Universidade Estadual de Londrina

TALITA LAIANE CARDOZO CEZAR

AVALIAÇÃO DO EFEITO DA 7(S) MARESINA 1 NA INFLAMAÇÃO E ESTRESSE OXIDATIVO INDUZIDOS PELA RADIAÇÃO UVB

Londrina
2017

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Dissertação apresentada ao Programa de Pós Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Londrina.

Orientador: Prof. Dra. Rúbia Casagrande.

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Londrina, 23 de fevereiro de 2017.

Dedico este trabalho:

A Deus, por estar presente em minha vida e proporcionar a realização de mais uma conquista.

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RESUMO

A pele é o maior órgão do corpo humano em extensão e a primeira barreira do organismo a fatores do meio externo. Entre estes, destaca-se à exposição à radiação UVB que pode causar aumento dos radicais livres, inflamação cutânea, a qual pode levar ao desenvolvimento de câncer e envelhecimento precoce. Neste contexto, os mediadores lipídicos encontrados endogenamente, são de grande importância por apresentarem ação anti-inflamatória/pró-resolução. Dentre os mediadores lipídicos, destaca-se a 7(S) Maresina 1, a qual tem demonstrado eliminar os radicais livres e inibir a inflamação. Assim, esta pesquisa teve como objetivo avaliar a eficácia terapêutica da MaR1 nos danos cutâneos induzidos pela radiação UVB em camundongos. Os resultados *in vivo* evidenciaram que MaR1 reduz a inflamação cutânea, diminuindo o edema de pele, recrutamento de neutrófilos, produção de citocinas inflamatórias, atividade de metaloproteinase-9, apoptose dos queratinócitos e espessamento da epiderme comparada com o grupo controle. A mesma comparação foi realizada para se determinar o efeito da MaR1 contra o estresse oxidativo induzido pela radiação UVB e foi demonstrado que este lipídio manteve os níveis de glutationa reduzida (GSH) e atividade da catalase a níveis basais, e ainda diminuíram a produção de hidroperóxidos lípidicos e de ânions superóxidos. Além disso, estes efeitos da MaR1 resultaram em melhora da capacidade antioxidante da pele pela manutenção do poder redutor do ferro e da capacidade em reduzir o radical ABTS. Dessa forma, os resultados sugerem que MaR1 é um potente aliado na prevenção/resolução da inflamação da pele e inibição do estresse oxidativo induzido pela radiação UVB.

Palavras-chaves: 7(S) Maresina 1. Antioxidante. Inflamação. Radiação UVB.

CEZAR, Talita Laiane Cardozo. **Evaluation of the effect of 7 (S) Maresine 1 on the inflammation and oxidative stress induced by UVB radiation.** 2016. 92 p. Dissertation (Master degree in Health Sciences) – Universidade Estadual de Londrina, Londrina, 2017.

ABSTRACT

The skin is the largest organ of the human body and it is the first barrier of the body with the external environment. Among the external factors, stands out the exposure to UVB radiation can cause an increase of free radicals, skin inflammation, which can bring cancer and premature aging. In this context, lipid mediators found endogenously, are great importance because they present anti-inflammatory / pro-resolution action. Among the lipid mediators, there is 7(S) Maresin 1, which has been shown to eliminate free radicals and inhibit inflammation. Thus, this study aimed at evaluate the therapeutic efficacy of MaR1 in skin damage induced by UVB radiation in hairless. The *in vivo* results showed that MaR1 have reduced skin inflammation, decreasing skin edema, neutrophil recruitment, production of inflammatory cytokines, matrix metalloproteinase-9, apoptosis keratinocytes and o epidermal thickness, activity compared with control group. The same comparison was performed to determine the effect of MaR1 against oxidative stress by UVB-irradiation-induced and it was shown that lipid maintained the levels of reduced glutathione (GSH) and catalase activity to basal levels, and even decreased production of lipid hydroperoxides and production of superoxide anions. Furthermore, these effects of MaR1 resulted in improved antioxidant capacity in the skin by maintaining the ferric reducing ability and ABTS radical reducing capacity. Thereby, the results suggest that MaR1 is a potent ally in the prevention/resolution of skin inflammation and inhibition of oxidative stress induced by UVB radiation.

Keywords: 7(S) Maresin 1. Antioxidant. Inflammation. UVB radiation.

