015) and nitrogen (RNS) species (Stabile et al., 2007). Thus, LPS-induced inflammatory response triggers a deleterious immune cell activation that causes tissue damage associated with the decline in overall clinical condition (Borges, 2018).

Specialized proresolving mediators (SPM) are a large group composed of different molecules that can be separated in four major families, such as maresins, protectins, resolvins and lipoxins (Serhan, 2014). In general, SPMs induce neutrophil apoptosis and reduce harmful inflammatory response through the inhibition of NF-κB, consequently decrease in pro-inflammatory cytokines and chemokines production, and neutrophil recruitment (Buckley et al., 2014; Romano et al., 2015; Serhan et al., 2014). Moreover, SPMs also induce the release of anti-inflammatory cytokines (*e.g*. IL-10 and IL-1ra), the expression of heme-oxygenase 1 enzyme, and antioxidant enzymes (*e.g.* superoxide dismutase, catalase and glutathione) which culminates in the reduction of

oxidative stress (Wang et al., 2020). SPMs also induce a resolution macrophage (mRES) that does not release inflammatory molecules, which resemble M2 macrophage but also have microbicidal activity, similarly to M1 macrophage (Buckley et al., 2014; Serhan et al., 2014).

The maresin (**ma**crophage mediators in **re**solving **in**flammation) family is composed by maresin 1, maresin 2 and maresin conjugated in tissue regeneration (MCTR). All these compounds are derived from docosahexaenoic acid (DHA) a metabolite of Omega-3 polyunsaturated fatty acid. Maresins are synthesized mainly by macrophages, however, an alternative route through the interaction of neutrophils and platelets has been also proposed (Abdulnour et al., 2014; Li et al., 2020). Maresin 1 (MaR1) has anti-inflammatory and antioxidant function, and it is known to control autoimmune diseases (Li et al., 2020) via the activation of resolution macrophages (Han et al., 2017), differentiation of naïve T cells into regulatory T cells (Krishnamoorthy et al., 2015), augment of cAMP levels, reduction of mitochondria damage, and inhibition of TLR4 receptors activation, and consequent decrease in MAPK and NF-κB signaling (Li et al., 2020). In fact, MaR1 is protective in a mouse sepsis model, decreased blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, and levels of pro-inflammatory cytokines (Li et al., 2016).

Maresin 2 (MaR2) is the second member described in the maresin family, with differences in molecular structure compared to MaR1 (Deng et al., 2014). To the date, it is known that MaR2 also limit the recruitment of neutrophils in a mouse model of zymosan-induced peritonitis. In this study, MaR2 enhanced zymosan particles phagocytosis by human macrophages (Deng et al., 2014; Maciejewska-Markiewicz et al., 2021). However, the effect of MaR2 over LPS endotoxemia are yet unknown. Therefore, this study aims to demonstrate the effects of MaR2 treatment in a mouse

model of LPS-induced endotoxemia.

3.2 Materials and Methods

3.2.1 Animals

The present study was conducted with female Swiss mice, approximately 2 months old, weighing between 20-25 g, from Universidade Estadual de Londrina animal facility (Londrina, PR, Brazil). The animals were in a climatized room (21°±1), light/dark cycle (12/12 h), and free access to water and food. The animals were divided into standard polypropylene cages measuring 41 x 34 x 16 cm (Insight®) with a maximum of 10 animals per cage. All procedures were performed in accordance with local, national, and international standards of ethics. Animal handling and welfare were approved by the Universidade Estadual de Londrina Ethics Committee for animal experimentation (n.052.2021). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010).

3.2.2 Administration of LPS and MaR2

MaR2 (13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-docosahexaenoic acid, Cayman Chemical, Ann Arbor, MI, USA) (1 and 10 ng/animal) or vehicle (0.1% Ethanol in saline) were administrated intraperitoneally 60 minutes before intravenously (i.v.- caudal vein) LPS (from Escherichia coli - Santa Cruz Biotechnology, 10mg/kg) injection or vehicle (saline, 0.9% NaCl solution). The LPS dose was chosen and adapted according to previous literate (Zhuo et al., 2018; Wang et al., 2020). Animals were divided into four equal sized groups consisting of 6 animals per group. Group 01: 0.1% ethanol in saline (i.p.) + saline (i.v.); Group 02: 0.1% ethanol in saline (i.p.)+ LPS

10 mg/kg (diluted in saline, i.v.); Group 03: MaR2 1 ng/animal (diluted saline - i.p.) + LPS 10 mg/kg (i.v.); Group 04: MaR2 10 ng/animal (i.p.) + LPS 10 mg/kg (i.v.). For all assays, samples were collected 6 h after LPS stimulation.

3.2.3 Determination of Liver Injury Markers Levels in the Plasma

Blood was collected by cardiac puncture under isoflurane anesthesia (3% v/v in oxygen) followed by an increase in isoflurane concentration for euthanasia (5% v/v in oxygen). Blood samples were placed in microcubes containing 50μL of porcine sodium heparin (5,000 IU/mL, Blau Farmaceutica S.A., SC, Brazil) and centrifuged (3500 rpm x 10 minutes x 4°C) for plasma separation. ALT and AST were quantitated in plasma samples by UV kinetic methodology (Gold Analise Diagnóstica – Minas Gerais – Brazil) using the Selectra automated equipment. Results were expressed in U/L.

3.2.4 Determination of Renal Injury Markers Levels in the Plasma

Blood was collected and processed as previously described . Levels of urea was determined by enzymatic method (Laborlab – São Paulo – Brazil) and creatinine by kinetic method (Gold Análise Diagnóstica – Minas Gerais – Brazil) using Selectra automated equipment from plasma samples. Results were expressed in mg/dL.

3.2.5 Myeloperoxidase (MPO) Activity Assay

MPO activity was used as an indirect measure of the presence of neutrophils in liver, kidney, and lung tissues. The samples were collected in 200 µl of K_2HPO_4 buffer solution (pH 6.0), containing 0.5% of HTAB, and were homogenized

with a tissue homogenizer. After, samples were centrifuged (14,000 rpm x 4°C x 2 min) and the supernatant was used for MPO determination.

Briefly, 200 µl of supernatant was added to 50 mM phosphate buffer solution (pH 6.0), containing 0.167 mg/ml of *o*-dianisidine dihydrochloride and 0.015% of hydrogen peroxide. Absorbances were recorded at 450 nm using a microplate spectrophotometer (ThermoScientific, MultskanGO). MPO activity was expressed as myeloperoxidase activity (number of neutrophils x10^3/mg of tissue) compared to a neutrophil standard curve (Casagrande et al., 2006).

3.2.6 N-Acetylglucosaminidase (NAG) Activity Assay

NAG activity was determined as described by Hohmann et al. (2013). Briefly, the enzyme activity was measured from the supernatant obtained in the MPO assay. Liver, kidney, or lung tissues supernatant were placed in a microplate well and 80 µL of 50 mM phosphate buffer (pH 6) was added. The reaction started after the addition of 2.24 mM of 4-nitrophenyl N-acetyl-β-D-glucaminide followed by incubation at 37°C for 10 minutes. Finally, 100 µL of 0.2M glycine buffer (pH 10.6) was added to the reaction. The enzyme activity was determined spectrophotometrically at 400 nm and the results are expressed as NAG activity (macrophages x 10^3/mg tissue) compared to the macrophage standard curve.

3.2.7 Cytokine Measurement

Liver, kidneys, and lungs were collected in phosphate buffered saline for the determination of cytokine levels. Samples were homogenized, centrifuged (3000 rpm, 4°C, 15 minutes), and the supernatant was used to measure the levels of TNF-α,

IL-1β, IL-6 and IL-10 by ELISA. The assay was conducted following manufacturer's instructions (BioLegend – San Diego, CA – USA).

3.2.8 Ferric Reducing Ability of Plasma (FRAP) Assay

Liver, kidney, and lung samples were collected and homogenized in 500 µL of 1.15% KCl and centrifuged (1500 rpm / 4 °C/ 10 min). Ferric Reducing Properties were analyzed as previously described (Menghini et al., 2018). Briefly, 150 µL of the FRAP reagent was added to the supernatant and the absorbance was determined at 595 nm. Results were compared to a Trolox standard curve (30 mM, final concentration) and are expressed as Trolox equivalent/mg of protein.

3.2.9 Determination of the Levels of Thiobarbituric Acid Reactive Substances (TBARS)

Free radicals, when in contact with lipids, destabilize these molecules and produce malondialdehyde (MDA). In turn, MDA amplifies the inflammatory process. Therefore, TBARS test was used to detect the presence of MDA in samples using thiobarbituric acid (TBA). TBA reacts with MDA forming a new compound, which can be detected spectrophotometrically. Sample were homogenized in 500 µL of 1.15% KCl, and the homogenates were incubated with 5 µl 1 mM FeCl3, 5 µl of 1 mM ascorbic acid, 50 µl of 50% trichloroacetic acid (TCA) and 50 µl of 1% TBA, at 95° C for 60 min or until the color changes to pink. Then, the absorbance was measure at 572 nm and 535 nm, and the results are expressed as 572 nm – 575 nm OD/mg of protein (Guedes et al., 2006).

3.2.10 Nitroblue Tetrazolium (NBT) Assay

Quantification of superoxide (O_2^-) production was performed using the

NBT assay as previously described with adaptations to be performed in microplates (Choi et al., 2006). Briefly, 50 µl of sample homogenate were used and incubated in microplates well with 100 µL of solution containing the NBT reagent (1 mg/ml) for 1 hour at room temperature and protected from direct light. After incubation, the entire contents of the microplate were removed and 120 µl of 2M KOH solution and 120 µl of pure DMSO were added. The relative production of O_2^- was measured spectrophotometrically at 600 nm. Results are expressed in OD/mg of protein.

3.2.11 Determination of Reduced Glutathione (GSH) Levels

GSH levels were determined as previously described (Hohmann et al. 2013). Liver, kidney, or lungs were homogenized in 500 µl of 0.02M EDTA. Two hundred µl of homogenate was added to 25 µl of 50% trichloroacetic acid, incubated for 15 min at room temperature, and centrifuged (4000 rpm/4°C/ 15 minutes). In the assay, 100 µl of the sample supernatant was mixed with 200 µl of 0.4M Tris-HCl pH 8.9 and 10 µl of 10 mM dithiobisnitrobenzoic acid (DTNB) was added to the mixture. After 5 minutes at room temperature, the absorbance was determined at 412 nm. Results are presented as nmol GSH/mg of protein compared to a GSH standard curve.

3.2.12 Histopathological Analysis

Samples from liver, lungs and kidneys were collected and fixed in 10% buffered formaldehyde. Subsequently, the samples were embedded in paraffin, sectioned, and the tissue sections (5 µm) were stained with hematoxylin-eosin (HE), and examined under an microscope (Olympus OX31, Olympus, Japan).

For histopathological evaluation, a score was adapted as previously described (Copelli et al., 2018). The score considered the presence of hydropic

degeneration [0 - absent; 1 - short (1% - 25%); 2 - moderate (25% - 75%) and 3 – abundant (> 75%)]; the presence of inflammatory cell infiltrate [0 - absent; 1 - short (< 25 cells); 2 - moderate (25 - 125 cells); 3 - severe (> 125 cells)]; the presence of necrosis, considering nuclear alterations/cytoplasmic alterations/loss of tissue arrangement [0 - absent; 1 - short (1% - 25%); 2 - moderate (26% - 50%); 3 - moderate/severe (51% - 75%) and 4 – severe (> 75%)]. In addition, the characteristics of the cellular infiltrate (polymorphonuclear, mononuclear and mixed) were considered. The final score (0 – no involvement; 1-3: short involvement; 4-6 moderate involvement, 7-10: severe involvement. Results are expressed as mean value (variation) for each group of 6 animals.

3.2.13 Statistical Analysis

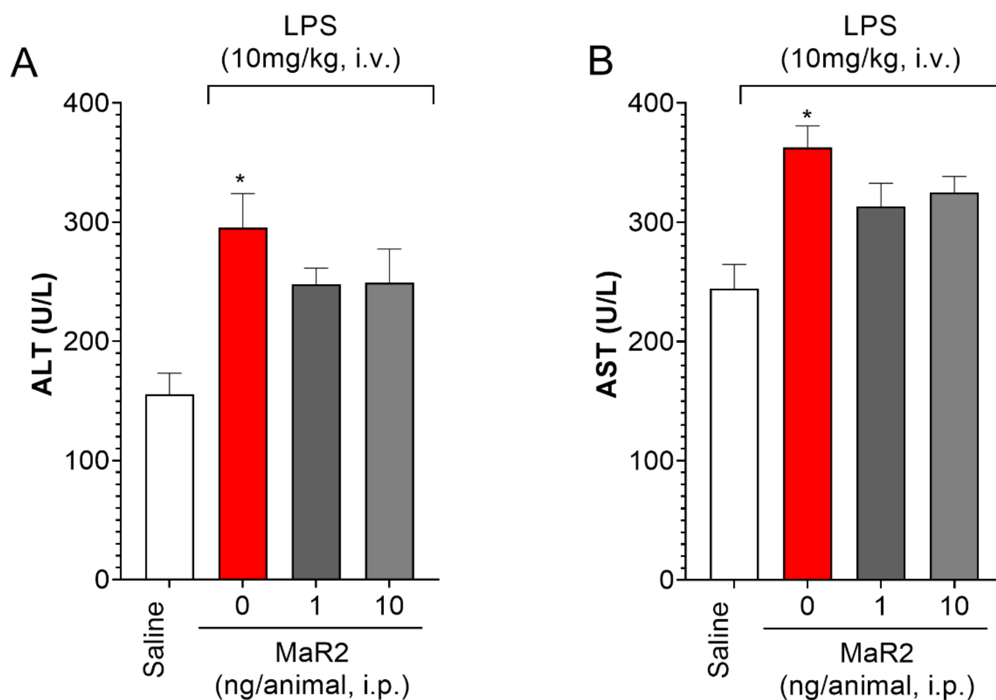
Assay results are presented as standard ± SEM (standard error of the mean) of 6 animals per group and are representative of one of 2 independent replicates. In case of normal distribution of the results and inhomogeneity variance, one-way ANOVA followed by Tukey's post-test was used and data was presented as mean ± SEM (standard error of the mean) of 6 animals per group. In case of non-normally distributed data or variance inhomogeneity, the non-parametric Kruskal-Wallis followed by Dunn´s test was used, and data presented as median and interquartile range (IQR). Differences were significant for $p < 0.05$.

3.3 Results

3.3.1 MaR2 Decreases Renal Injury Markers

Firstly, we examined liver and kidneys function by measuring the plasma

levels of ALT and AST, urea, and creatinine, respectively. Systemic LPS administration increased blood levels of ALT (Fig. 1A), AST (Fig. 1B), urea (Fig. 1C), and creatinine (Fig. 1D). The treatment with MaR2 (1 or 10 ng/animal) did not reduce serum levels of liver injury markers (AST and ALT). However, animals treated with MaR2 at the dose of 10 ng presented a significant decrease in urea levels (Fig. 1C). Both doses of MaR2 decreased LPS-induced levels of creatinine in the plasma (Fig. 1D). Thus, this result indicates that MaR2 have kidney protective effects.



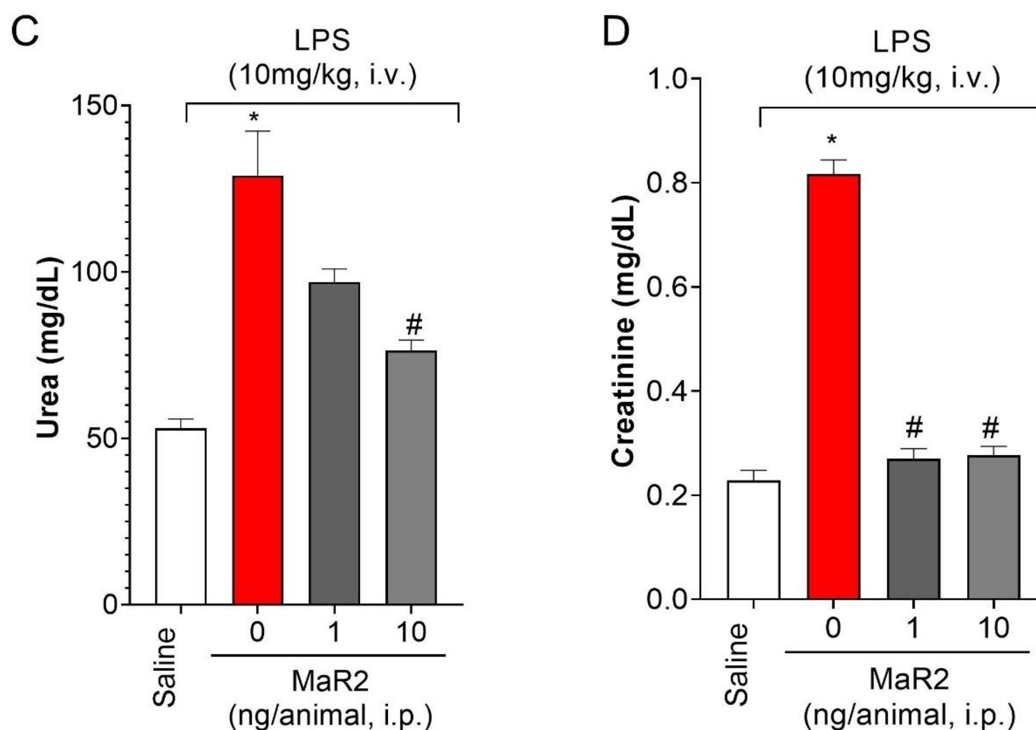
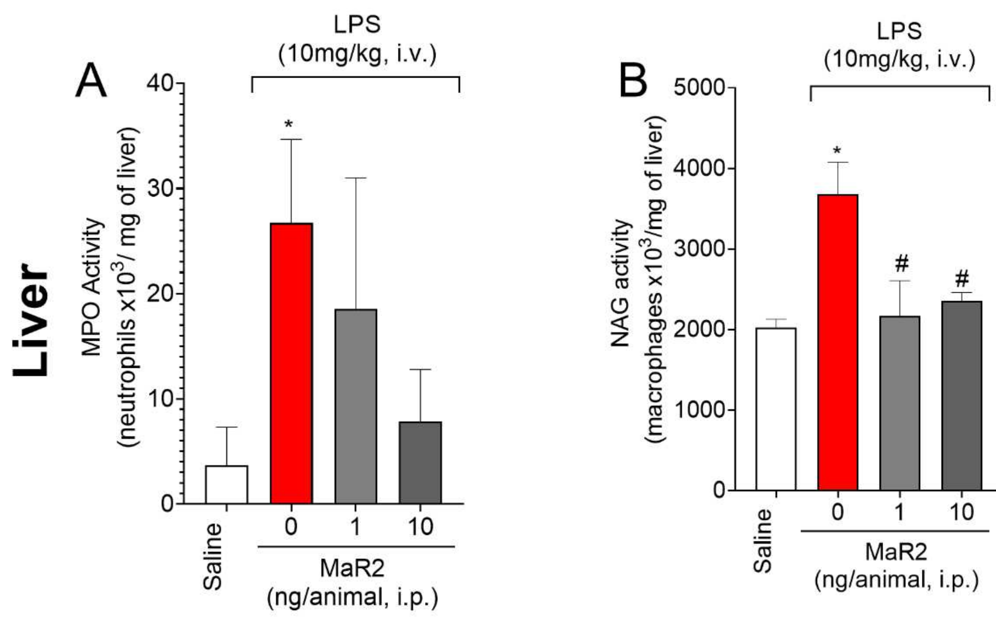


Figure 1 – **MaR2 decreased renal injury markers**. Blood was harvested by cardiac punction six hours after LPS administration (10 mg/kg, i.v.). The levels of ALT (A), AST (B), urea (C) and creatine (D) were measured. Results are presented as mean ± SEM (standard error of the mean) of 6 animals per group, and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

3.3.2 MaR2 Decreases Renal MPO and NAG Activities

Leukocyte recruitment is a hallmark of inflammation. Thus, we next determined the level of MPO and NAG activity in liver, lung, and kidney tissue. Systemic administration of LPS increased MPO and NAG activity in all tested tissues (Fig. 2). The treatment with MaR2 decreased LPS-induced MPO (Fig. 2E) and NAG (Fig. 2F) activity in the kidneys at both tested doses. MaR2 also reduced liver NAG activity (Fig. 2B). No effects on MPO activity were observed in the liver or lungs (Fig. 2A, C), nor in the levels of NAG in the lung (Fig. 2D). Thus, these results corroborate that MaR2 has a more prominent effect on MPO and NAG activities in kidneys.