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LISTA DE ABREVIATURAS

ABTS	2,2’ azinobis (3-etilbenzotiazolina-6-ácido sulfônico)
ANOVA	Análise de variância
AP-1	Ativador de proteína-1
APS	Persulfato de amônio
$CaCl_2$	Cloreto de calico
CAT	Catalase
COX-2	Ciclooxigenase-2
DNA	Ácido desoxirribonucleico
DPPH	2,2-difenil-1-picrilidrazil
DTNB	Ácido 5,5’-ditio-bis-(2-nitrobenzóico)
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Ensaio imunoenzimático
EPM	Erro padrão da media
EROs	Espécies reativas de oxigênio
Fe^{2+}	Ferro
$FeCl_3.6H_2O$	Cloreto férrico hexahidratado
FRAP	Poder antioxidante de redução férrica
Gapdh	Gliceraldeído-3-fosfato desidrogenase
GPx	Glutationa peroxidase
GSH	Glutationa reduzida
HCl	Ácido clorídrico
HO•	Radical hidroxil
HO-1	Hemeoxigenase-1
H&E	Hematoxilina e eosina
HTAB	Brometo de hexadecil trietil amônio
H_2O	Água
H_2O_2	Peróxido de hidrogênio
IL	Interleucina
KCl	Cloreto de potássio
K_2HPO_4	Fosfato de potássio dibásico
KH_2PO_4	Fosfato de potássio monobásico
KOH	Hidróxido de potássio

LOOH Hidroperóxidos lipídicos
LPO Peroxidação lipídica
MaR1 7(S) Maresina 1
MMPs Metaloproteinases da matriz
MPO Mieloperoxidase
NaCl Cloreto de sódio
NADPH Nicotinamida adenina dinucleotídeo fosfato
NBT Azul de nitrotetrazólico
NF-κB Fator nuclear-Kb
Nrf2 Fator nuclear eritróide 3 relacionado ao fator 2
O_2 Oxigênio molecular
$O_2^{\bullet -}$ Ânion superóxido
OH Radical hidroxil
PBS Tampão fosfato salino
RNAm Ácido ribonucléico mensageiro
SDS Duodecil sulfato de sódio
SDS-PAGE Eletroforese em gel de poliacrilamida com duodecil sulfato de sódio
SOD Superóxido dismutase
TCA Ácido tricloro acético
TNF-α Fator de necrose tumoral
TPTZ 2,4,6 tripiridil-S-triazina
Tris Hidroximetil aminometano
UV Ultravioleta
UVB Ultraviolet B

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1. INTRODUÇÃO

1.1 PELE E A RADIAÇÃO ULTRAVIOLETA (UV)

A pele do ser humano, que corresponde a 15% de seu peso corporal, é um órgão que reveste e delimita o organismo, sendo uma interface biológica com o meio ambiente continuamente exposta a fatores que ameaçam a integridade de estruturas celulares. Essencialmente dinâmica, a pele apresenta alterações constantes, sendo dotada de grande capacidade renovadora e de reparação (Afaq *et al.*, 2003; Hiramoto *et al.*, 2012). Com organização estratificada é composta por três camadas - a hipoderme, a derme e epiderme. A hipoderme, camada mais profunda, é composta por tecido adiposo e conjuntivo, funcionando como depósito de calorias, protegendo o organismo de traumas e variações de temperatura, modela o corpo e permite a mobilidade da pele em relação as estruturas subjacentes. A derme é constituída de tecido conjuntivo e fica acima da hipoderme; nela se situam os vasos sanguíneos e linfáticos, terminações nervosas, folículos pilosos, glândulas sebáceas e sudoríparas. Já a epiderme está disposta em quatro camadas: basal, espinhosa, granulosa e estrato córneo, sendo esta última a camada mais externa da epiderme e a principal responsável pela função barreira que a pele desempenha (Maibach, 1984; Kohen and Gati, 2000; Menon, 2002).

A radiação ultravioleta (RUV) é o fator físico mais importante que atinge a pele e uma das principais causas de danos, que resulta em lesões pré-cancerosas, cancerosas e aceleração do envelhecimento cutâneo. A RUV atinge diferentes camadas da pele dependendo do comprimento de onda, e assim interage com diferentes células localizadas em profundidades distintas do tecido cutâneo (Saija, 2000; Casagrande *et al.*, 2007; Touitou and Godin, 2008; Quan *et al.*, 2009).

A RUV pode ser dividida em UVA (320 a 400nm), UVB (290 a 320nm) e UVC (200 a 290nm) (Figura 1). Aproximadamente 95% da RUV que chega à superfície da Terra é formada por UVA e somente 5% por UVB (Afaq *et al.*, 2005).

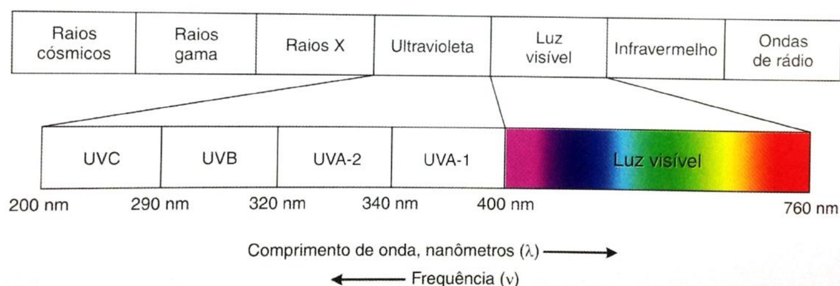

Figura 1: Espectro eletromagnético (Azulay *et al.,* 2013).

A UVA consegue atravessar a camada de ozônio e pode causar queimadura solar, envelhecimento precoce da pele e até supressão da função imune. Já a UVB sub-região do RUV é bloqueada pela camada de ozônio, porém pode causar queimadura na pele. E a UVC é totalmente filtrada pela camada de ozônio do planeta Terra (Shetty *et al.*, 2015) (Figura 2). As radiações ao atingirem a pele, são parcialmente refletidas, refratadas e, em parte absorvidas. A molécula que absorve essa radiação é conhecida como cromóforo. Quando o cromóforo absorve o fóton, a molécula deixa o estado de repouso e torna-se excitada e, portanto, é capaz de reagir com moléculas do meio biológico, levando à formação de fotoprodutos, ou, ainda a modificações bioquímicas, ou mesmo a alterações celulares e, finalmente a alterações teciduais (Xu e Fisher, 2005). Ambas as radiações UVA e UVB induzem a formação de fotoprodutos e causam danos cutâneos, porém a UVB é mais relevante para a fotocarcinogênese, uma vez que ela também causa danos diretos ao DNA, RNA, proteínas e outros componentes (Emri *et al.*, 2006).

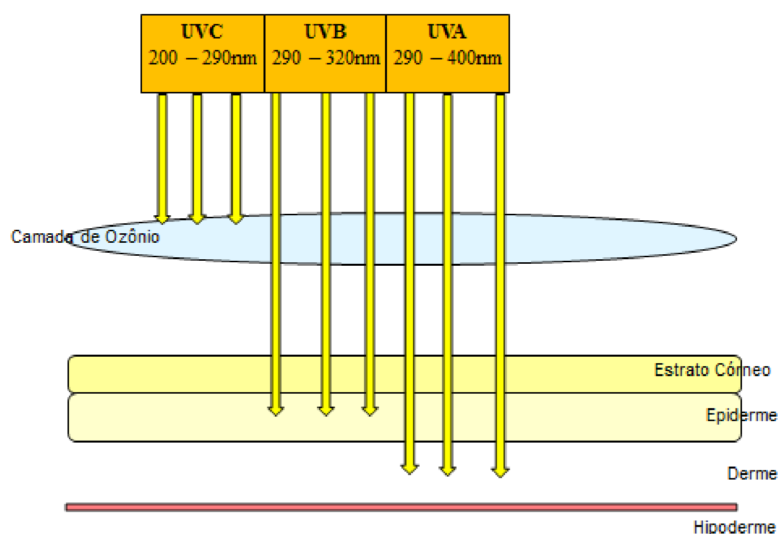

Figura 2: Absorção da RUV pelas camadas da pele

1.2 ESTRESSE OXIDATIVO E RESPOSTA INFLAMATÓRIA INDUZIDA PELA RADIAÇÃO UVB

A radiação UVB apresenta comprimento de onda curto, sendo mais energética, logo é absorvida predominantemente pela epiderme, afetando diretamente os queratinócitos. Já a radiação UVA é de comprimento de onda longo, sendo menos energética quando comparada a UVB, portanto é capaz de penetrar profundamente a pele, interagindo assim tanto com os queratinócitos da epiderme, quanto com os fibroblastos dérmicos (Paz *et al.*, 2008). Dessa forma, as moléculas que estão presentes na pele (lipídios, proteínas, ácidos nucléicos) e apresentam dupla ou tripla ligações de carbono com carbono em sua estrutura, podem absorver a radiação ultravioleta, sendo então denominadas de cromóforos (Figura 3) (Tewari *et al.*, 2013). Ambas as radiações UVA e UVB induzem a formação de EROs e causam danos cutâneos, porém a UVB causa também danos diretos ao DNA, RNA proteínas e outros componentes da célula, sendo absorvida principalmente pelo DNA dos queratinócitos, e desta forma é mais relevante para a fotocarcinogênese (Emri *et al.*, 2006).