Liver
A
LPS
(10mg/kg, i.v.)
MPO Activity
(neutrophils x10³/ mg of liver)
40
30
20
10
0
*
Saline
0
1
10
MaR2
(ng/animal, i.p.)
B
LPS
(10mg/kg, i.v.)
NAG activity
(macrophages x10³/mg of liver)
5000
4000
3000
2000
1000
0
*
#
#
Saline
0
1
10
MaR2
(ng/animal, i.p.)

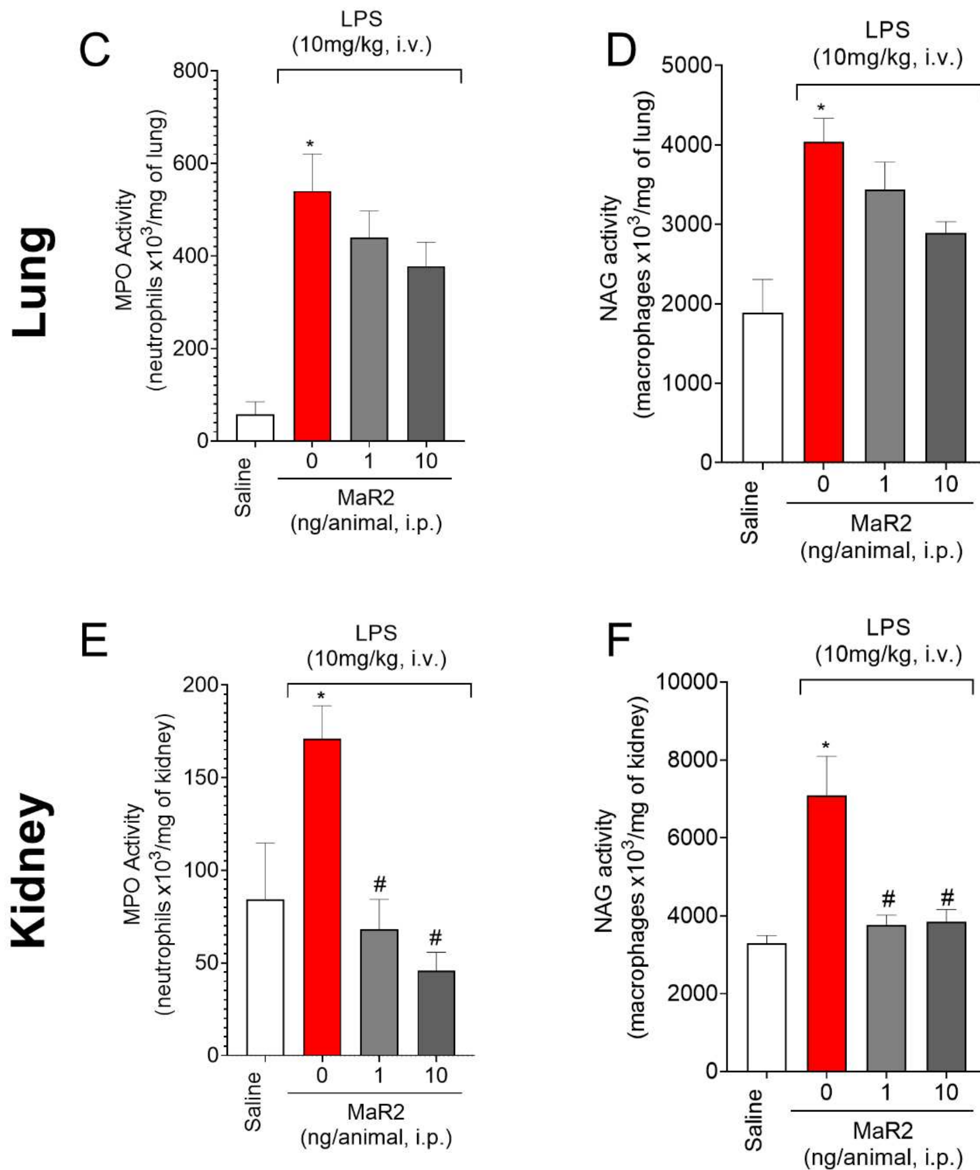


Figure 2 – **MaR2 decreased MPO and NAG activity in the kidneys.** Six hours after treatment with Mar2 (1 ng or 10 ng, i.p.) and LPS injection (10mg/kg, i.v.) MPO and NAG activity were determined. MPO activity in liver (A), lungs (C) and kidneys (E). NAG activity in the liver (B), lungs (D) and kidneys (F). Results are presented as mean ± SEM of 6 animals per group, and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

3.3.3 MaR2 Decreases the Production of Pro-Inflammatory Cytokines

LPS systemic administration triggered the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in the liver, lungs, and kidneys. All the tested doses of MaR2 reduced IL-1β production in the liver (Fig.3A), lungs (Fig.3D) and kidneys (Fig.

3G), as well TNF-α levels (Fig. 3B, E and H), and IL-6 levels (Fig. 3C, F and I). Furthermore, MaR2 treatment also reduced the levels of IL-10 in all evaluated organs (Fig. 4A – C).

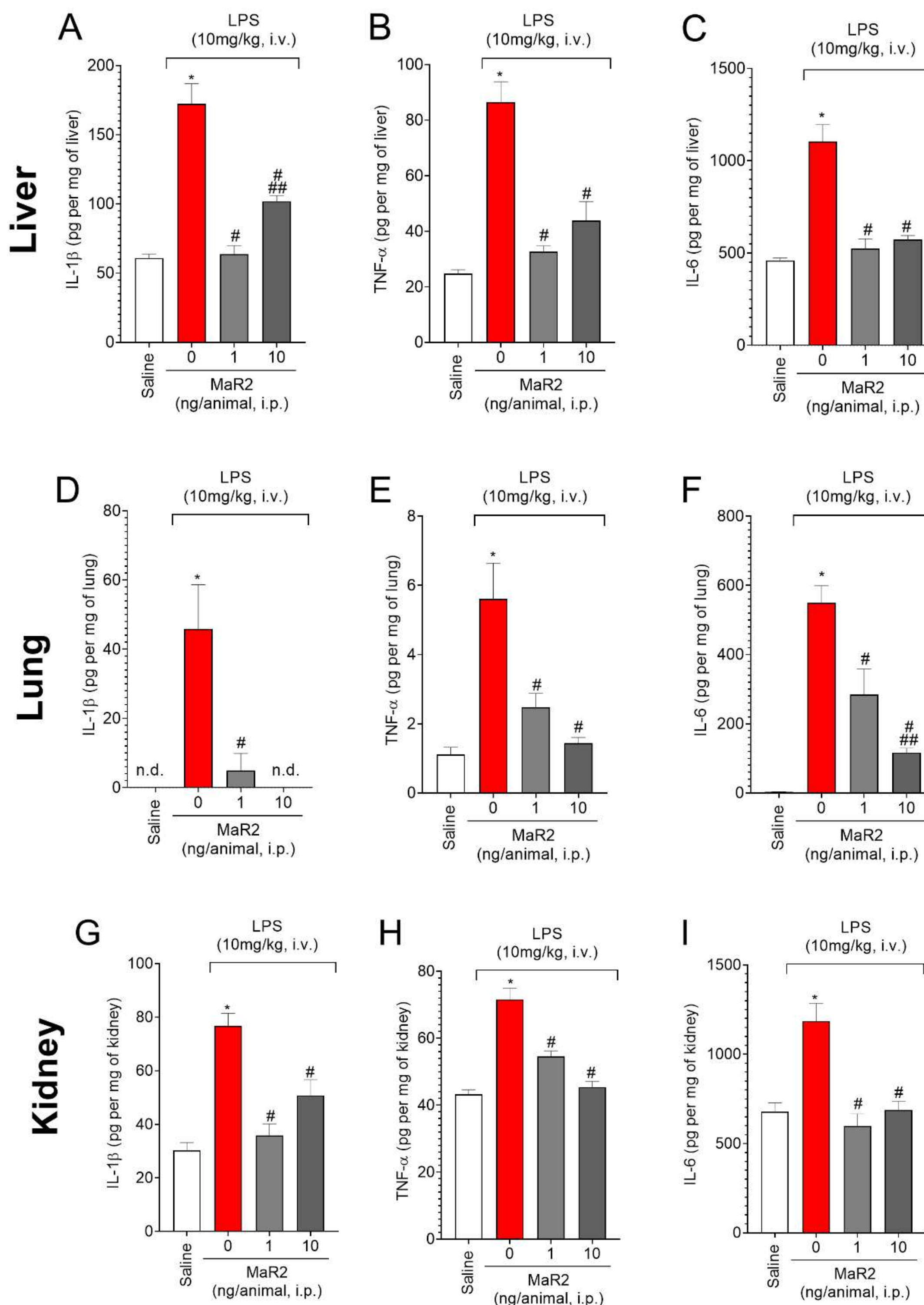


Figure 3 – MaR2 reduced LPS-induced IL1-β, TNF-α and IL-6 levels production in the lungs,

viver and kidneys. Tissue were collected, homogenized and the supernatant was used to measure IL1-β in liver (A), lungs (D) and kidneys (G); TNF-α in all three organs (B, E and H), and IL-6 (C, F, and I). Results are presented as mean ± SEM of 6 animals per group, and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; ## P < 0.05 vs. MaR2 10ng/animal. one-way ANOVA followed by Tukey's post-test).

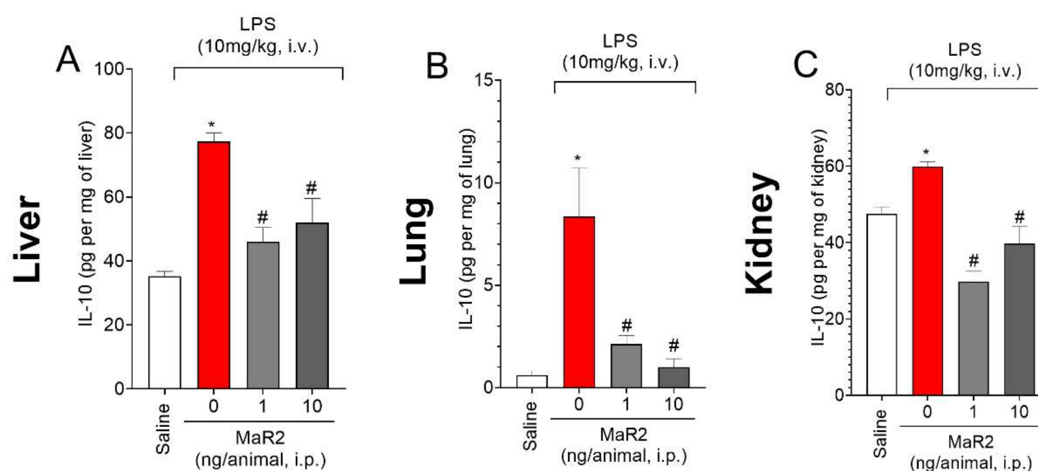
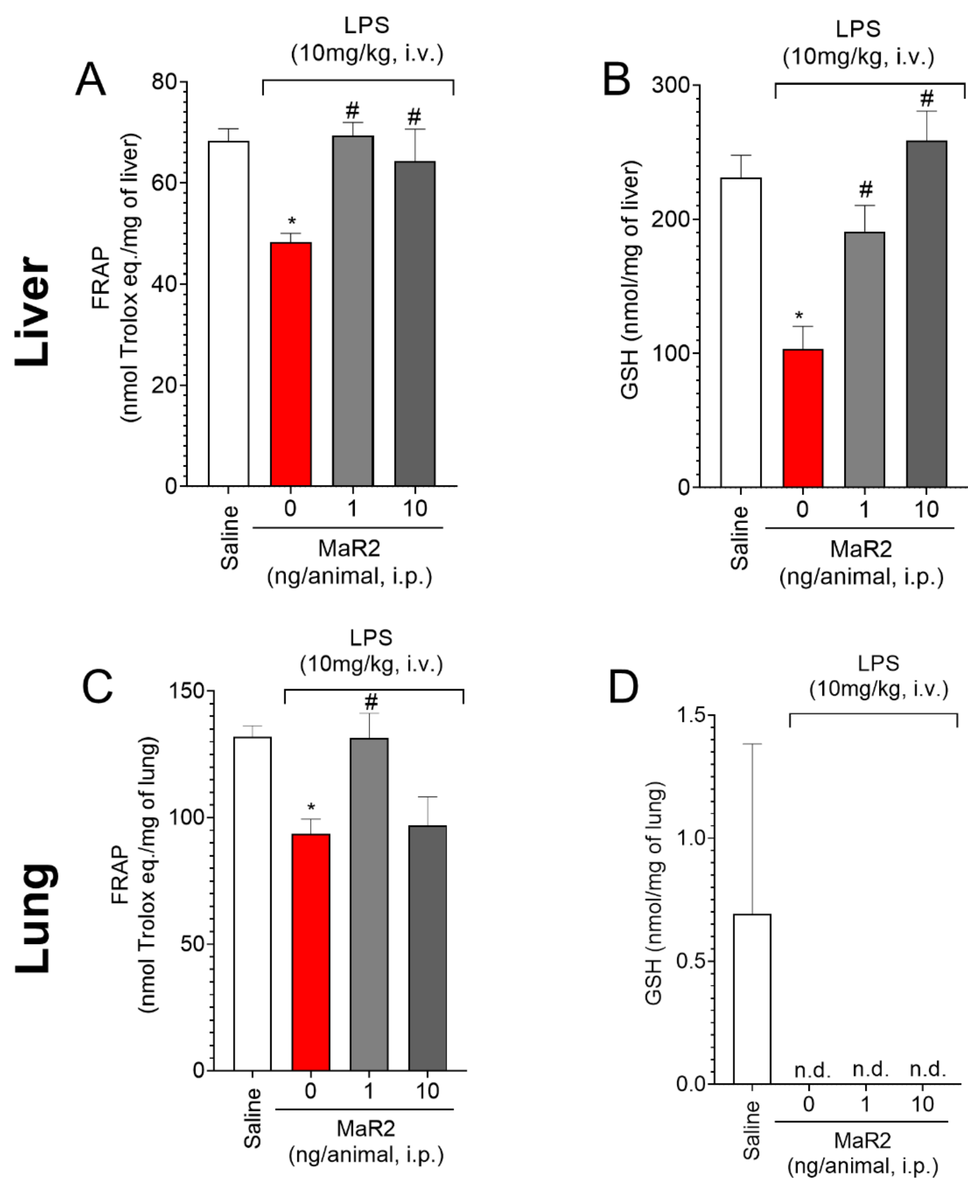


Figure 4 – MaR2 reduces LPS-induced IL-10 levels in the liver, lungs and kidneys. The anti-inflammatory cytokine IL-10 was measured in liver (A), lungs (B) and kidneys (C). Results are presented as mean ± SEM (mean error) of 6 animals per group and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

3.3.4 MaR2 Reestablishes Antioxidant Capacity

The Ferric Reducing Antioxidant Power (FRAP) is an indirect method that evaluates the antioxidant capacity of the sample through the reduction of Fe^{3+}. The systemic administration of LPS induced a decrease in the antioxidant capacity from the analyzed organs. The treatment with MaR2 reestablished the antioxidant capacity in the liver (Fig. 5A) and in the lungs (Fig. 5C) at 1ng /animal. Antioxidant capacity was not restored in the kidneys (Fig.5E). After stimulation with LPS, levels of GSH also reduced in liver and kidneys (Fig. 5B and 5F). MaR2 prevented the decrease of GSH levels in the liver (Fig. 5B) and kidney (Fig. 5F) in a dose-dependent manner. In the lungs the levels of GSH were not detected after LPS stimulus nor in MaR2 treated groups (Fig.5D).



Liver
Lung
A
LPS
(10mg/kg, i.v.)
FRAP
(nmol Trolox eq./mg of liver)
80
60
40
20
0
*
#
#
Saline
0
1
10
MaR2
(ng/animal, i.p.)
B
LPS
(10mg/kg, i.v.)
GSH (nmol/mg of liver)
300
200
100
0
*
#
#
Saline
0
1
10
MaR2
(ng/animal, i.p.)
C
LPS
(10mg/kg, i.v.)
FRAP
(nmol Trolox eq./mg of lung)
150
100
50
0
*
#
Saline
0
1
10
MaR2
(ng/animal, i.p.)
D
LPS
(10mg/kg, i.v.)
GSH (nmol/mg of lung)
1.5
1.0
0.5
0.0
n.d.
n.d.
n.d.
Saline
0
1
10
MaR2
(ng/animal, i.p.)