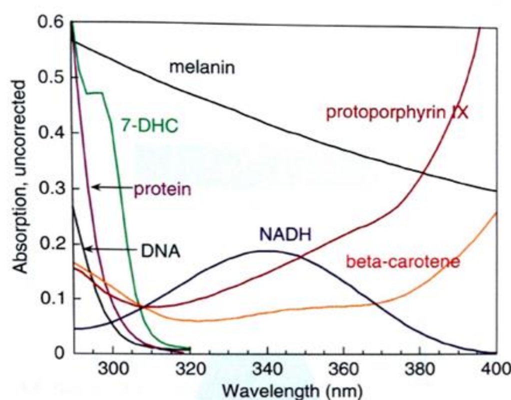

Figura 3: Absorção da radiação UV por diferentes cromóforos da pele (Lim *et al.*, 2007).

A hipótese provável para o aparecimento de patologias de pele devido à exposição à radiação solar é a formação de espécies reativas de oxigênio (EROs). As EROs são moléculas que possuem um ou mais elétrons desemparelhados, altamente instáveis, com meia-vida curtíssima e quimicamente muito reativas (Aruoma, 1998). Nas últimas décadas, as EROs têm chamado a atenção de pesquisadores devido a sua participação em vários eventos patológicos como em processos inflamatórios, câncer e envelhecimento precoce (Ji, 2007). A exposição à RUV aumenta diretamente a formação de radicais livres, ou indiretamente por meio da resposta inflamatória induzida pela RUV (Wei *et al.*, 2002).

Com o aumento da geração de EROs, a regeneração de antioxidantes endógenos torna-se insuficiente ocasionando a sua depleção (Vicentini *et al.*, 2008). O resultado do desequilíbrio entre EROs e antioxidantes (estresse oxidativo) modula vias de transdução de sinais celulares e expressão de genes, com consequente dados nos lipídios de membrana, proteínas e ácidos nucléicos das células epidémicas e dérmicas, que interrompem as funções celulares e em última instância leva a morte das células (Georgetti *et al.*, 2006; Vicentini *et al.*, 2008; Circu and Aw, 2010).

Estudos recentes ratificaram a associação entre o estresse oxidativo e o desenvolvimento de câncer de pele (Venza *et al.*, 2015; McAdam, Brem and Karran, 2016; Soliman *et al.*, 2016).

A exposição à RUV induz fatores de transcrição de genes de metaloproteinases, enzimas proteolíticas produzidas principalmente por células polimorfonucleares, macrófagos, queratinócitos, fibroblastos e células tumorais (Fisher *et al.*, 2009a). O aumento da produção de metaloproteinases pelos queratinócitos epidermais e fibroblastos da derme resulta na degradação do colágeno e outras proteínas da matriz extracelular. Este processo é seguido pelo fotoenvelhecimento e danos severos à pele (Rieger, 1999; Quan *et al.*, 2009). Além disso, após a exposição à radiação ultravioleta os queratinócitos expressam uma ampla variedade de citocinas e quimiocinas, sendo por isso consideradas como as principais células que iniciam a resposta inflamatória (Barker *et al.*, 1991). Os queratinócitos alteram sua morfologia apresentando cromatina condesada e citoplasma eosinofílico. Com isso, essas células entram em apoptose para eliminar o DNA danificado e este processo é proporcional ao tempo de exposição à RUV. Em termos práticos, os efeitos da radiação UVB na pele podem ser divididos em resposta imediata, com aparecimento de eritema, edema e infiltrado leucocitário, além da diminuição dos níveis dos antioxidantes endógenos como a glutationa reduzida (GSH), que é um tripeptídeo sensível ao estresse oxidativo mediado pela radiação UVB (Montenegro *et al.*, 1995; Carini *et al.*, 2000; Meloni and Nicolay, 2003; Casagrande *et al.*, 2006; Sawane *et al.*, 2011) e tardios com danos às fibras elásticas, no colágeno e nas glicosaminoglicanas da matriz extracelular da derme, perdendo a elasticidade, e contribuindo para o fotoenvelhecimento da pele e pôr fim à fotocarcinogênese.

Porém, uma grande quantidade de antioxidantes enzimáticos como a superóxido dismutase, a catalase e glutationa peroxidase assim como, antioxidantes hidrofílicos e lipofílicos de baixo peso molecular, glutationa reduzida, vitamina C e E e carotenóides estão presentes na pele para protegê-la dos danos oxidativos. A maior parte destes se concentra na epiderme, visto ser a camada mais exposta às agressões (Fuchs, 1998).

Um sistema endógeno que auxilia a limitar os danos teciduais causados pela radiação UVB é do fator de transcrição Nrf2 (fator nuclear [derivado eritróide-2] tipo 2). Este fator regula a transcrição de genes para moléculas antioxidantes e anti-inflamatórias como a hemeoxigenase-1 (HO-1), a qual quebra o heme em biliverdina que apresenta efeito antioxidante e em monóxido de carbono que apresenta efeito anti-inflamatório (Choi *et al.*, 2013).

Dentre os efeitos tardios da exposição à radiação UVB, a fotocarcinogênese é a mais preocupante. É bem conhecido o efeito deletério da radiação UVB sobre as proteínas e membranas celulares, levando consequentemente a geração de EROs. Entretanto, sabe-se que o efeito deletério principal associado à radiação UVB está relacionado à dimerização das bases de pirimidina do DNA. Esta dimerização gera distorção da estrutura do DNA, e o reparo incorreto dessas lesões conduz a mutações, como também ao bloqueio da replicação do DNA e da divisão celular, e a interrupção da transcrição do DNA necessário para a síntese de RNA mensageiro (Emri, Horkay and Remenyik, 2006).

Conforme descrito acima, vários são os danos que a radiação RUV pode causar na pele. Os efeitos podem ser agudos como o eritema, inflamação local e até mesmo crônico como o fotoenvelhecimento (Quan *et al.*, 2009; Azulay *et al.*, 2013). Por isso, é necessário o estudo de novas substâncias antioxidantes com o intuito de prevenir a pele contra a ação da radiação (Gowell *et al.*, 2015).

1.3 MEDIADOR LIPÍDICO ANTI-INFLAMÓRIO/PRÓ-RESOLUÇÃO 7(S) MARESINA 1

A inflamação causada na pele pela RUV resulta em um aumento de células como neutrófilos e macrófagos, estes também produzem grandes quantidades de EROs, como o ânion superóxido (Halliday, 2005), gerado como produto da nicotinamida adenina dinucleotídeo fosfato (NADPH) oxidase. As células inflamatórias também produzem mediadores inflamatórios, como citocinas e quimiocinas, as quais atuam ativando células e recrutando mais células inflamatórias para o foco primário da inflamação e induzindo produção de EROs. Portanto, o estresse oxidativo e a inflamação, induzidos pela RUV estão intimamente relacionados e levam a um círculo vicioso, que resultará em danos teciduais e em longo prazo, pode conduzir a carcinogênese (Reuter *et al.*, 2010).

A resolução da inflamação é considerada atualmente como um processo ativo e finamente controlado, com liberação de mediadores anti-inflamatórios e substâncias que são denominadas de pró-resolutivas. A finalidade da liberação destes mediadores ocorre devido à necessidade de redução no calibre dos vasos, alteração da população e características celulares para um estado anti-inflamatório, drenagem de células que migraram para espaços extravasculares e para a limpeza de células mortas, para que o tecido retome sua função normal (Martinez *et al.*, 2006; Serhan *et al.*, 2007; Serhan *et al.*, 2008; Serhan *et al.*, 2005).

Diante desse quadro é promissor desenvolver terapias para o controle da inflamação e estresse oxidativo induzidos pela radiação UVB, tendo como alvo seus mecanismos fisiopatológicos. Estas terapias podem ser consolidadas baseando-se na redução ou resolução da inflamação tratando o processo desencadeado pela radiação UVB com mediadores lipídicos anti-inflamatórios/pró-resolução que incluem 4 classes de moléculas principais: lipoxinas, protectinas, resolvinas e maresinas. De maneira geral, essas moléculas inibem a produção de citocinas, recrutamento de células pró-inflamatórias, aumentam o recrutamento de células anti-inflamatórias, inibem a ativação do NFκB, inibem a expressão/atividade da NADPH oxidase e aumentam a expressão do Nrf2 e OH-1.

Entre esses mediadores destacamos a Maresina 1 (MaR1) que é sintetizada por macrófagos através da transformação do ácido graxo docosahexaenóíco (DHA), que pode ser convertido através de reação de 14-lipoxigenação em intermediários do (DHA), culminando com a produção do ácido 7,14-dihidroxidocosa-4Z,8,10,12,16Z,19Z-hexaenóico, denominado meniador de macrófago (Fredman and Serhan, 2011).

As características estruturais da MaR1 incluem: duplas conjugadas e OH livres, o que confere a estrutura a capacidade de doar elétrons e estabilizar compostos como radicais livres (Figura 3).