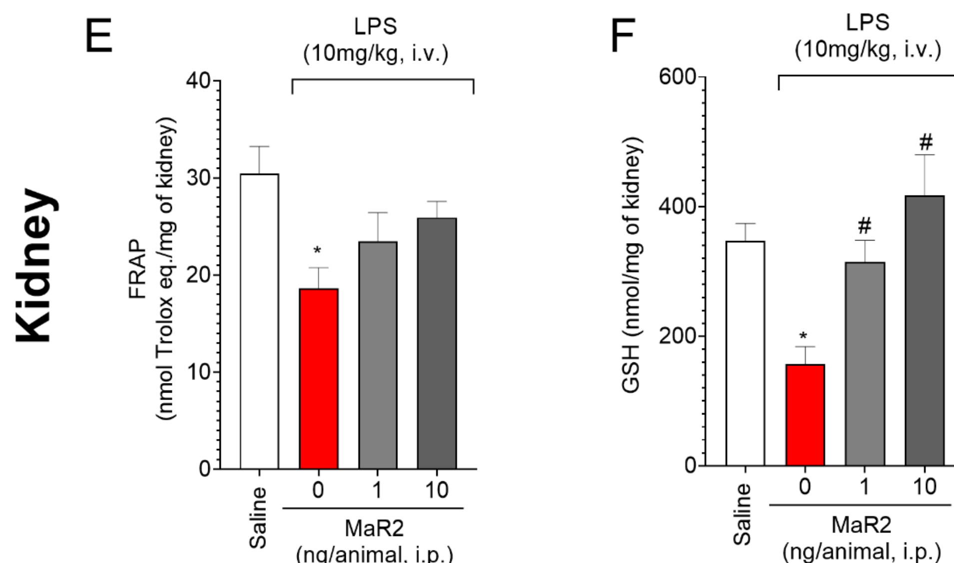
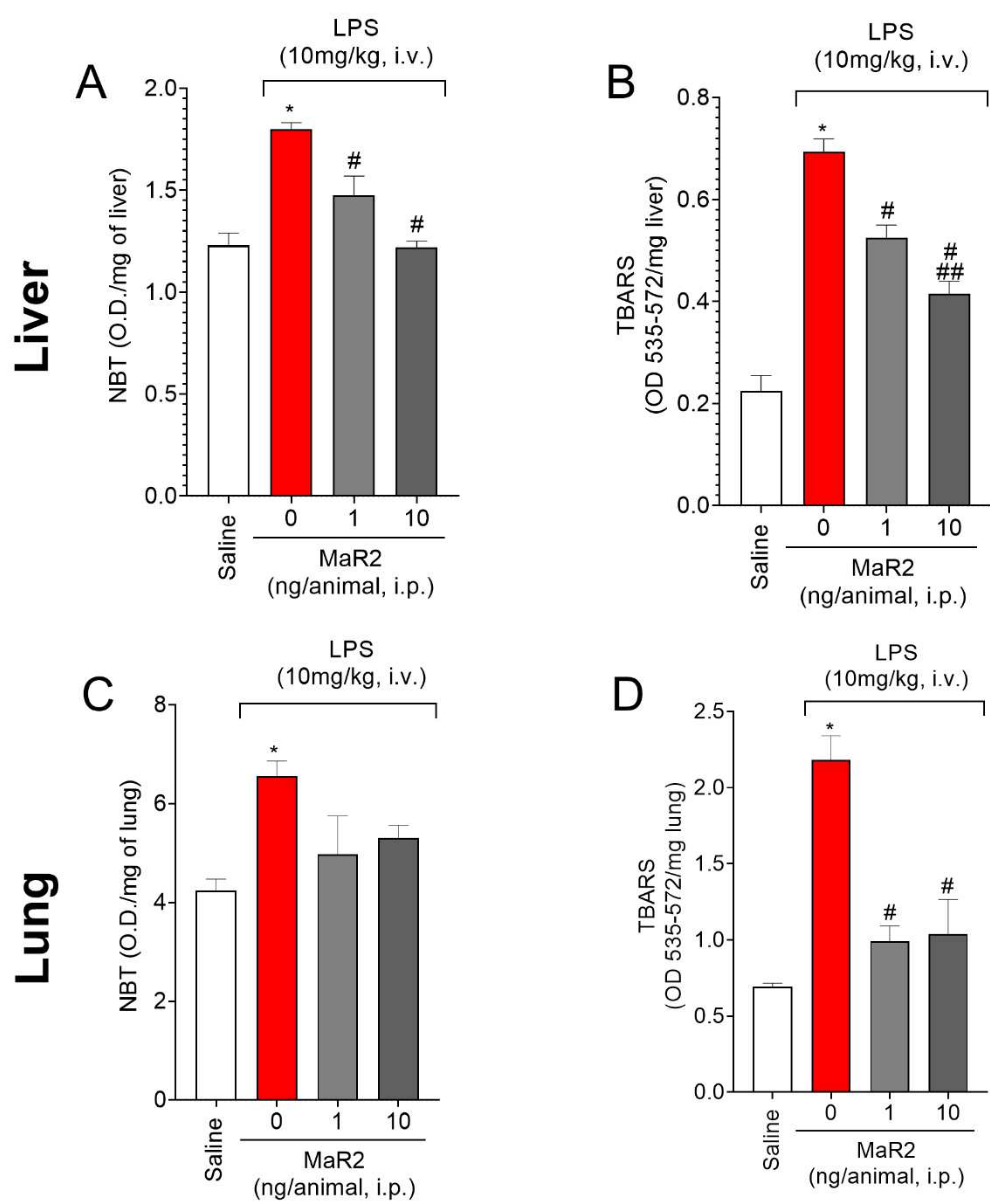


Figure 5 – **MaR2 reestablishes tissue antioxidant capacity.** Tissue was collected six hours after treatment (MaR2 0.1, or 1 ng/animal, i.p.) and LPS administration and were used to measure antioxidant capacity by FRAP method in liver (A), lungs (C) and kidneys (E) and to measure GSH levels on liver (B), lungs (D) and kidneys (F). Results are presented as mean ± SEM of 6 animals per group and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

3.3.5 MaR2 Decreases Oxidative Injury

As expected, systemic administration of LPS increased lipid peroxidation observed at TBARS test in the liver, lungs, and kidneys. The treatment with MaR2 decrease the lipid peroxidation products in the liver (Figure 6B) and lungs (Figure 6D) in both tested doses. Moreover, in the liver the effect was dose-dependent manner. In the kidneys (Figure 6F), MaR2 at 10 ng/animal decreased lipid peroxidation.

Corroborating with these findings, LPS enhances superoxide anion levels, as determined per NBT assay, in all tested organs. Further, both doses of MaR2 decreased superoxide anion levels in a dose-dependent manner in the liver (Figure 6A). MaR2 at 10 ng/animal also reduced superoxide anion levels in the kidneys (Figure 6E). No significant changes in the groups treated with MaR2 were observed in the lungs (Figure 6C).


Liver
Lung
A
B
C
D
LPS
(10mg/kg, i.v.)
NBT (O.D./mg of liver)
TBARS
(OD 535-572/mg liver)
NBT (O.D./mg of lung)
(OD 535-572/mg lung)
Saline
0
1
10
MaR2
(ng/animal, i.p.)
*
#
##

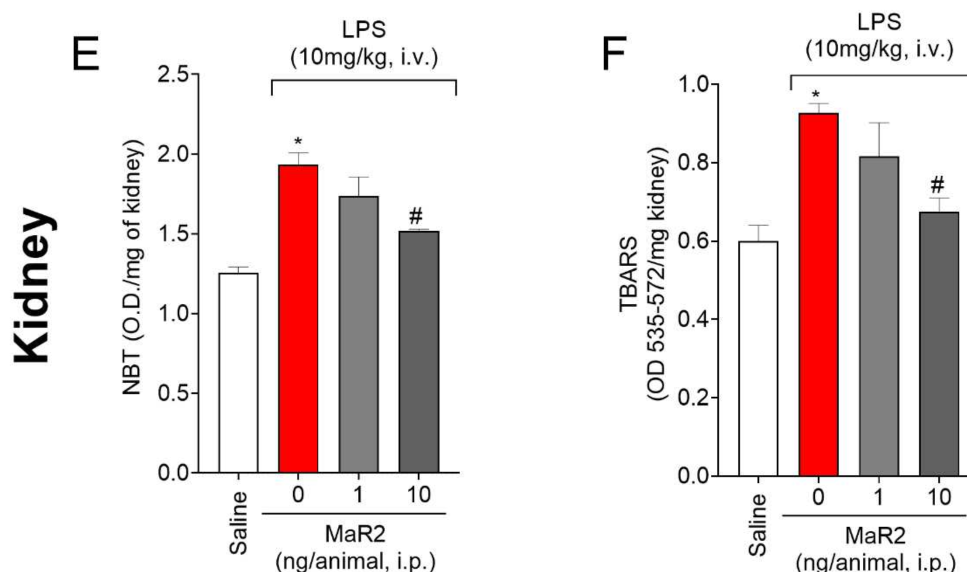
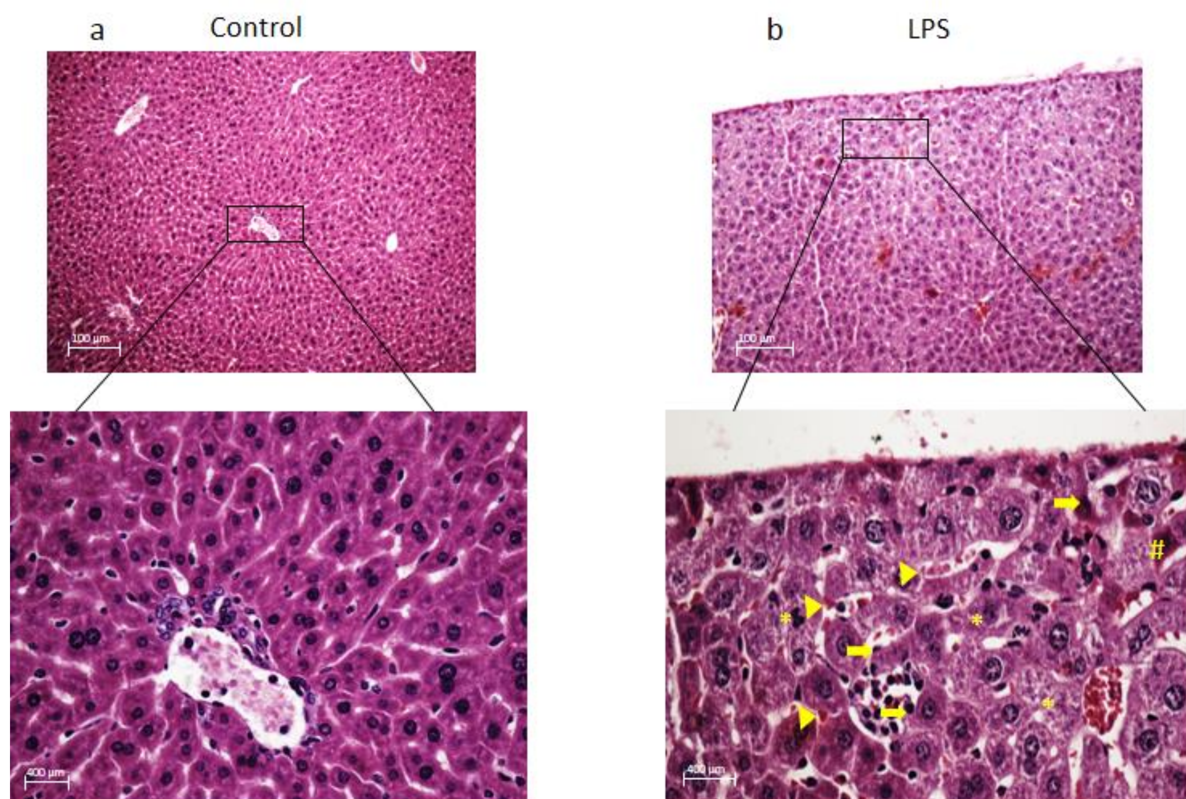


Figure 6 – **MaR2 decreased oxidative stress and reduces lipid peroxidation and superoxide anion levels.** LPS-induced lipid peroxidation and superoxide anion levels in all examined issues. MaR2 decrease TBARS level in the liver (B), lungs (D) and kidneys (F). The treatment also reduced superoxide anion levels of in liver (A) and kidneys (E). No effect from the treatment were observed in the lung (D). Results are presented as standard ± SEM (mean error) of 6 animals per group and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

3.3.6 MaR2 Decreases Histopathological Score, Leukocyte Infiltrate, and Necrosis in the Kidney

Samples of liver, lung, and kidney were processed and stained with H&E for histopathological score determination, Figures 7 - 9 present our findings. In the liver, LPS systemic administration increased the presence of mixed infiltrate cells (<25 cell), red blood cells extravasation into the parenchyma (Figure 7B). Moreover, it was also observed the presence of nuclear alterations suggestive of a necrotic process, with subtle presence of hydropic degeneration (Figure 7B).Both doses of Mar2 (1 ng and 10 ng) protected the liver tissue from damage (Fig. 7C – D, respectively), with minor hydropic degeneration, decreased inflammatory infiltrate and red blood cells in liver parenchyma. Moreover, it was not observed necrotic regions.


a
Control
b
LPS
100 µm
400 µm
100 µm
400 µm
#
*
*
*

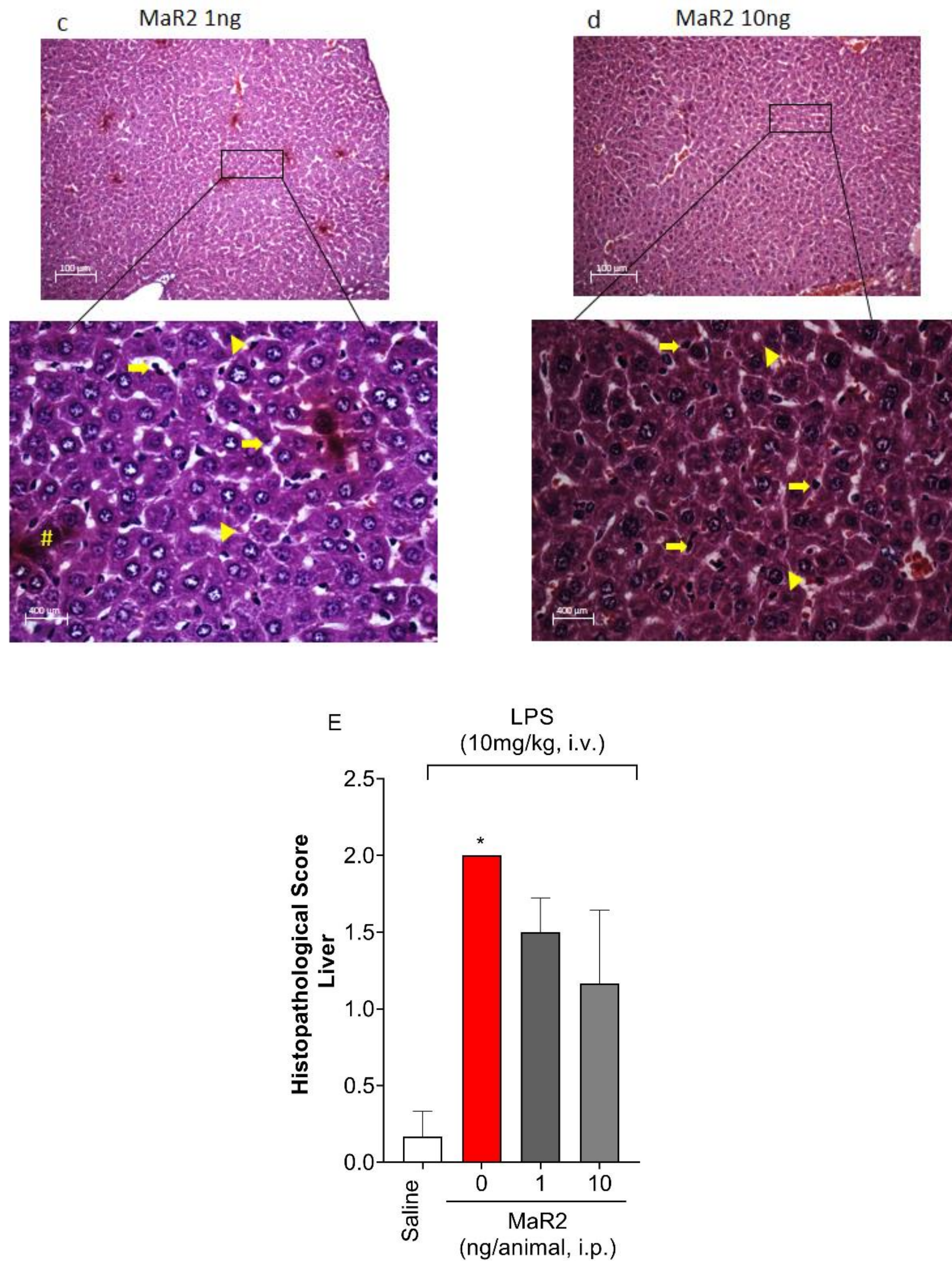
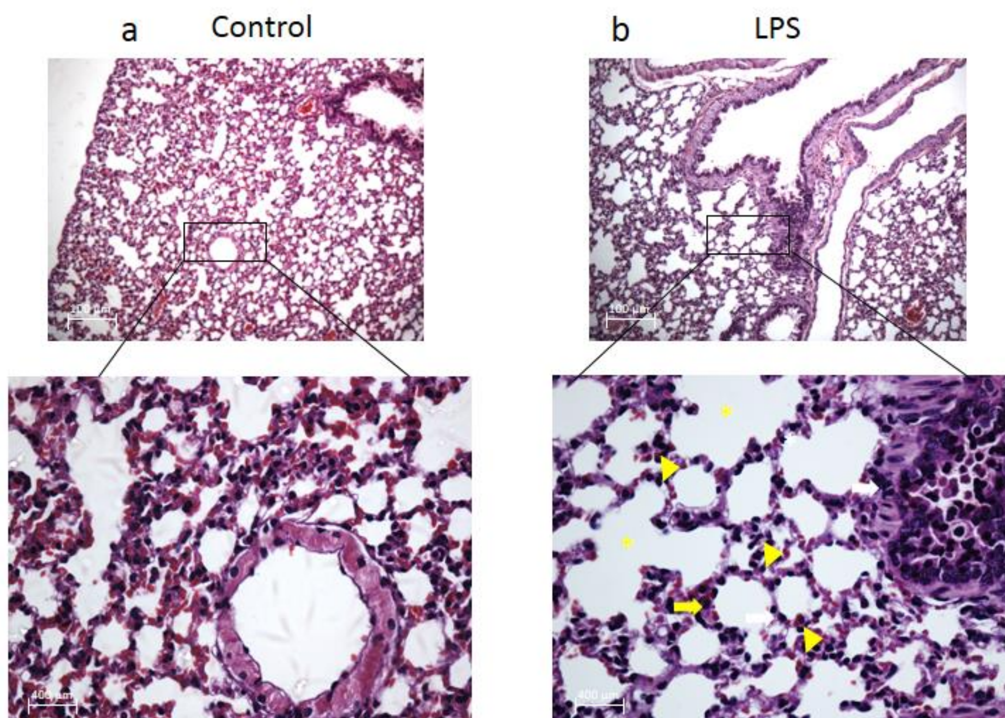


Figure 7 – MaR2 preserved liver architecture after LPS stimulus. Representative photomicrographs of hepatic histology (haematoxylin and eosin, magnification 400×), from control (A), LPS + vehicle (B) (10mg/kg – iv), MaR2 at 1 ng (C) and 10 ng (D). Arrow: inflammatory infiltrate; Arrowhead: red blood cells; *necrotic changes; #bleeding focus. Histopathological score (E). **Fig.7E:** *P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; # P < 0.001 vs. MaR2 0.01 ng; Kruskal-Wallis followed by Dunn´s test.

Similarly, in the lung tissue was observed a slight increase in the inflammatory infiltrate, small presence of red blood cells in the parenchyma, increased alveolar space, with minor involvement of the organ. MaR2 exert protective effects in the lung (Fig. 8C – D). Although there were no significant differences among vehicle and MaR2 doses a protective effect in the lung tissue was observed. MaR2 prevents LPS-induced tissue damage by decreasing inflammatory infiltrate, hydropic degeneration, red blood cells spot in the parenchyma, and no necrotic region (Fig. 8E).