O tratamento com MaR1 em modelo de inflamação induzida por extrato de poeira resultou em menor produção de citocinas IL-6 e IL-8 em células brônquicas epiteliais (Nordgren *et al.*, 2013). A MaR1 também reduz a inflamação em modelo de colite por ácido sulfônico 2,4,6-trinitrobenzeno (TNBS) e por sulfato de dextrana sódico (DSS) por reduzir a produção de citocinas (TNFα, IL-1β, IL-6 e IFNγ dependendo da fase da doença) e indução de macrófagos com perfil M2 devido a inibição da ativação do NFκB (Marcon *et al.*, 2013). De maneira semelhante, a MaR1 reduz a inflamação pulmonar induzida pelo LPS (lipopolissacarídeo), atuando pela inibição da produção de citocinas como TNFα, IL-1β, IL-6 e das quimiocinas KC, MCP-5, MIP-1α e MIP-1γ, este efeito resulta em menor expressão de moléculas de adesão ICAM-1, P-selectina e CD24 e em menor atividade de mieloperoxidase pulmonar e menor recrutamento de neutrófilos (Gong *et al.*, 2014). Esses mecanismos reforçam o potencial benéfico deste mediador no tratamento de inflamações cutâneas e a proteção contra o fotoenvelhecimento.

É importante mencionar que apesar do estabelecimento do papel endógeno e efeito anti-inflamatório e pró-resolução, em vários modelos de doença, não existem evidências do papel ou efeito terapêutico dessa molécula na inflamação e estresse oxidativo induzido pela radiação UVB.

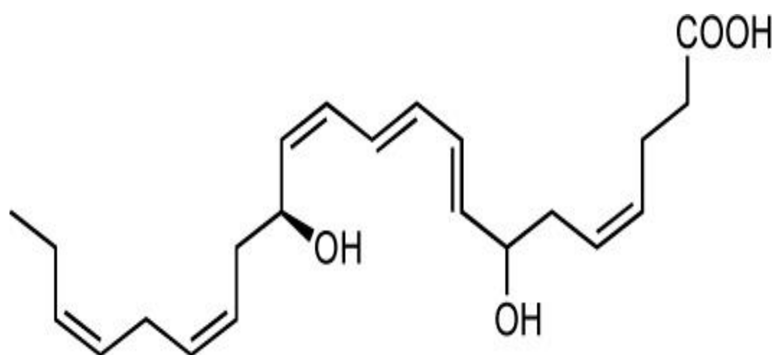

Figura 4. Estrutura molecular 7(S) Maresina 1

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito anti-inflamatório/pró-resolução do lipídeo MaR1 em lesões cutâneas induzidas pela radiação UVB em camundongos sem pelo.

2.2 OBJETIVOS ESPECÍFICOS

Avaliar os efeito protetor do lipídio MaR1 quando administrado via intraperitoneal em camundongos sem pelo, no modelo de lesão cutânea induzida pela radiação UVB com enfoque na (o):

- Edema de pele;
- Infiltrado leucocitário (ensaio da mieloperoxidase);
- Atividade/secreção de metaloproteinase-9;
- Produção de citocinas pró-inflamatórias (TNFα, IL-1β) e na produção da citocina anti-inflamatória (IL-10)
- Níveis dos antioxidantes endógenos GSH e catalase;
- Produção de ânion superóxido;
- Capacidade antioxidante global cutânea por meio dos ensaios de ABTS (2,2' azinobis (3-etilbenzotiazolina-6-ácido sulfônico) e FRAP (Poder antioxidante de redução férrica);
- Histopatologia do tecido com enfoque na apoptose de queratinócitos e espessura da epiderme.

3. MATERIAIS E MÉTODOS

3.1 MATERIAIS

3.1.1 Reagentes, padrões e polímeros

Ácido cítrico, hidróxido de sódio, ácido clorídrico e fosfato de potássio, Merck (grau analítico, Darmstadt, Hessen, Alemanha); tween 20®, Santa Cruz Biotechnology Inc (Dallas, Texas, USA); terc-butil hidroperoxido, Azul brilhante R, glutationa reduzida (GSH), brometo de hexadecil trietil amônio (HTAB), n-etilmaleimida, *o*-dianisidina, fluoreto de fenilmetilsulfonila, fenantrolina, 2,2' azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), 2,4,6 tripiridil-S-triazina (TPTZ), Trolox e ácido 5,5'-ditio-bis-(2-nitrobenzóico) (DTNB), bisacrilamida, *nitroblue tetrazolium* (NBT) foram obtidos da Sigma-Aldrich (St. Louis, MO, USA). Xilene cianol e hidroximetil aminometano (Tris) da Amresco (Solon, OH, USA). Kits de Ensaio imunoenzimático (ELISA) para dosagem das citocinas da eBioscience (San Diego, CA, USA). Acrilamida, dodecil sulfato de sódio (SDS) (Superscrip III). Todos os outros reagentes utilizados foram de grau analítico.