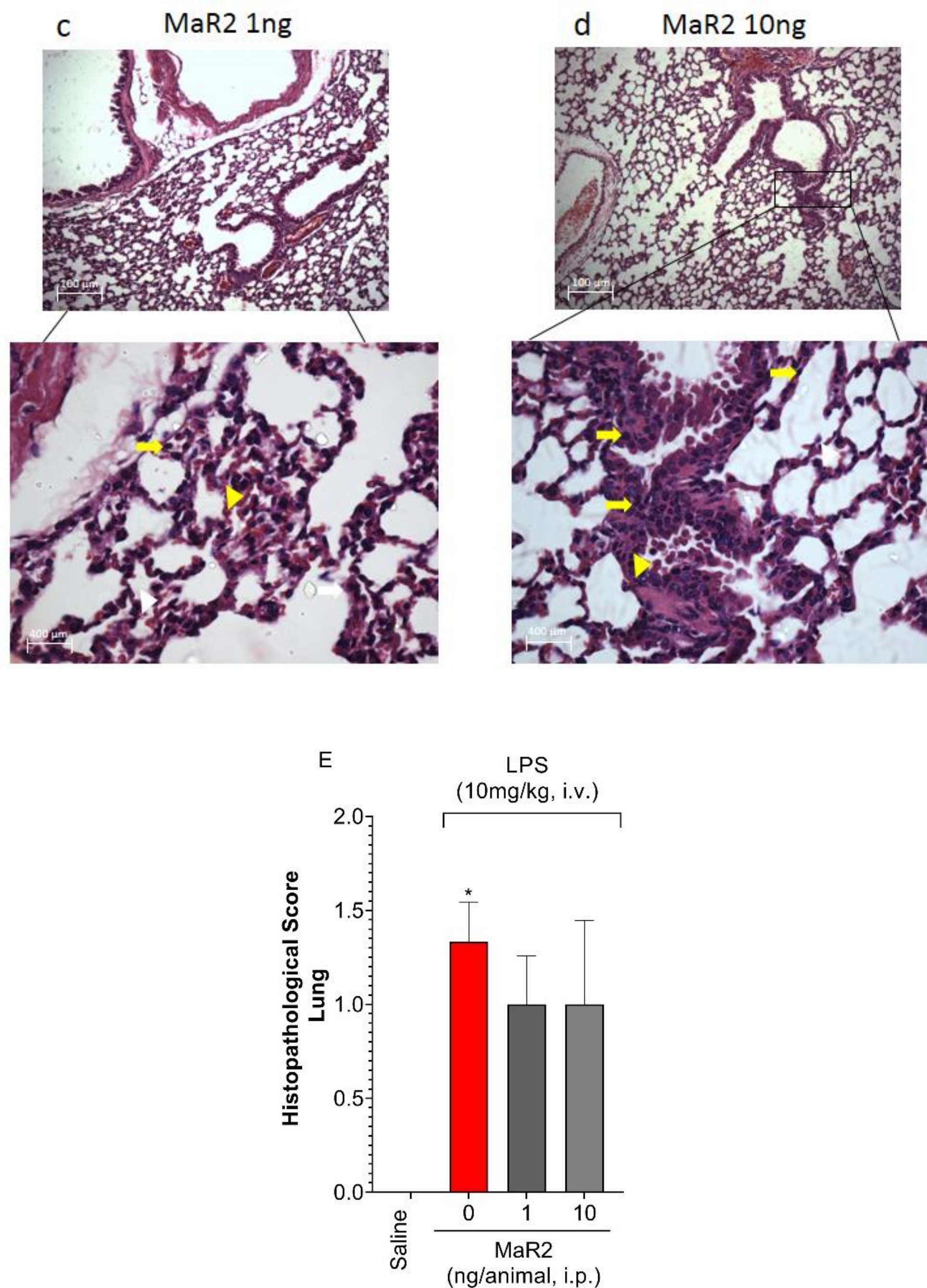
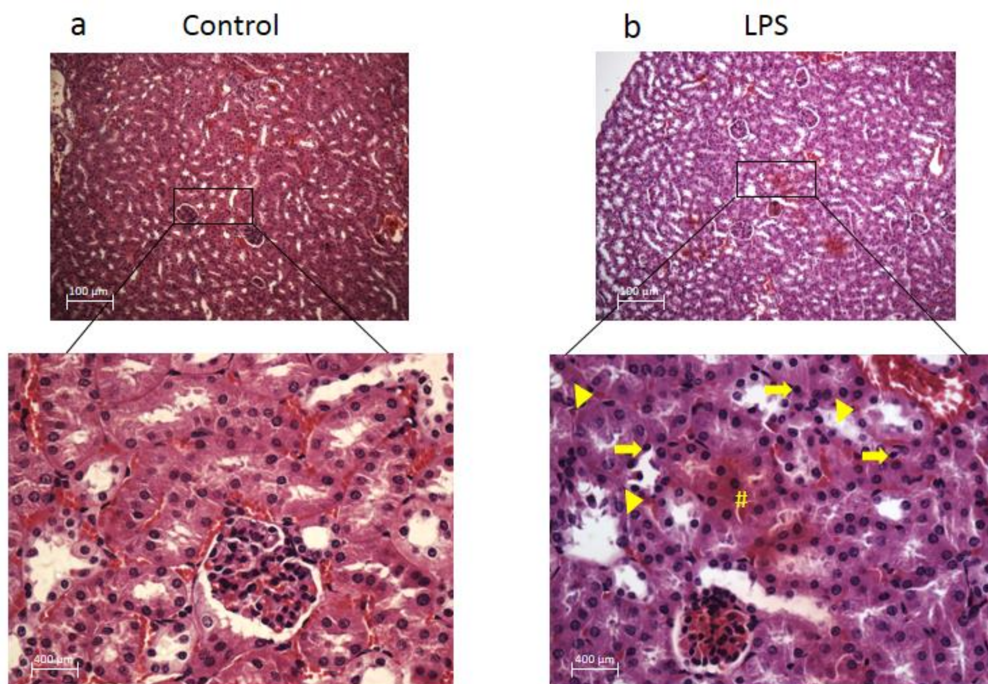
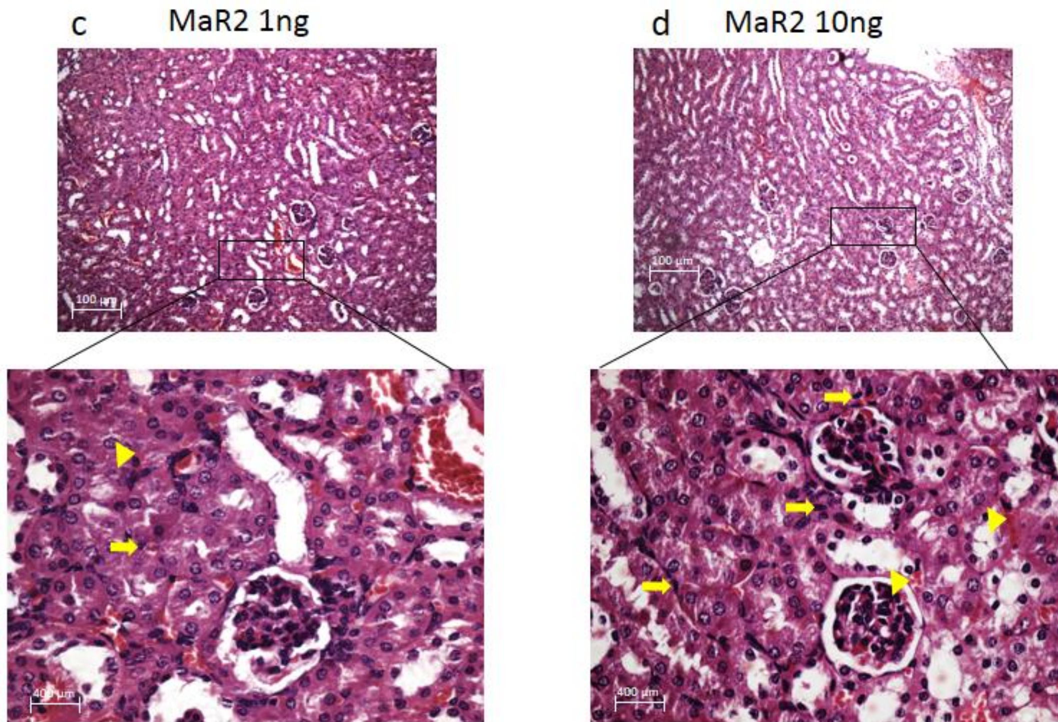


Figure 8 – MaR2 preserved lung architecture after LPS stimulus. Representative photomicrographs of pulmonary histology (haematoxylin and eosin, magnification 400×). Control (A), LPS + vehicle (B), MaR2 (i.p.) at 1 ng (C) and 10 ng (D). Arrow: inflammatory infiltrate; Arrowhead: red blood cells; *increased alveolar space; #bleeding focus. € Histopathological score. **Fig. 8E:** *P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; # P < 0.001 vs. MaR2 0.01 ng; Kruskal-Wallis followed by Dunn´s test.

In the kidneys LPS systemic administration also induced the presence of inflammatory infiltrate, whit mild presence of red blood cells in parenchyma and presence of a small amount of hydropic degeneration (Fig. 9B).

The treatment with MaR2 at 1 ng (Fig. 9C) or 10 ng (Fig. 10D) decreased the inflammatory findings in renal tissue, without degenerative representations. It was observed the presence of hydropic degeneration, with a decrease in the inflammatory infiltrate, without necrotic representations and no significant structural losses (Fig. 9E).





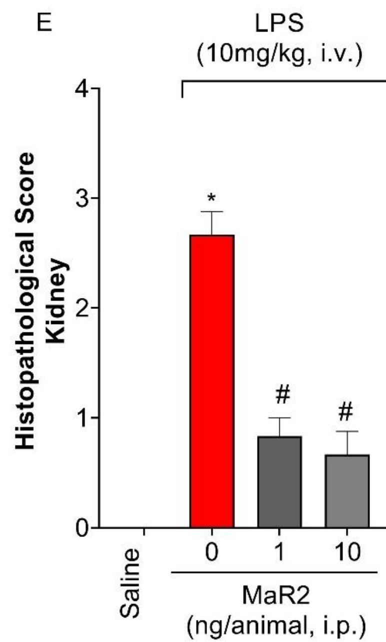


Figure 9 – MaR2 preserved kidney architecture after LPS stimulus. Representative photomicrographs of renal histology (haematoxylin and eosin, magnification 400×). Control (A), LPS + vehicle (B) (10mg/kg, i.v.), MaR2 treated at 1 ng (C) and 10 ng (D). Arrow: inflammatory infiltrate; Arrowhead: red blood cells; *necrotic changes; #bleeding focus. (E) Histopathological score. **Fig.9E:** *P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; # P < 0.001 vs. MaR2 0.01 ng; Kruskal-Wallis followed by Dunn´s test).

3.4 DISCUSSION

In this study we investigated the effects of MaR2 treatment in a model of endotoxemia induced by systemic administration of LPS in mice. As demonstrated, animals treated only with LPS have increased serum levels of liver and kidney damage markers and worsen histopathological scores in the liver, lungs, and kidneys. Moreover, LPS also increased inflammation, by inducing the production of pro-inflammatory cytokines, and depleted antioxidant capacity and oxidative stress in the analyzed tissues. MaR2 reduced inflammation and oxidative stress parameters in all investigated organs, however, the effects in the kidneys were outstanding.

The animal model of LPS systemic administration used in this study, induced the endotoxemic phenotype, as expected. Corroborating that endotoxemia facilitates the production of pro-inflammatory cytokines, the production of free radicals, with consequent damage and dysfunction of organs and systems (Gong et al., 2015; Li et al., 2021). However, this whole context can be reversed and avoided by decreasing or inhibiting inflammatory and oxidative activities, favoring tissue, and improving metabolism (Hao et al., 2019; Li et al., 2020; Rodriguez et al., 2021). In addition, upregulation of TLR4 and inhibition of NF-κB signaling pathways also favored lower expression of pro-inflammatory cytokines and decreased production of superoxide anion and oxidative damage (Principe et al., 2017).

Other studies demonstrated the clinical and metabolic signs of endotoxemia in animals submitted to intravenous LPS administration. The signs consist of reduced blood pressure, increased heart rate, enhanced serum lactate levels, reduced expression of the antioxidant enzyme Gpx3 (Blang-Letheule et al., 2021) and increased recruitment of tissue leukocytes (Wang et al., 2019). Moreover, mice treated with LPS may also show increased MPO activity in the liver (Bekpinar et

al., 2013), enhanced phosphorylation of IKK, IκB and NF-κB p65, raising liver injury markers, pro-inflammatory cytokines levels and generating reactive oxygen species (Yun et al., 2010; Guo et al., 2014). Those effects lead to the increase in inflammatory infiltrate and consequent liver injury (Zhong et al., 2016).

In the present study, MaR2 decreased NAG activity and the production of pro-inflammatory cytokines in the liver. Furthermore, it reestablished the antioxidant capacity of this organ and reduced the production of the superoxide anion and lipid peroxidation. The treatment reduced inflammatory infiltrate and protected the tissue from necrosis.

The lungs are considered the most vulnerable organs and the first compromised during endotoxemic shock (Xiao et al., 2021). Alteration in pulmonary function enable an increase in capillary permeability with consequent organ edema and alveolar septum thickening (Tseng et al., 2012). Therefore, the lungs are organs of interest in endotoxemic animal models. LPS administration promotes alveolar septum thickening, decreased alveolar spaces, and increased inflammatory infiltrate in lung tissues (Xie et al., 2017). In addition, LPS induces an increase in the total leukocyte count in the blood, elevate levels of pro-inflammatory cytokines and nitric oxide in the lung (Tseng et al., 2012; Xie et al., 2017). Furthermore, LPS also increases oxidative stress injury and depletes antioxidant enzyme levels in this organ (Zhang et al., 2014). We demonstrated that MaR2 decreased pulmonary levels of pro-inflammatory cytokines, reestablished the antioxidant capacity and decreased lipid peroxidation in this tissue. In addition, treatment with MaR2 protected lung tissue and avoid tissue damage.

The kidneys are also an organ worth of investigation, since it is known that renal function might be compromised during endotoxemia. LPS administration

triggers the production of nitric oxide and the reduction of renal vascular resistance, which culminates in a decrease of glomerular filtration rate (Bansal et al., 2009) and leads to the installation of an acute renal failure condition (Boffa et al., 2004). In a model of acute renal failure, LPS increased serum urea and creatinine levels, enhanced MPO activity, promoted tubular lumen dilation, brush border loss and renal tubular derangement (Sun et al., 2019; Li et al., 2021; Xiao et al., 2021). In addition, there may be an increase in the production of reactive oxygen species, worsening of lipid peroxidation and depletion of antioxidant enzymes (Li et al., 2021; Xiao et al., 2021).

In our study, we demonstrated that MaR2 decreased serum levels of urea and creatinine, which suggest that this SPM might promote renal protection during LPS-induced endotoxemia. Furthermore, MaR2 decreased MPO and NAG activity suggesting tha MaR2 regulates the recruitment and activation of neutrophils and macrophages in the kidney. Nevertheless, MaR2 reduced renal levels of TNF-α, IL-1β, IL-6, and IL-10. Moreover, MaR2 inhibited tissue damage, in which was observed lower scores of involvement and inflammatory infiltrate in both doses, strengthening the suggestion of an anti-inflammatory and renal protective effect in this model. Despite MaR2 did not restore total renal antioxidant capacity, the treatment increased GSH levels, decreased lipid peroxidation and the production of the superoxide anion, suggesting the importance of its antioxidant role in endotoxemia.

Therefore, we demonstrated that MaR2 has anti-inflammatory and antioxidant proprieties. Furthermore, the reduction of kidney markers was accompanied with protective histopathological effects. Despite the protective effect in the kidneys, there were not observed significant evidence of liver or lung tissue protection effects. However, in general, our data suggest that MaR2 exert its protective

role via the reduction of pro-inflammatory cytokines and oxidative stress parameters, which were reduced in all tested organs, liver, lung, and, specially, kidneys. Nevertheless, our data suggest that MaR2 might be an alternative therapeutic approach for the treatment of endotoxemic shock.

3.5 ACKNOWLEDGEMENT

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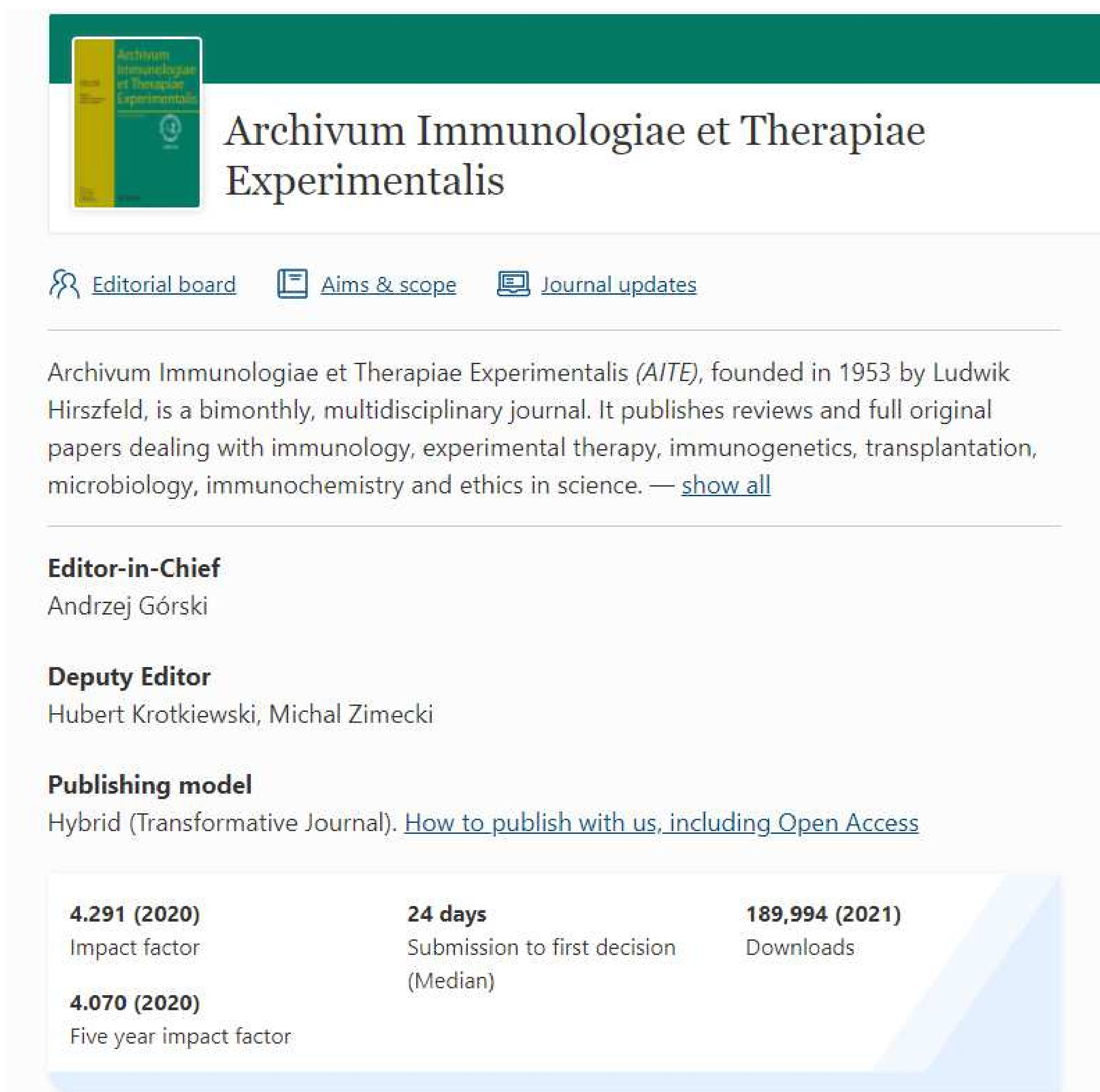
4 ARTIGO RESOLVINA D5 (RvD5)



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Resolvin D5 reduces LPS-induced endotoxemia in mice: Protection of liver, lungs and kidneys

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Abstract

LPS (lipopolysaccharide) is considered a potent activator of the inflammatory response that can lead to a systemic imbalance promoting an exacerbated endotoxemic response. Resolvin D5 (RvD5) is a specialized pro-resolution lipid mediator (SPM) with anti-inflammatory and antioxidant property that could protected mice against endotoxemia. Animals were treated with LPS (10mg/kg - iv.) 60 minutes before administration of RvD5 (0,1ng; 1ng or 10ng/animal - i.p). Serum levels of ALT, AST, Urea and Creatinine were evaluated. Activity of MPO, NAG and the production of pro-inflammatory cytokines in the liver, kidneys and lungs were observed. Antioxidant capacity and the levels of lipid peroxidation in these samples were also measured, as well as histological analysis. We demonstrated that RvD5 performs renal and hepatic protective activity, observed with the improvement of ALT, AST, urea and creatinine. Besides that, improvement of histological parameters, reduced expression of pro-inflammatory cytokines, diminished lipid peroxidation and superoxide anion production. RvD5 acted positively by decreasing NAG and MPO activity and restored antioxidant levels, as well as GSH. Our data suggest that RvD5 would be a therapeutic possibility for endotoxemia and endotoxemic shock.