3.1.2 Equipamentos

Agitador mecânico, Fisatom®; Balança analítica, HR-120, A&d®, precisão de 4 casas; Banho-maria, 314/2 DN, Nova Ética®; Banho-maria 100, Fanem; Centrífuga refrigerada, Rotina 46R, Hettich Zentrifugen®; Câmara de madeira projetada para irradiação; Deionizador de água, Purebal Option-Q, Elga®; Estufa 0-120°C, De Leo & Cia®; Fonte elétrica para eletroforese MS 300V, Major Science®; Homogeneizador de tecidos Ultra Turrax®, T18 basic, IKA; Lâmpada ultravioleta fluorescente PHILIPS TL/12 40W RS-UVB, MedicalHoland®; Leitor de microplaca, Asys Expert Plus, Biochrom®; Leitor de microplaca, Enspire, Perkin Elmer®; Leitor de microplaca, Multiskan GO, Thermo Scientific; pHmetro, Tec-3MP, TECNAL®; Radiômetro IL 1700 Research Radiometer. Detectores: SED240 – filtro UVB (290nm), SED005 – filtro UV (350nm); Sistema de eletroforese Mini Vertical, Mini drier, model SD-05; Ultra-som, TSO, Thornton®.

3.2 MÉTODOS

3.2.1 Animais experimentais

Foram utilizados camundongos sem pelo da linhagem HRS/J, de ambos os sexos, adultos e com massa de 20 a 30 g. Os camundongos foram mantidos no Biotério do Centro de Ciências da Saúde da Universidade Estadual de Londrina com temperatura controlada de 22± 2°C, ciclo claro/escuro de 12 horas e com livre acesso a água e ração. Os experimentos foram realizados conforme as normas da Comissão de Ética no uso de Animais (CEUA) da Universidade Estadual de Londrina (registrado no Ofício Circular CEUA nº017/2015, processo CEUA n° 1447.2015.10.

3.2.2 Sistema e fonte de radiação UVB

A fonte de luz utilizada nos experimentos para indução das lesões fotoxidativas foi uma lâmpada UVB fluorescente modelo PHILIPS TL/12 40W RS (Medical). A lâmpada emite radiação na faixa λ de 270 a 400 nm com pico máximo de emissão em torno de 313 nm. A medida da irradiância foi realizada utilizando-se um radiômetro (IL 1700) com detectores para radiação UV (SED 005) e, especialmente, para UVB (SED 240) (Carini *et al.*, 2000; Casagrande *et al.*, 2006). A lâmpada foi instalada numa câmara de madeira desenvolvida especificamente para a indução do estresse oxidativo e inflamação. Os camundongos foram separados em caixas de plástico e cobertos com tela plástica para garantir a total exposição da região dorsal. Foi realizado rodízio com as caixas contendo os animais devido às variações de radiação ao longo da lâmpada. Os animais foram colocados a 20 cm de distância da lâmpada UVB e expostos à radiação à temperatura ambiente com livre movimentação na caixa (Casagrande *et al.*, 2006). A dose de radiação utilizada para indução da inflamação e do estresse oxidativo foi de 4,14 J/cm^2 (Campanini *et al.*, 2013; Ivan *et al.*, 2014; Martinez, Pinho-Ribeiro, Steffen, Caviglione, Vignoli, Barbosa, *et al.*, 2015). Y Shindo, Witt and Packer, 1993 demonstraram que a dose de 25 J/cm^2 equivale a 4-5 horas de exposição ao sol de outono na latitude da Califórnia (38°N).

3.2.3 Protocolo experimental para administração de MaR1 pela via intraperitoneal

Os camundongos foram distribuídos em cinco grupos com seis animais cada:

- Grupo 1: controle não irradiado;
- Grupo 2: controle irradiado;
- Grupo 3: irradiado e tratado pela via intraperitoneal com 0,1 ng/Kg de MaR1;

- Grupo 4: irradiado e tratado pela via intraperitoneal com 1,0 ng/Kg de MaR1;
- Grupo 5: irradiado e tratado pela via intraperitoneal com 10,0 ng/Kg de MaR1.

Os camundongos foram tratados pela via intraperitoneal, com 200 µL para atingir as doses de 0,1, 1,0 e 10,0 ng/Kg de MaR1, 10 minutos antes do início da sessão de irradiação UVB (Figura 4) (Martinez *et al.*, 2016). As doses da MaR1 foram escolhidas com base em estudos previamente publicados (Serhan *et al.*, 2012a; Serhan *et al.*, 2009). Doze horas após o final da irradiação os animais foram eutanasiados e as amostras de pele foram coletadas para os seguintes ensaios: edema; atividade de mieloperoxidase, avaliação de FRAP; capacidade em reduzir o radical ABTS; níveis de GSH e atividade de metaloproteinase (MMP-9), capacidade em reduzir apoptose dos queratinócitos e capacidade em reduzir o espessamento da epiderme. O segundo tempo de irradiação foi realizado com a mesma quantidade de animais e mesmo esquema de tratamentos, com a eutanásia ocorrendo 2 horas após o final da irradiação e a pele coletada foi utilizada para os seguintes ensaios: teste de produção de ânion superóxido e atividade da catalase. Já a terceira irradiação, seguiu os mesmos parâmetros descritos, porém a eutanásia ocorreu 4 horas após o final da irradiação e as amostras de pele foram usadas para analisar níveis de citocinas, e produção de hidroperóxido lipídico (LOOH).