Keywords: resolvin D5; lipopolysaccharide; endotoxemia; inflammation.

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Abbreviations

5-LOX	5-Lipoxygenase
15-LOX	15-Lipoxygenase
AA	Arachidonic acid
ADAM-17	Desintegrin and Metalloprotease 17
CCL5	Chemokine (C-C motif) ligand 5
CLP	Cecal Ligation Puncture
DHA	Docosapentaenoic acid
DPA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
IL-6	Interleukin 6
LPS	Lipopolysaccharide
NF-kB	Nuclear factor-kappa B
RvD	Resolvin D
RvE	Resolvin E
RvD5	Resolvin D5
SPM	Specialized Proresolving Mediator
THP-1	THP-1 Cell line

4.1 INTRODUCTION

LPS (lipopolysaccharide), the main component of the outer wall of gram-negative bacteria, is considered a potent activator of the inflammatory response (Mohammad; Thiemermann, 2021). TLR4 recognizes bacterial LPS and activates intracellular transcription pathways such as NF-κB (nuclear factor-kappa B), triggering the production of pro-inflammatory cytokines (Lu et al., 2008). However, if an excessive activation of these pathways occurs, a systemic inflammatory imbalance may happen, promoting a possible exacerbated septic response of the host, with high potential for morbidity and lethality (Mohammad; Thiemermann, 2021).

Specialized proresolving lipid mediators (SPMs) are molecules that actively promote the resolution of the inflammatory process (Serhan; Savill, 2005; Serhan, 2014). These lipid mediators can down-modulate NF-κB activation, reduce its signaling pathways, decreasing the expression of pro-inflammatory cytokines and overall inflammatory response (Jordan; Werz, 2021).

In a sepsis model in mice induced by cecal ligation and puncture, it was observed that SPM protected vital organs during systemic septic response, decreased inflammatory parameters, enabling an improvement in the overall survival rate of these animals (Li et al., 2016). Thus, the resolution of the inflammatory process is an event orquestrated by SPMs, which can reduce excessive neutrophil infiltration and perform tissue regeneration and bacterial clearance (Serhan, 2014; Jordan; Werz, 2021).

Resolvins are SPMs derived from the omega-3 fatty acids EPA (Eicosapentaenoic acid) and DHA (Docosapentaenoic acid), which are considered primary substrates of these biomolecules, which can be subdivided according to the precursos molecule into Resolvins – E (RvE) and Resolvins – D (RvD) (Molaei et al., 2021) dreved from EPA and DHA, respectively. DHA-derived RvDs are synthesized by different cells (polymorphonuclear cells and macrophages) through the action of 15-lipoxygenase (15-LOX) which transforms DHA into 17S-hydroperoxy-DHA, and this intermediate, in turn, undergoes the action of 5-lipoxygenase (5-LOX) generating six RvD, which differ structurally according to position, chirality and the number of hydroxyl residues (Demarquoy; Borgne, 2014; Dartt et al., 2020; Molaei et al., 2021), including the RvD5.

Resolvin D5 (7(S),17(S)-diHDHA) in an *in vitro* model decreased the expression of IL-6 and CCL5 in THP-1 cells challenged with LPS, by inhibiting ERK

phosphorylation and the nuclear translocation of p65 and p50 subunits of NF-κB (Chun et al., 2020). And in a cecal ligation and puncture (CLP) sepsis model, RvD5 protected mice against endotoxemia and accelerated the resolution of inflammation and the return of tissue homeostasis through activation of the ADAM-17 pathway. This is a complex condition since there are concomitant bacterial proliferation and inflammation response (Zhou et al., 2019). On the other hand, by using a systemic dose of LPS we can study specifically endotoxemia. Thus, in the present study we focused on the activity of RvD5 in a systemic model of endotoxemia induced by LPS in mice.

4.2 MATERIALS AND METHODS

4.2.1 Animals

The present study was conducted with female Swiss mice (8-weeks old, weighing between 20-25 g). Animals were prevenient from Universidade Estadual de Londrina animal facility (Londrina, PR, Brazil). The animals were housed in a climatized room (21°±1), with free access to water and food, and light/dark cycle (12/12 h). The animals were kept into standard polypropylene cages measuring 41 x 34 x 16 cm (Insight®) with a maximum of 10 animals per cage. All procedures were performed in accordance with local, national, and international standards of ethics. Animal handling and welfare were approved by the Universidade Estadual de Londrina Ethics Committee for animal experimentation (CEUA-UEL, n.052.2021). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010).

4.2.2 Administration of LPS and RvD5

RvD5(7S,17S-dihydroxy-4Z,8E,10Z,13Z,15E,19Z- docosahexaenoic acid), Cayman Chemical, Ann Arbor, MI, USA, 0,1; 1 and 10 ng/animal) or vehicle (0.1% ethanol in saline) were administrated intraperitoneally 60 minutes after mice

received intravenously (i.v.- caudal vein) LPS (10 mg/kg, from Escherichia coli - Santa Cruz Biotechnology) or vehicle (0.9% saline solution). The LPS dose was chosen and adapted according to previous literate (Zhuo et al., 2018; Wang et al., 2020). Animals were divided into five equal sized groups consisting of 6 animals per group. Group 01: 0.1% ethanol in saline (i.p.) + saline (i.v.); Group 02: 0.1% ethanol in saline (i.p.)+ LPS 10 mg/kg (diluted in saline, i.v.); Group 03: RvD5 0.1 ng/animal (diluted saline - i.p.) + LPS 10 mg/kg (i.v.); Group 04: RvD1 1 ng/animal (i.p.) + LPS 10 mg/kg (i.v.), and Group 05: RvD1 10 ng/animal (i.p.) + LPS 10 mg/kg (i.v.). For all assays, samples were collected 6 h after stimulation.

4.2.3 Determination of Liver Injury Markers Levels in the Plasma

Blood was collected by cardiac puncture under isoflurane anesthesia (3% v/v in oxygen) followed by an increase in isoflurane concentration (5%) for animal euthanasia. Blood samples were placed in microcubes containing 50µL of porcine sodic heparin (5000 IU/mL) and centrifuged (3500 RPM x 10 minutes x 4°C) for plasma separation. ALT and AST were quantitated in plasma samples by UV kinetic methodology (Gold Analise Diagnóstica – Minas Gerais – Brazil) using the Selectra automated equipment. Results were expressed in U/L.

4.2.4 Determination of Renal Injury Markers Levels in the Plasma

Blood was collected as previously described (item 4.2.3). The determination of plasma urea levels was performed by enzymatic method (Laborlab – São Paulo – Brazil) and creatinine by kinetic method (Gold Análise Diagnóstica – Minas Gerais – Brazil) using Selectra automated equipment. Results were expressed in mg/dL.

4.2.5 Myeloperoxidase (MPO) Activity Assay

MPO activity was used as an indirect measure of neutrophils in the liver, kidney, or lung tissues. Samples were collected in 200 µl of K_2HPO_4 buffer solution (pH 6.0), containing 0.5% of HTAB and were homogenized with tissue homogenizer. After, samples were centrifuged (14,000 RPM x 4°C x 2 min) and the supernatant was separated. The supernatant of each tissue was added to 200 µl of 50 mM phosphate buffer solution (pH 6.0), containing 0.167 mg/ml of *o*-dianisidine dihydrochloride and 0.015% of hydrogen peroxide. Absorbances were recorded with a microplate spectrophotometer (ThermoScientific, MultskanGO) at 450 nm. MPO activity was expressed as myeloperoxidase activity (number of neutrophils x10^3/mg of tissue) compared to a neutrophil standard curve (Casagrande et al., 2006).

4.2.6 N-Acetylglucosaminidase (NAG) Activity Assay

NAG activity was determined as described by Hohmann et al. (2013). Briefly, the enzyme activity was measured from the supernatant obtained in the MPO assay. Samples were placed in a microplate and 80 µL of 50 mM phosphate buffer (pH 6) was added. The reaction started after the addition of 2.24 mM of 4-nitrophenyl N-acetyl-β-D-glucaminide followed by incubation at 37°C for 10 minutes. Finally, 100 µL of 0.2M glycine buffer (pH 10.6) was added to the reaction. The enzyme activity was determined spectrophotometrically at 400 nm and the results are expressed as NAG activity (macrophages x 10^3/mg tissue) compared to the macrophage standard curve.

4.2.7 Cytokines Measurement

Liver, kidneys, and lungs were collected in phosphate buffered saline for cytokines levels determination. Samples were homogenized, centrifuged (3000 rpm x 4°C x 15 minutes), and the supernatant was used to measure the levels of TNF-α, IL-1β, IL-6 and IL-10 by ELISA. The assay was conducted following manufacturer's instructions (BioLegend – San Diego, CA – USA).

4.2.8 ABTS and FRAP Assay

Liver, kidney, and lung samples were collected in 500 μL of 1.15% KCl buffer, homogenized, and centrifuged (1500 rpm / 4 °C/ 10 min). To measure the ability of each sample to resist damage from stress oxidative, the modified ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) method primarily described by Re et al. (1999) was used. ABTS reagent were added to the supernatant, allowed to incubate for 6 min at room temperature and the samples were read in a microplate spectrophotometer with at 730 nm. To evaluate the FRAP (ferric reducing ability of plasma), 150 μL of the FRAP reagent was added to the supernatant and read at 595 nm. Results were compared to a Trolox standard curve (30 mM, final concentration) and expressed as Trolox equivalent/mg tissue at both evaluations.

4.2.9 Determination of Thiobarbituric Acid Reactive Substances (TBARS) Levels

Free radicals, when in contact with lipids, destabilize these molecules and produce malondialdehyde (MDA). In turn, MDA amplifies the inflammatory process. Therefore, TBARS test was used to detect the presence of MDA in samples using thiobarbituric acid (TBA). TBA reacts with MDA forming a new compound, which can be detected spectrophotometrically. Sample were homogenized in 500 μL of 1.15% KCl, and the homogenates were incubated with 5 μl 1 mM FeCl3, 5 μl of 1 mM

ascorbic acid, 50 µl of 50% trichloroacetic acid (TCA) and 50 µl of 1% TBA, at 95° C for 60 min or until the color changes to pink. Then, the absorbance was measure at 572 nm and 535 nm, and the results are expressed as 572 nm – 575 nm OD/mg of protein (Guedes et al., 2006).

4.2.10 Nitroblue Tetrazolium (NBT) Assay

Quantification of superoxide (O_2^-) production was performed using the NBT assay as previously described adapted to be performed in microplates (Choi et al., 2006). Briefly, 50 µl of sample homogenate were used and incubated in microplates with 100 µL of solution containing the NBT reagent (1 mg/ml) for 1 hour at room temperature and protected from the direct light. After incubation, the entire contents of the microplate were removed and 120 µl of 2M KOH solution and 120 µl of pure DMSO were added. The relative production of O2- was measured spectrophotometrically at a 600 nm. Results are expressed in OD/mg of protein.

4.2.11 Determination of Reduced Glutathione (GSH) Levels

GSH levels were determined as previously described (Hohmann et al. 2013). Liver, kidney, or lungs were homogenized in 500 µl of 0.02M EDTA. Two hundred µl of homogenate was added to 25 µl of 50% trichloroacetic acid, incubated for 15 min at room temperature, and centrifuged (4000 rpm x 4° x 15 minutes). For the assay 100 µl of the sample supernatant was mixed with 200 µl of 0.4M Tris-HCl pH 8.9 and 10 µl of dithiobisnitrobenzoic acid (DTNB) 10mM. After 5 minutes at room temperature, the absorbance was determined at 412 nm. Results are presented as nmol GSH/mg of protein compared to a GSH standard curve.

4.2.12 Histopathological Analysis

Samples from each organ were collected and fixed in 10% buffered formaldehyde. Subsequently, the samples were embedded in paraffin, sectioned, and the tissue sections (5 μm) were stained with hematoxylin-eosin (HE), and examined under an optical microscope (Olympus OX31, Olympus, Japan). For histopathological evaluation, a score was adapted as previously described (Copelli et al., 2020). The score considered the presence of hydropic degeneration [0 - absent; 1 - short (1% - 25%); 2 - moderate (25% - 75%) and 3 – abundant (> 75%)]; the presence of inflammatory cell infiltrate [0 - absent; 1 - short (< 25 cells); 2 - moderate (25 - 125 cells); 3 - severe (> 125 cells)]; the presence of necrosis, considering nuclear alterations/cytoplasmic alterations/loss of tissue arrangement [0 - absent; 1 - short (1% - 25%); 2 - moderate (26% - 50%); 3 - moderate/severe (51% - 75%) and 4 – severe (> 75%)]. In addition, the characteristics of the cellular infiltrate (polymorphonuclear, mononuclear and mixed) were considered. The final score (0 – no involvement; 1-3: short involvement; 4-6 moderate involvement, 7-10: severe involvement. Results are expressed as mean value (variation) for each group of 6 animals.

4.2.13 Statistical Analysis

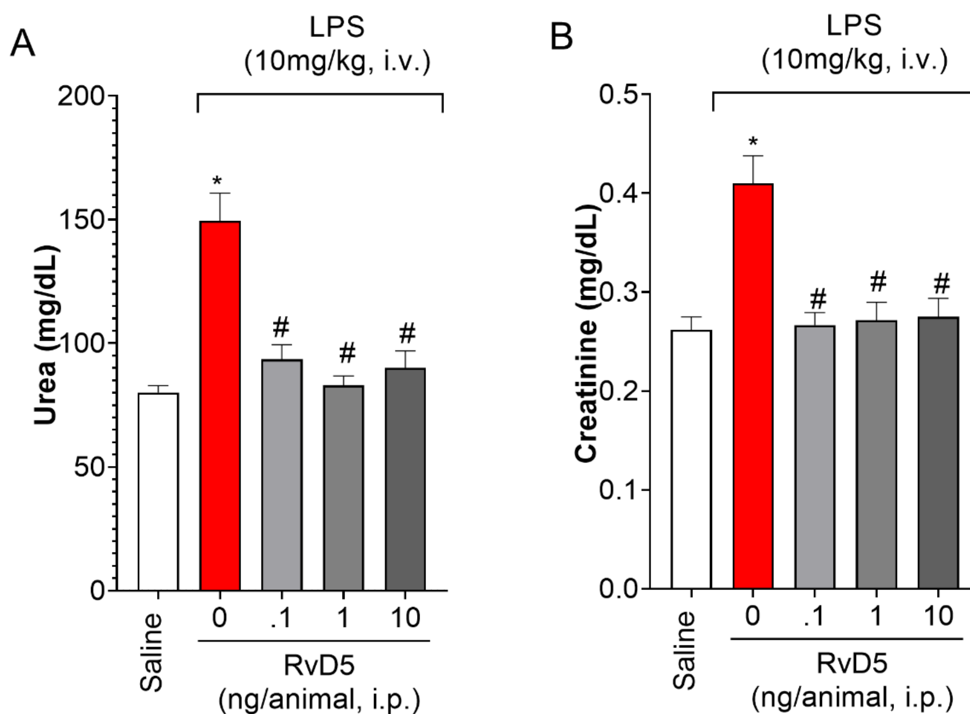
Assay results are presented as standard ± SEM (standard error of the mean) of 6 animals per group and are representative of one of 2 independent replicates. In case of normal distribution of the results, one-way ANOVA followed by Tukey's post-test was used and data was presented as mean ± SEM (standard error of the mean) of 6 animals per group. In case of non-normally distributed data, the non-parametric Kruskal-Wallis followed by Dunn´s test was used, and data presented as

median and interquartile range (IQR). Differences were significant for $p < 0.05$.

4.3 RESULTS

4.3.1 RvD5 Decreases Renal and Hepatic Injury Markers

Blood levels of liver (ALT and AST) and kidney (urea and creatinine) injury markers were measured 6 hours after LPS stimulation (7 h after RvD5 treatment) in the plasma. The systemic administration of LPS (10 mg/kg, i.v.) significantly increased serum levels of urea (Fig. 1A), creatinine (Fig. 1B), ALT (Fig. 1C) and AST (Fig. 1D). Treatment with RvD5 (0.1; 1 or 10 ng/animal, i.p.) significantly decreased plasma levels of those markers (Fig. 1 A-D). Therefore, these results suggest the protective activity of RvD5 in kidneys and liver.



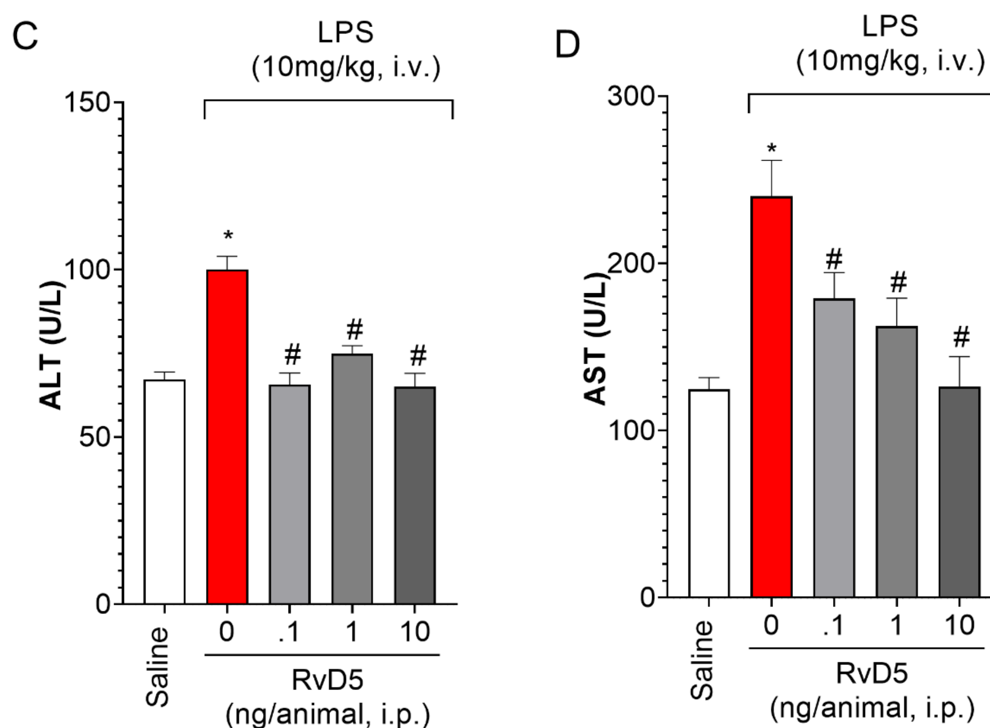


Figure 1 – RvD5 decreased renal and hepatic injury markers. Blood was harvested by cardiac punction six hours after LPS administration (10 mg/kg, i.v.). The levels of urea (A), creatinine (B), ALT (C) and AST (D) were measured. Results are presented as mean ± SEM (standard error of the mean) of 6 animals per group and are a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

4.3.2 RvD5 Decreases MPO and NAG Activities

Leukocytes activation and recruitment to the affected tissue are crucial points in the development of inflammatory process. Thus, we determined the levels of MPO and NAG activity in the liver, lungs, and kidneys. We observed a significant increase in MPO and NAG activities after systemic administration of LPS in all reported organs (Fig. 2). RvD5 decreases, in all treated doses, MPO and NAG activity in the liver and kidneys (0.1; 1 or 10 ng/animal) (Fig. 2A, B, E and F). In the lungs, RvD5 also reduced MPO (Fig. 2C) and NAG (Fig. 2D), in a dose-dependent manner. However, no significant effect was observed in the dose of 0.1ng/animal in lungs tissue.

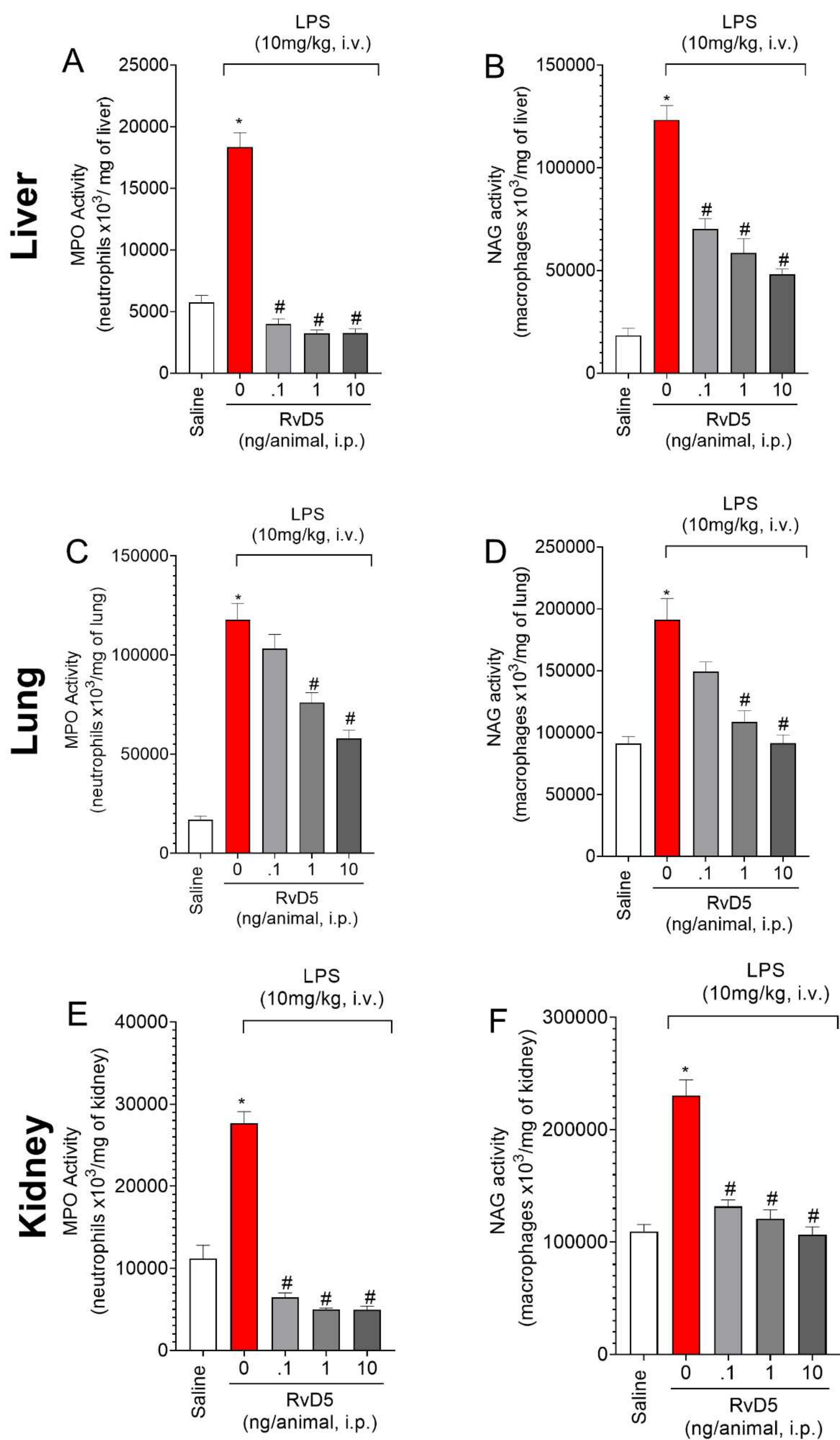

Liver
A
LPS
(10mg/kg, i.v.)
MPO Activity
(neutrophils x10³/ mg of liver)
Saline
0
.1
1
10
RvD5
(ng/animal, i.p.)
B
NAG activity
(macrophages x10³/mg of liver)
Lung
C
MPO Activity
(neutrophils x10³/mg of lung)
D
NAG activity
(macrophages x10³/mg of lung)
Kidney
E
MPO Activity
(neutrophils x10³/mg of kidney)
F
NAG activity
(macrophages x10³/mg of kidney)

Figure 2 – **RvD5 decreased MPO and NAG activity in the liver, kidneys and lungs.** Six hours after treatment with RvD5 (0,1 ng, 1 ng or 10 ng, i.p.) and LPS injection (10mg/kg, i.v.) MPO and NAG activity were determined. MPO activity in liver (A), lungs (C) and kidneys (E). NAG activity in the liver (B), lungs (D) and kidneys (F). Results are presented as mean ± SEM of 6 animals per group and are a representative of two independent replicates. (*$P < 0.05$ vs. saline, # $P < 0.05$ vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

4.3.3 RvD5 Decreases the Production of Pro-Inflammatory Cytokines

Systemic administration of LPS, induced the production of IL-1β, TNF-α and IL-6 in all analyzed organs (liver, lungs and kidneys) (Fig.3). All doses of RvD5 decreased the levels of IL-1β (Fig. 3A), TNF-α (Fig. 3B) and IL-6 (Fig. 3C) in the liver. In the lungs, RvD5 decreased IL-1β levels (Fig 3D) at all administered doses, with emphasis on the dose of 10ng/animal that reached undetectable levels. RvD5 also decreased TNF-a to undetectable levels in the lungs (Fig. 3E), as well as reduced IL-6 levels in a dose-dependent manner (Fig. 3F). Finally, in the kidneys, the levels of all three were decrease with RvD5 treatment at the doses of 1 and 10 ng/animal (i.p.). Furthermore, no changes were observed in IL-10 levels with RvD5 administration. Thus, suggesting that RvD5 does not affect the production of this anti-inflammatory cytokine (Fig. 4A - C).

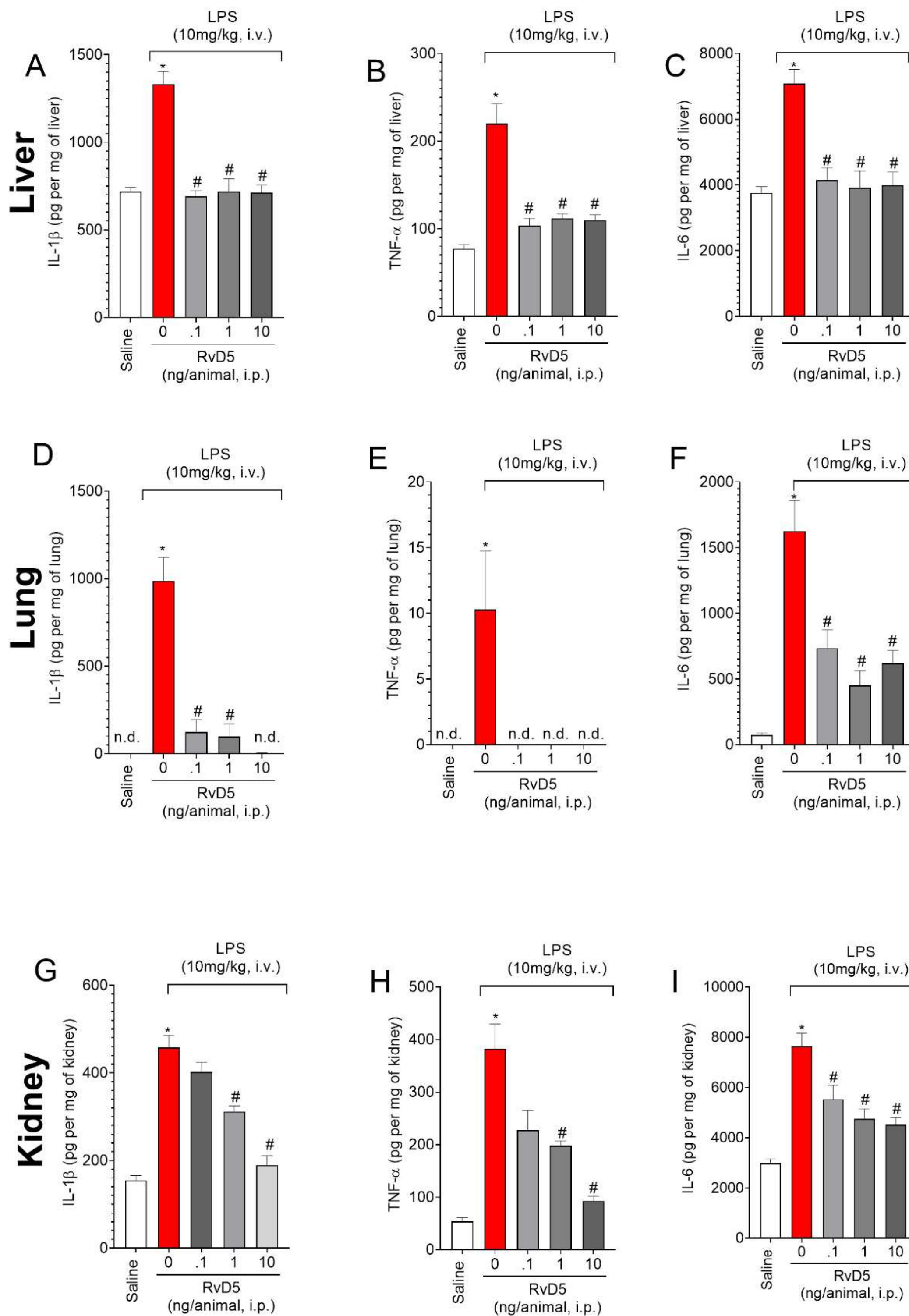


Figure 3 – **RvD5 reduced LPS-induced IL1-β, TNF-α and IL-6 levels production in the liver, lungs and kidneys.** Tissue were collected, homogenized and the supernatant was used to measure IL1-β in liver (A), lungs (D) and kidneys (G); TNF-α in all three organs (B, E and H), and IL-6 (C, F and I)). Results are presented as mean ± SEM of 6 animals per group, and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

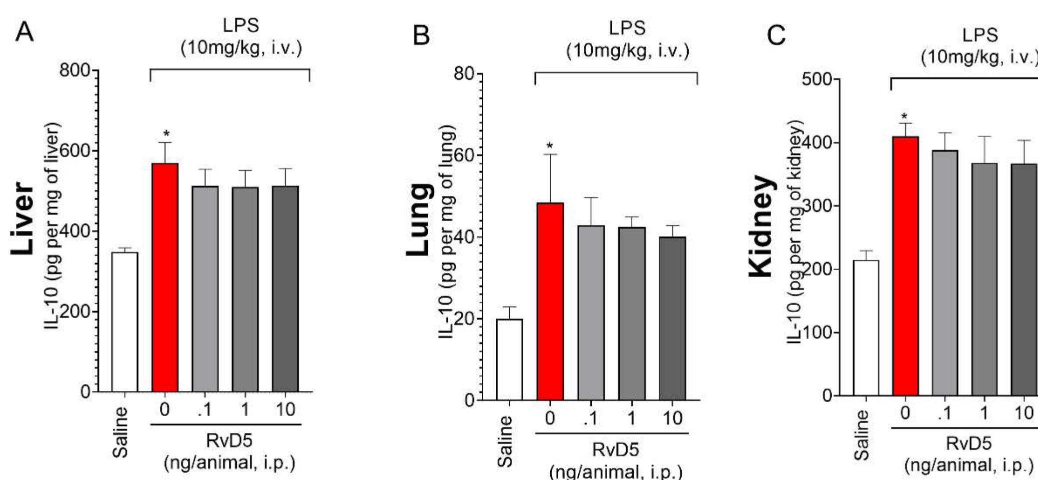
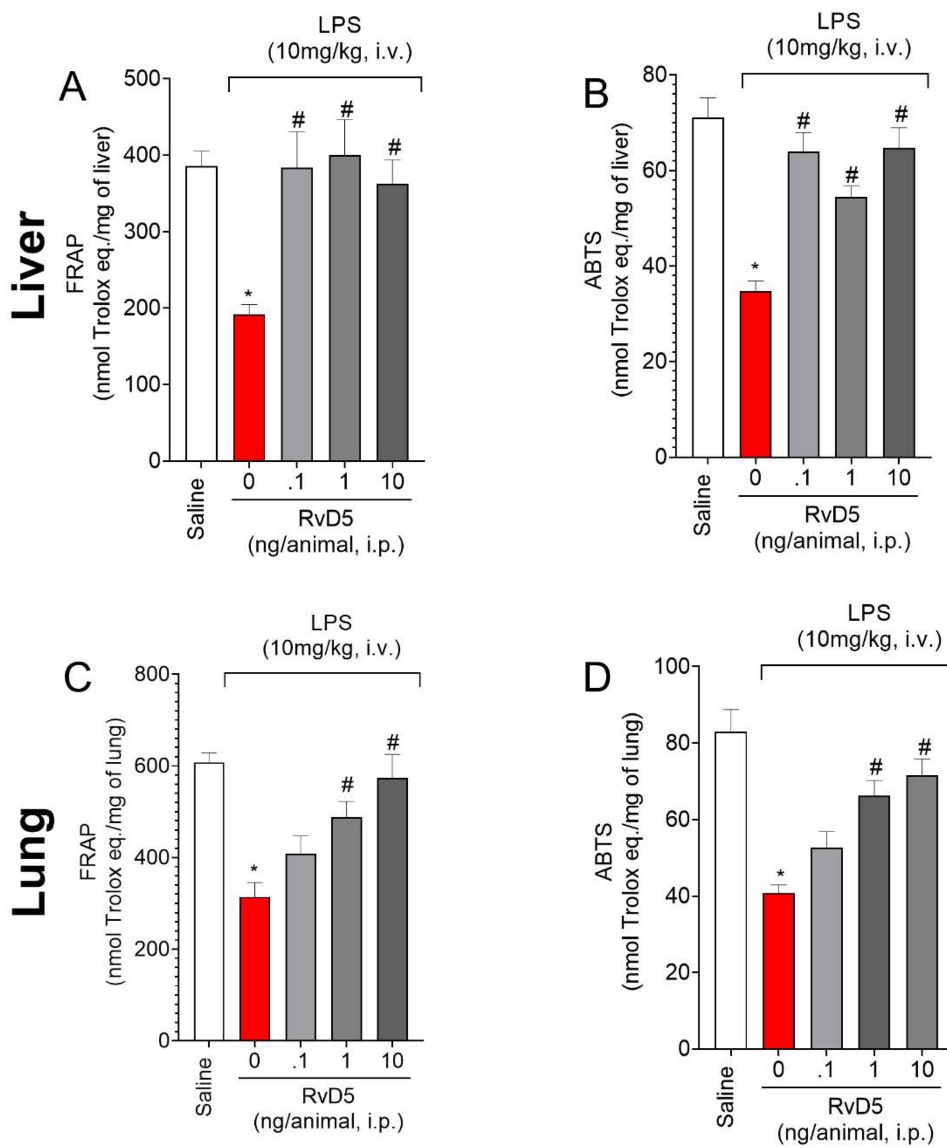


Figure 4 – RvD5 did not change IL-10 levels in the liver, lungs and kidneys. The anti-inflammatory cytokine IL-10 was measured in liver (A), lungs (B) and kidneys (C). Results are presented as mean ± SEM (mean error) of 6 animals per group, and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

4.3.4 RvD5 Reestablishes Antioxidant Capacity

The ABTS method (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and the FRAP method (Ferric Reducing Antioxidant Power) are indirect methods to evaluate the total antioxidant capacity of a sample. Systemic administration of LPS decreased antioxidant capacity as per FRAP and ABTS methods (Fig. 5), and decreased GSH levels (Fig. 6) in all analyzed organs. In the liver, all doses of RvD5 reestablished antioxidant capacity (Fig. 5A-B) and GSH levels at 1ng and 10 ng/aniaml (Fig. 6A). On the other hand, in the lungs, the lower dose of 0.1 ng was not effective in restore tissue antioxidant capacity (Fig. 5C-D). Moreover, no effect was observed in any of the tested doses for GSH restoration to basal levels (Fig. 6B). In the kidneys, RvD5 reestablished the total antioxidant capacity at all tested doses of RvD5 (Fig.5E-F) and restored GSH levels at 1ng and 10ng (Fig. 6C).



Liver
Lung
A
B
C
D
LPS
(10mg/kg, i.v.)
FRAP
(nmol Trolox eq./mg of liver)
ABTS
(nmol Trolox eq./mg of liver)
FRAP
(nmol Trolox eq./mg of lung)
ABTS
(nmol Trolox eq./mg of lung)
Saline
0
.1
1
10
RvD5
(ng/animal, i.p.)

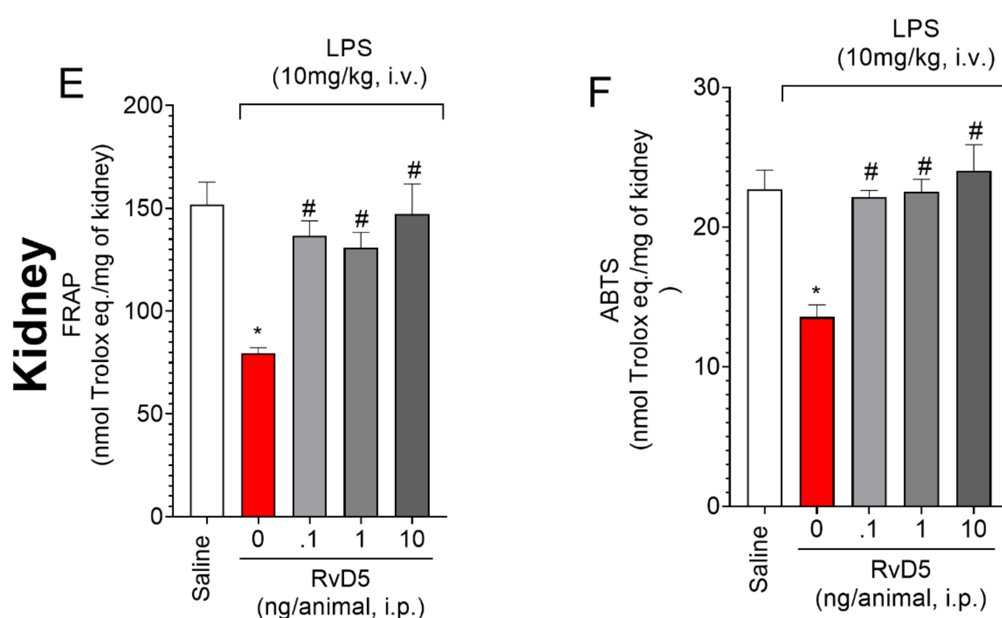


Figure 5 – RvD5 reestablishes tissue antioxidant capacity. Tissue was collected six hours after treatment (RvD5 0.1, 1 or 10 ng/animal, i.p.) and LPS administration and were used to measure antioxidant capacity by FRAP method in liver (A), lungs (C) and kidneys (E) and ABTS method on liver (B), lungs (D) and kidneys (F). Results are presented as mean ± SEM of 6 animals per group and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

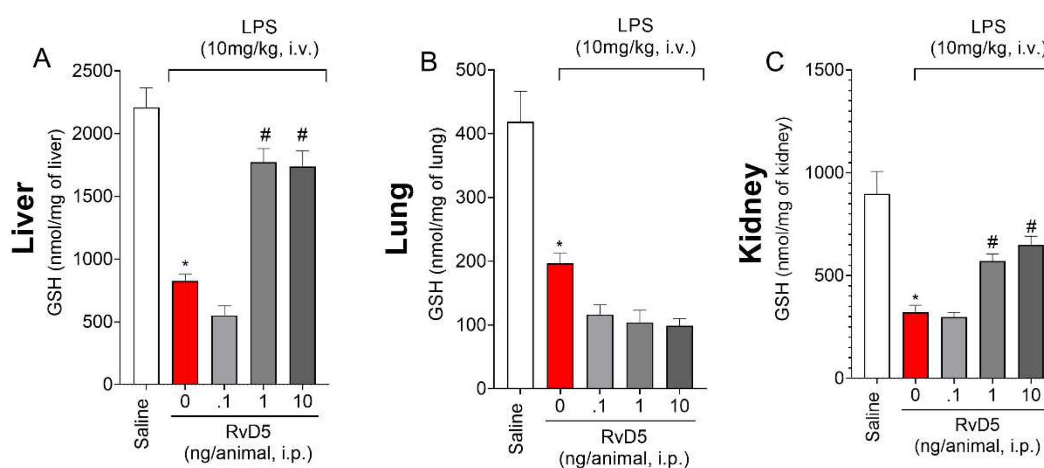
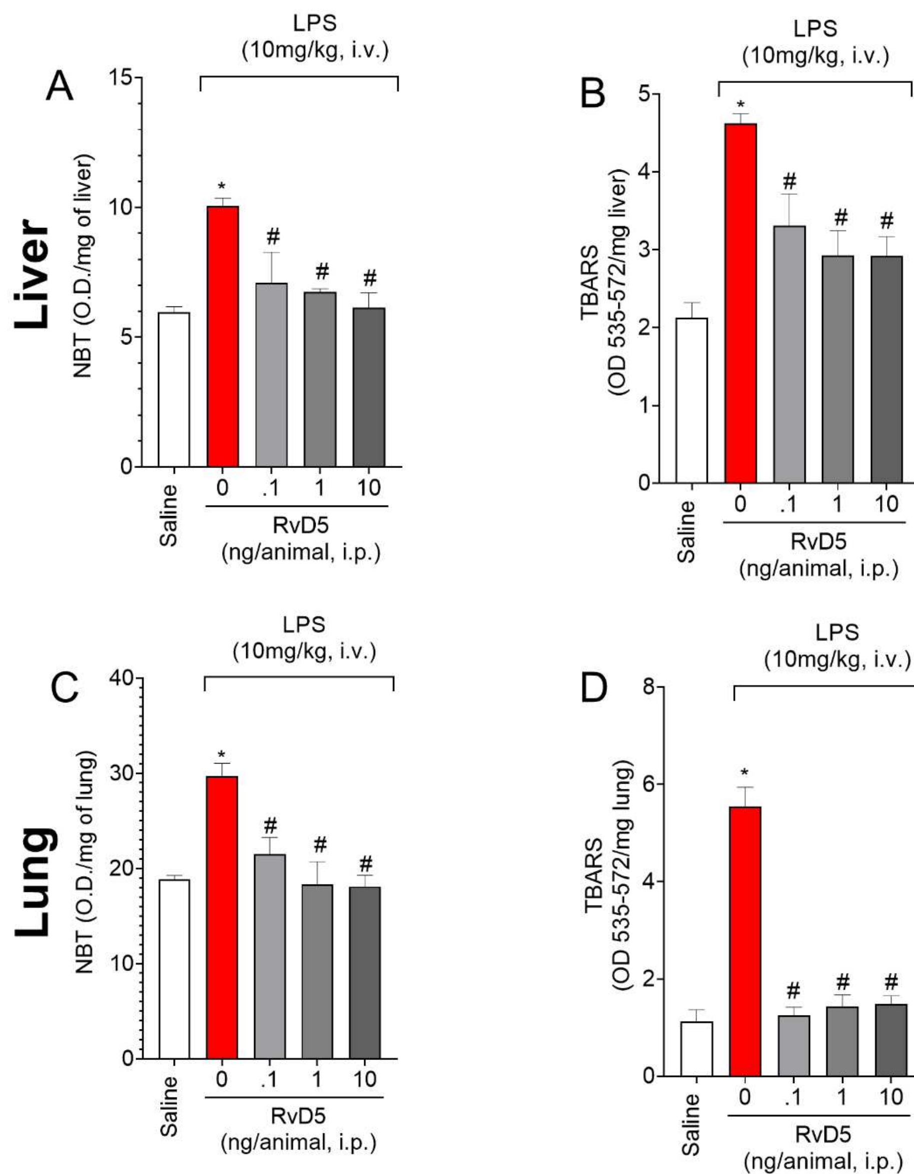


Figure 6 – RvD5 enhanced GSH levels in liver and kidneys without effect in the lungs. Tissue was collected six hours after treatment (RvD5 0.1, 1 or 10 ng/animal, i.p.) and LPS administration and were used to measure GSH levels on liver (A), lungs (B) and kidneys (C). Results are presented as mean ± SEM of 6 animals per group and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

4.3.5 RvD5 Decreases Oxidative Stress

After LPS administration, lipid peroxidation levels (TBARS) and superoxide anion (NBT) levels increased in the liver, lungs, and kidneys (Fig. 7). RvD5 at all doses decreased NBT and TBARS levels in the liver (Fig. 7 A-B), lungs (Fig. 7 C-D) and kidneys (Fig. 7 D-E).



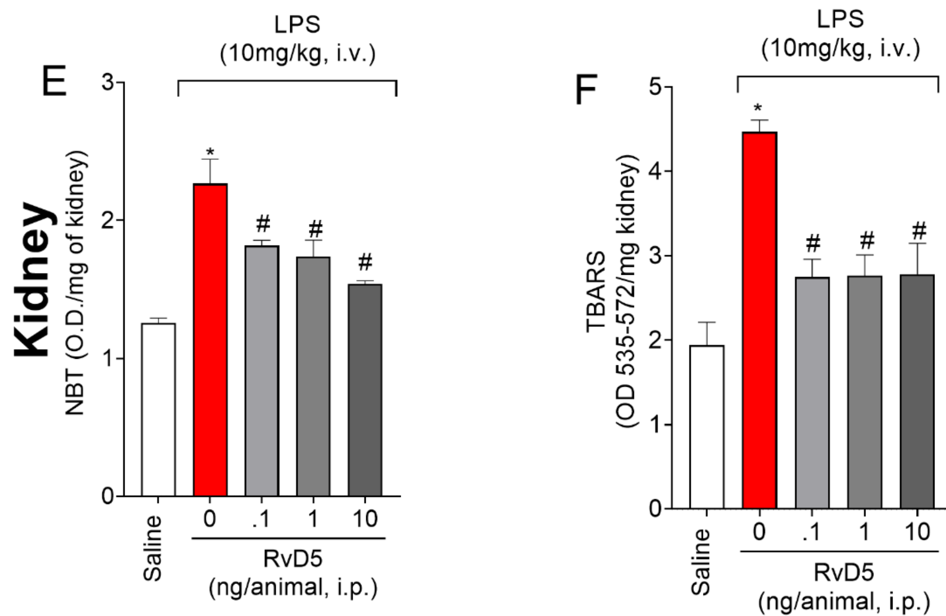
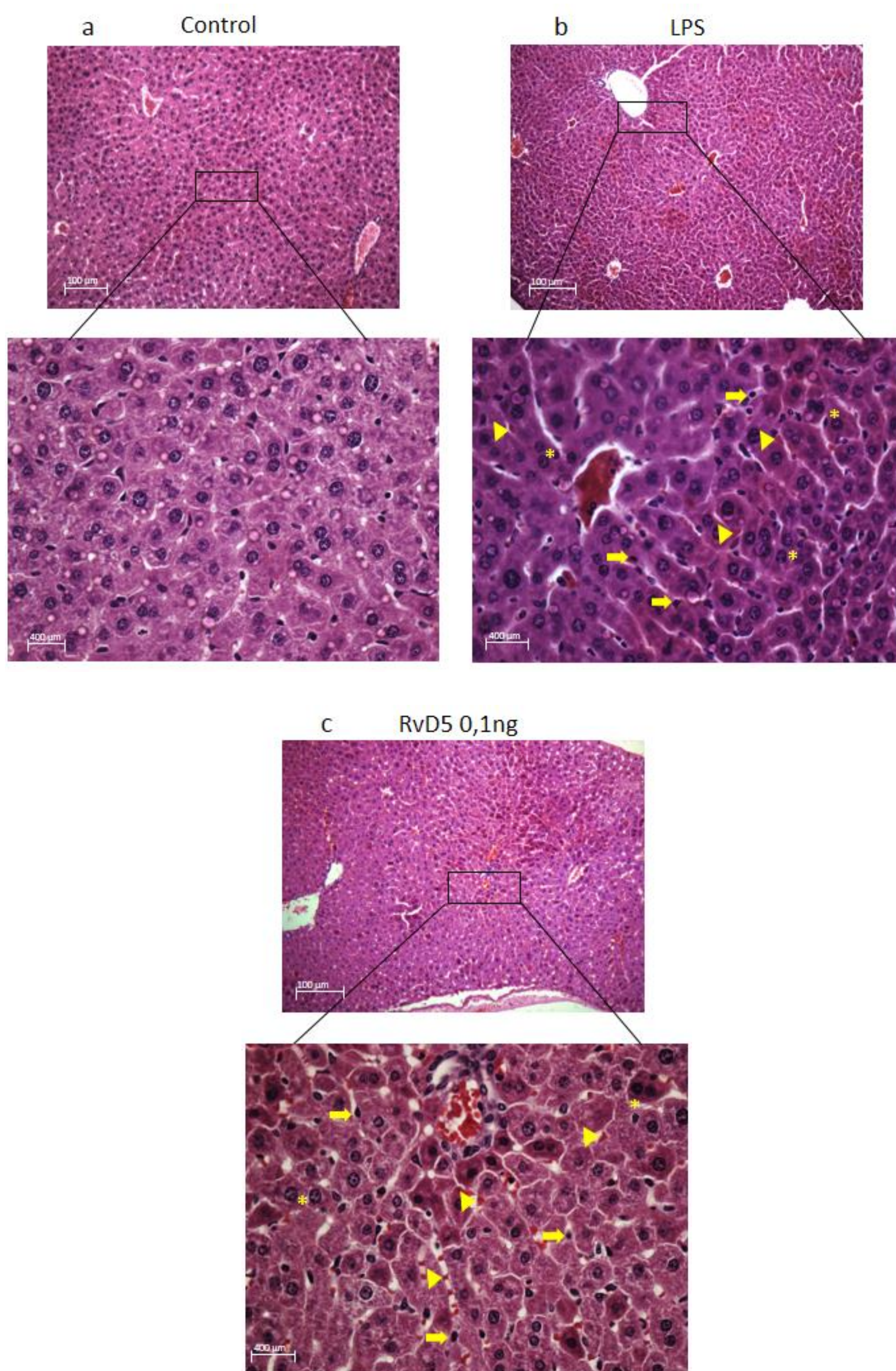
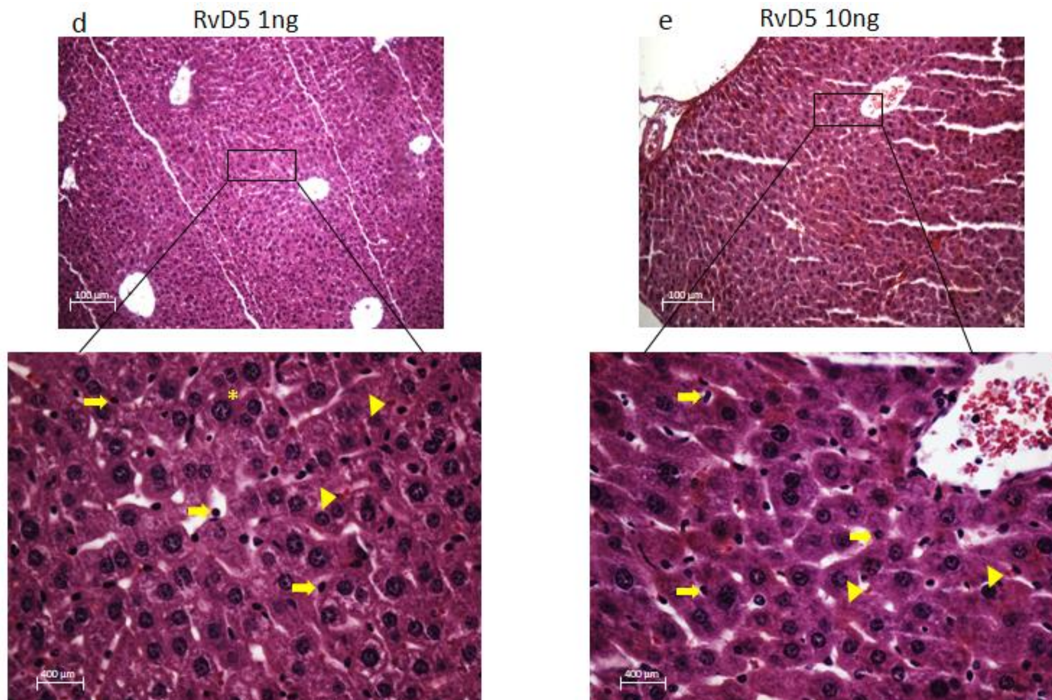


Figure 7 – RvD5 reduces lipid peroxidation and superoxide anion levels. LPS-induced lipid peroxidation and superoxide anion levels in all examined issues. RvD5 decrease NBT levels in the liver (A), lungs (C) and kidneys (E) and reduces TBARS levels in the liver (B), lungs (D) and kidneys (F). Results are presented as standard ± SEM (mean error) of 6 animals per group and is a representative of two independent replicates. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; one-way ANOVA followed by Tukey's post-test).

4.3.6 RvD5 Decreases Histopathological Score, Leukocyte Infiltration, and Necrosis in the Liver, Lungs, and Kidneys

Liver, lungs, and kidneys samples were processed and stained with H&E. In the liver after administration of systemic LPS (Fig. 8B) a moderate increased in the inflammatory infiltrate (25-125 cells) with a predominance of polymorphonuclear cells and necrotic findings of mild intensity were observed. Besides that, short presence of hydropic degeneration and parenchymal hemorrhagic foci and congestion of the portal space also were observed. At the dose of 0.1 ng (Fig. 8C) a maintenance of necrotic involvement and absence of congestion in the portal space were observed. At higher doses, RvD5 protected the liver with a significant decrease in several histopathological parameters (Fig. 8D-F).


a
Control
b
LPS
c
RvD5 0,1ng
100 µm
400 µm



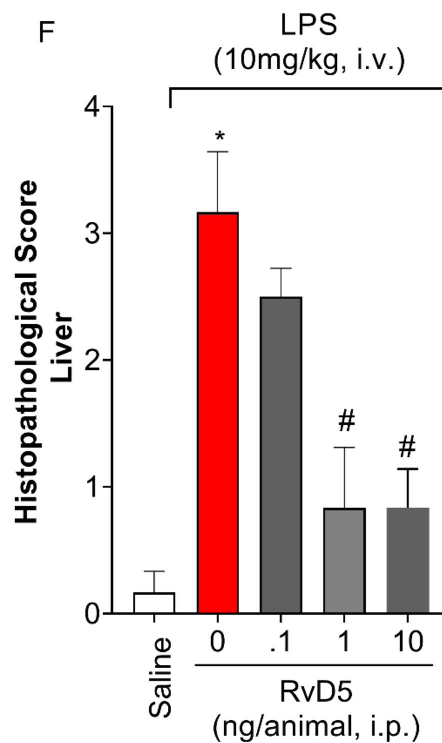
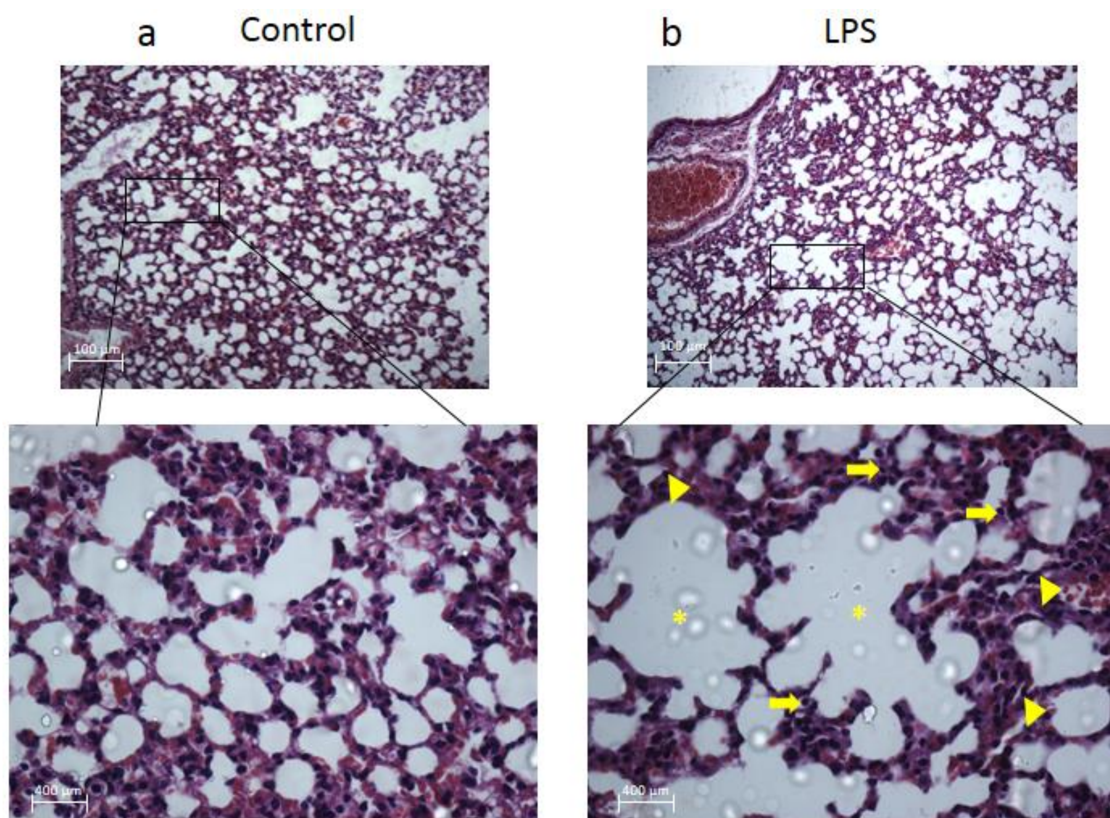
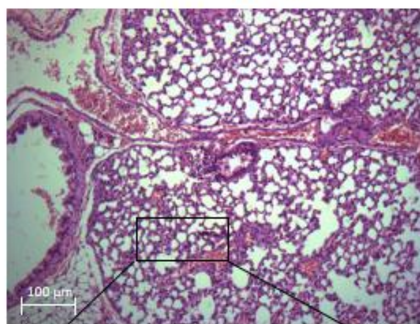


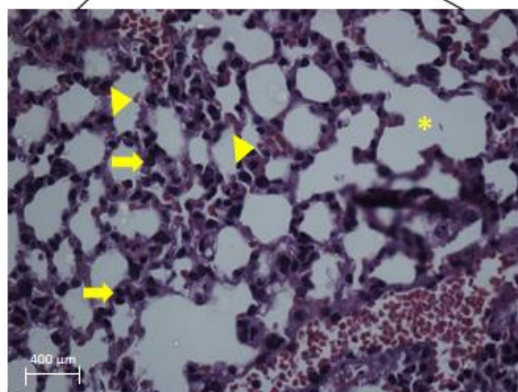
Figure 8 – RvD5 preserves liver architecture in LPS endotoxemia. Representative photomicrographs of hepatic histology (hematoxylin and eosin, magnification 400×), from control (A), LPS + vehicle (B) (10mg/kg – iv), RvD5 at 0,1ng (C), 1 ng (D) and 10 ng (E). Arrow: inflammatory infiltrate; Arrowhead: red blood cells; *necrotic changes; #bleeding focus. Histopathological score (F). *P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; Kruskal-Wallis followed by Dunn´s test.

At lung, a similar effect was observed, with the presence of moderate mixed inflammatory cells, an increased alveolar space and the presence of few red blood cells after LPS administration (Fig. 9B). While all doses of RvD5 (Fig. 9 C-E) decreased the presence of inflammatory foci and reduces alveolar changes.

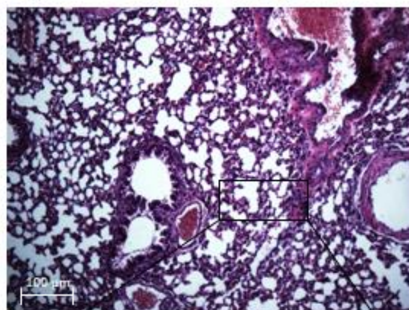


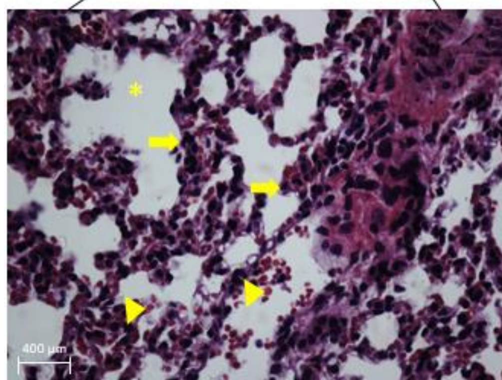
c RvD5 0,1ng



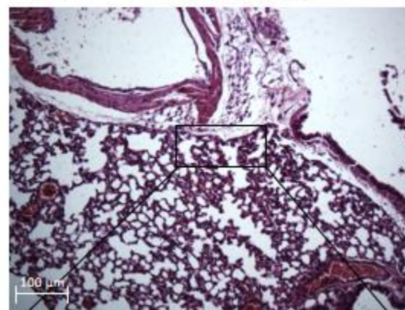


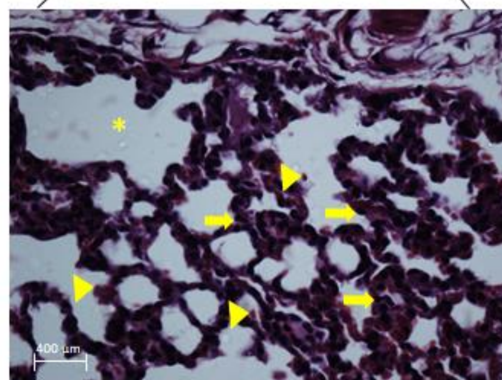
d RvD5 1ng





e RvD5 10ng





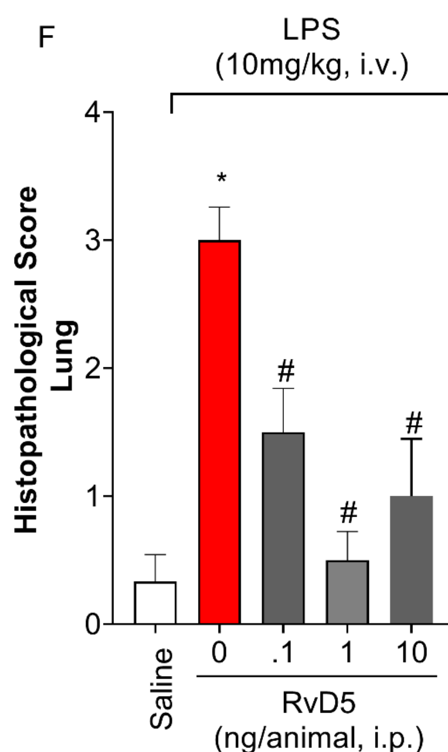
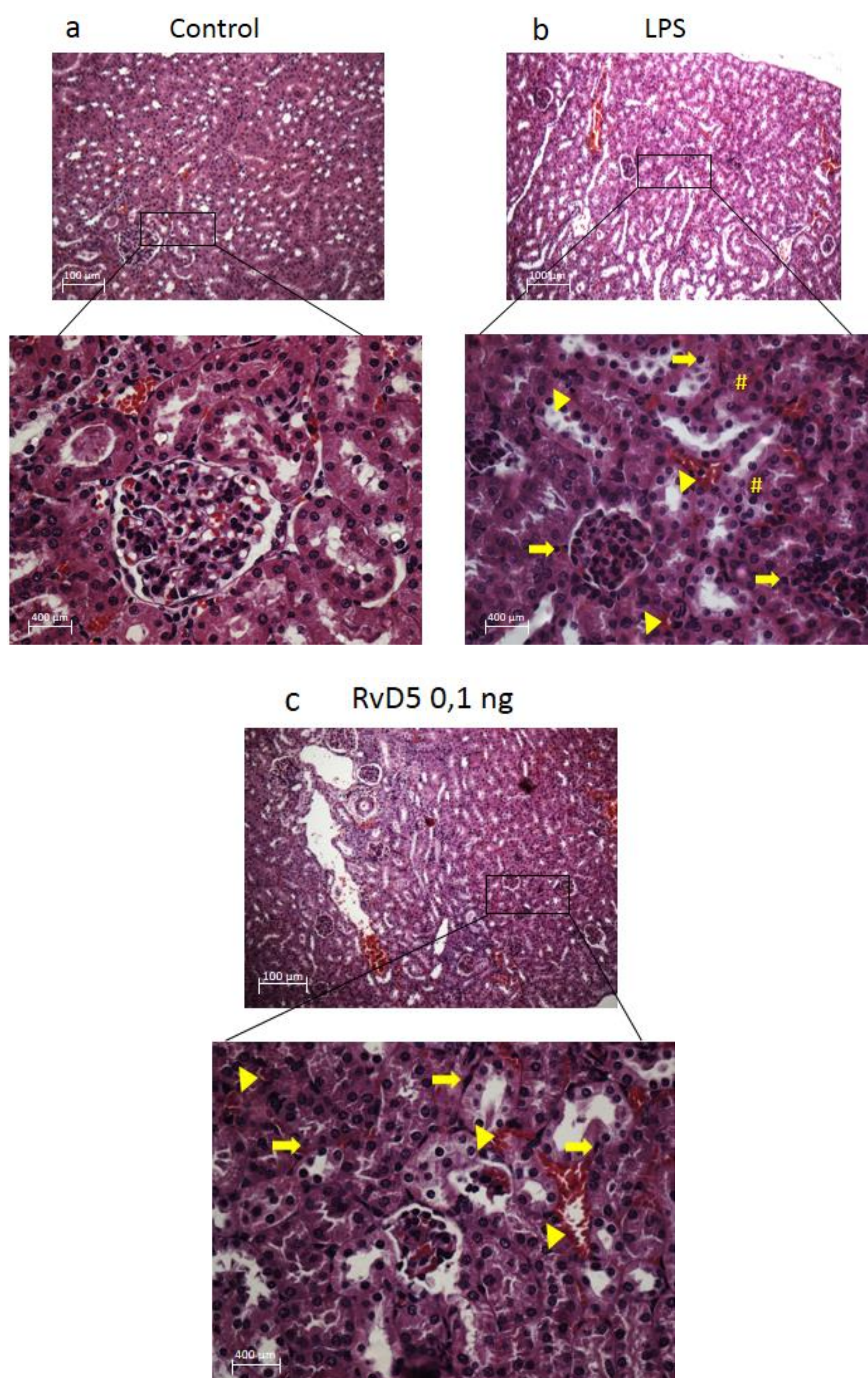


Figure 9 – RvD5 preserves lung architecture during LPS endotoxemia. Representative photomicrographs of pulmonary histology (hematoxylin and eosin, magnification 400×). Control (A), LPS + vehicle (B), MaR2 (i.p.) at 0,1 ng (C), 1 ng (D) and 10 ng (E). Arrow: inflammatory infiltrate; Arrowhead: red blood cells; *increased alveolar space; #bleeding focus. (E) Histopathological score. *P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; Kruskal-Wallis followed by Dunn´s test.

After LPS administration (Fig. 10B), the same pattern was maintained in kidneys, with moderate presence of hydropic degeneration, mild mixed inflammatory cells (<25 cells), mild necrotic involvement (up to 25%) and presence of blood cells in the parenchyma. The lowest dose of RvD5 (Fig. 10C) was not different from the group stimulated with LPS and treated with vehicle. On the other hand, treatment with higher doses of RvD5 (Fig. 10D-E) decreased the number of inflammatory cells, with absence of necrotic signs and maintenance of hydropic degeneration.


a
Control
100 µm
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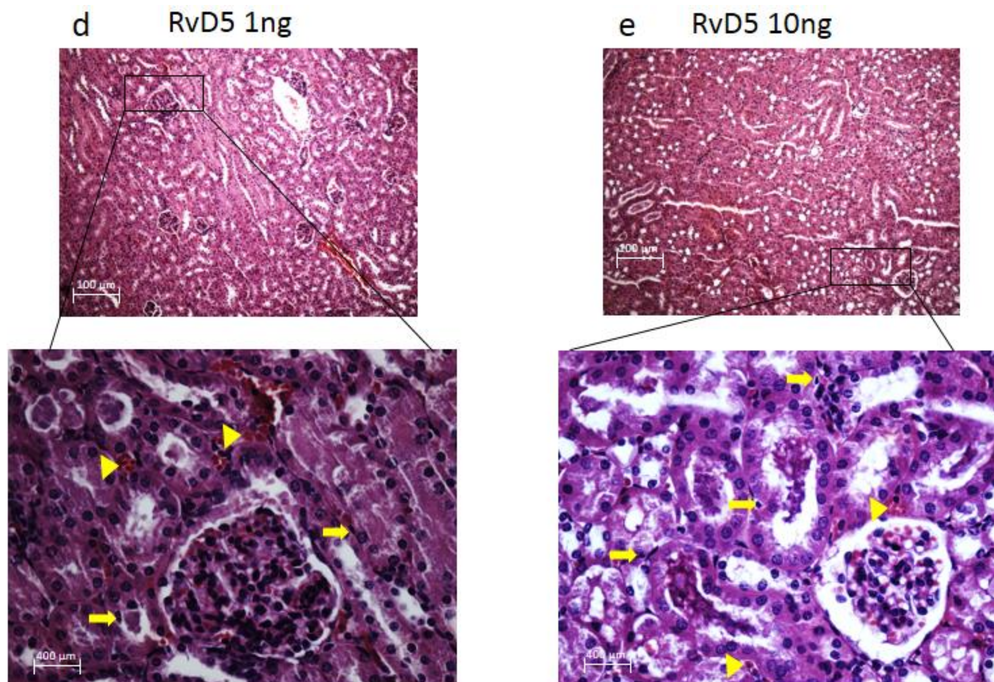
b
LPS
100 µm
400 µm
#
#
c
RvD5 0,1 ng
100 µm
400 µm



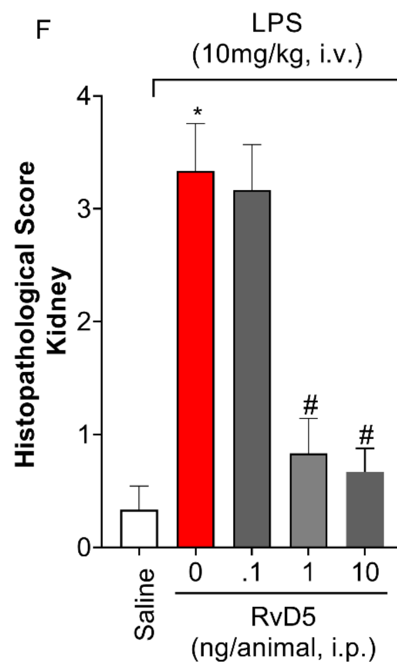


Figure 10 – RvD5 preserved kidney architecture after LPS stimulus. Representative photomicrographs of renal histology (hematoxylin and eosin, magnification 400×). Control (A), LPS + vehicle (B) (10mg/kg, i.v.), RvD5 treated at 0,1 ng (C), 1 ng (D) and 10 ng (E). Arrow: inflammatory infiltrate; Arrowhead: red blood cells; *necrotic changes; #bleeding focus. (E) Histopathological score. (*P < 0.05 vs. saline, # P < 0.05 vs. LPS 10mg/kg; Kruskal-Wallis followed by Dunn´s test).

4.4 Discussion

In the present study, we analyzed the activity of RvD5 in an LPS-induced

endotoxemia model in mice. We demonstrated that the administration of LPS potentially increased tissue injuries in the liver, lungs, and kidneys, as it increases histopathological changes. Systemic administration also induced an increase in plasma levels of kidney (urea and creatinine) and liver (ALT and AST) markers, corroborating the observed histopathological findings. In addition, LPS increased the production of pro-inflammatory cytokines, leucocytes recruitment (MPO and NAG activities), oxidative damage, depleted the levels of antioxidant enzymes, and total antioxidant capacity of the analyzed samples. On the other hand, treatments with RvD5 protected the liver, lungs and kidneys from all observed LPS-induced deleterious effects. RvD5 decreased inflammation, oxidative stress, and protected the tissue from damage.

Endotoxemic models are used to simulate an acute inflammatory response with potential evolution to sepsis (Dickson; Lehmann, 2019). In an pre-clinical scenario, LPS is the most used molecule to establish the endotoxemic inflammatory process (Hurley et al., 2015; Dickson; Lehmann, 2019).. LPS administration, either intravenously or intraperitoneally, induces systemic inflammation, resulting in immune cell activation, release of inflammatory mediators, with consequent vascular/hemodynamic changes, generation of reactive oxygen and nitrogen species, which may lead to organ and tissue dysfunction, followed by potential multiple organs failures (Copeland et al., 2005; Rybicki et al., 2018; Dickson; Lehmann, 2019).

Endotoxemia at robust levels (endotoxemic shock) can evoke organ and tissue failure, such as the liver after LPS-induced derangement of hepatic sinusoids with a significant increase in leukocytes recruitment to the tissue, enhanced levels of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), expression of Bax and

phosphorylation of NF-κB, with high levels of reactive oxygen species in tissue and blood samples (Li et al., 2021). In addition, Xiong et al. (2017) demonstrated that LPS induces a significant increase in serum levels of ALT, AST and bilirubin, suggesting the occurrence of liver damage after immune activation via LPS (Kim, et al., 2021).

In the present study the proposed model mimicked all clinicals signs of endotoxemia, allowing the evaluation of RvD5 as a therapeutics candidate. We observed that RvD5 protected the liver, preventing the unbalance in levels of pro-inflammatory cytokines, decreasing the activity of NAG and MPO, reducing levels of oxidative, and maintaining tissue total antioxidant capacity. Therefore, RvD5 decreased liver damage, demonstrated by the decrease in LPS-induced ALT and AST plasma levels and histopathological changes.

Acute lung injury is one of the first changes in the development of inflammatory endotoxemic, mainly with loss of the alveoli capillary barrier and the promotion of lung edema, as consequence (Matthay et al., 2012). In a pulmonary endotoxemic model, induced by LPS in mice, an increased in MPO activity was observed within an upregulated expression of adhesion molecules (Li et al., 2016; Phander et al., 2021). In addition, the use of LPS increases alveolar spaces and promotes the thickening of alveolar septa, facilitating the presence of pulmonary edema and hemorrhage, with increase of expression of pro-inflammatory cytokines associated with the generation of reactive oxygen species and the depletion of endogenous antioxidant molecules (Zhang et al., 2014; Li et al., 2016; Xie et al., 2017; Phander et al., 2021). Herein, we demonstrated that RvD5 exert pulmonary protection by reducing the levels of inflammatory cytokines, MPO and NAG activity, lipid peroxidation, and the production of superoxide anion. Moreover, the treatment maintained total antioxidant levels, except for GSH. All positive findings reported

reduced lung injury, especially at 1 and 10 ng/animal.

It is known that kidneys can also be affected in endotoxemic processes, which could escalate to acute renal failure. In this case, renal falure is triggered mainly by renal vascular reduction resulted from uncontrolled production of nitric oxide with an exponentially decreasing of glomerular filtration rate (Boffa et al., 2004; Bansal et al., 2009). In endotoxemic renal models, swelling and tubular degeneration have been demonstrated, with an increased expression of pro-inflammatory cytokines, inflammatory cell influx, and high serum levels of urea, creatinine, and free radicals after stimulation with LPS (Hang et al., 2017; Wang et al., 2020). In addition, LPS increased the expression of HMGB1, iNOS, Bcl-2, BAX, caspase-3, and phosphorylated forms of NF-κB (p65) and MAPK (p38) (Ren et al., 2020).

In the present study, we demonstrated that RvD5 also performs a renal protective activity observed with the improvement of serum urea and creatinine levels. Improvement of histological parameters, decreased expression of pro-inflammatory cytokines, reduced levels of lipid peroxidation and superoxide anion production. In addition, RvD5 decreased NAG and MPO activity, restored total antioxidant, and reestablished GSH levels.

RvD5 treatment in vitro in THP-1 cells reduced LPS-induced production of IL-6 and CCL5 through the modulation of MAPK and NF-kB, suggesting the anti-inflammatory pathway activity of RvD5 (Chun et al., 2020). This anti-inflammatory activity was also demonstrated in our study through decreased in NAG and MPO activities and decrease of pro-inflammatory cytokines levels.

Therefore, we demonstrated that RvD5 has anti-inflammatory and antioxidant activity, which allowed significant protection of the liver, lungs, and kidneys. Our data suggest that RvD5 would be a very promising therapeutic candidate for

endotoxemia/endotoxemic shock.

4.4.1 Acknowledments

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5 CONCLUSÃO

Demonstrou-se que a MaR2 e a RvD5 possuem atividades protetivas, anti-inflamatórias e antioxidantes em modelo de endotoxemia, visualizada através da redução dos níveis de marcadores séricos de lesão renal e hepática, pelo controle inflamatório visível pela diminuição do recrutamento de células inflamatórias e pela menor expressão de citocinas pró-inflamatórias. Além disso, ambas moléculas diminuíram os níveis de estresse oxidativo, mantiveram a capacidade antioxidante total das amostras e apresentaram diminuição das alterações histológicas nos órgãos avaliados. Sendo assim, nossos dados sugerem que a MaR2 e a RvD5 podem ser uma abordagem terapêutica alternativa para o tratamento do endotoxemia e choque endotoxêmico.

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