As amostras foram divididas para os diferentes testes e armazenadas a -80°C para as análises. Apenas o teste de edema cutâneo foi realizado no mesmo dia em que a pele foi retirada. Para os testes de 12 horas os animais foram terminalmente anestesiados com 3% de isofurano ou anestesiados seguido de decapitação para os testes de 2 e 4 horas após o término da radiação UVB (4,14 J/cm^2). As peles foram lavadas com solução de NaCl 145 mM em água deionizada (CASAGRANDE *et al.,* 2006a; IVAN *et al.,* 2014).

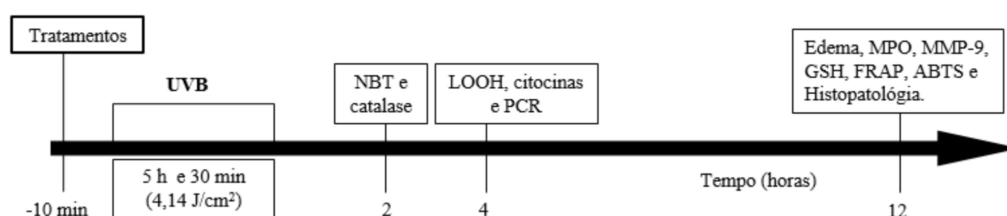

Figura 5. Fluxograma dos tempos de tratamento com os protocolos experimentais para avaliação da eficácia da MaR1 administrada intraperitonealmente. Os animais foram irradiados com radiação UVB durante 5 horas e 30 min (tempo necessário para atingir 4,14. J/cm^2). Dez minutos antes do início da radiação os animais foram tratados com as soluções de MaR1 para cada grupo (0,1, 1,0 e 10,0 ng/Kg). Os animais foram eutanasiados e

amostras de pele foram coletadas 2 horas (produção do ânion superóxido [NBT] e atividade da catalase), 4 horas (dosagem de citocinas e hidropéroxido lipídico) e 12 horas (avaliação do teste de edema, atividade de metaloproteinase-9 [MMP-9], atividade de mieloperoxidase [MPO], níveis de glutationa reduzida [GSH], avaliação do poder antioxidante redutor de ferro [FRAP], transferência de elétrons ao radical [ABTS]) e análise histopatológica após o fim da radiação UVB.

3.2.4 Avaliação do edema de pele

A exposição em demasia da radiação UVB sobre a pele pode causar expressiva reação inflamatória levando, entre outras consequências, ao edema (Martinez *et al.*, 2015).

As amostras de pele do dorso de cada animal foram coletadas com auxílio de um molde com área fixa de 5 mm de diâmetro e posteriormente foram pesadas (Boller *et al.*, 2010; Ivan *et al.*, 2014; Martinez *et al.*, 2015). O efeito dos tratamentos no edema cutâneo causado pela radiação UVB foi mensurado pelo aumento do peso de pele na região dorsal. A análise foi feita comparando-se o peso de pele entre os diferentes grupos. Os resultados foram expressos em mg de pele.

3.2.5 Avaliação da atividade da enzima mieloperoxidase

Os grânulos azurófilos dos neutrófilos contêm a enzima mieloperoxidase (MPO), que, na presença de sais como o íon cloreto, convertem o peróxido de hidrogênio a hipoclorito. Para o estudo da quantificação de MPO foi adicionado no meio reacional o substrato o-dianisidina, o que resultou em uma solução colorida que foi analisada pelo espectrofotômetro em 540 nm (Bradley *et al.*, 1982; Casagrande *et al.*, 2006; Martinez, Pinho-Ribeiro, Steffen, Caviglione, Vignoli, Baracat, *et al.*, 2015).

As amostras de pele dos animais (aproximadamente 30 mg) foram coletadas em microtubos contendo 400 µL de tampão fosfato de potássio 0,05 M (pH 6,0) com 0,5% de brometo de hexadecil trietil amônio (HTAB) e mantidas a - 80 °C até o uso. Para realização do ensaio, as amostras foram homogeneizadas com auxílio do homogeneizador de tecidos Tissue-Tearor (Biospec®). O homogenato foi centrifugado a 16.100 g por 2 minutos a 4°C e o sobrenadante foi retirado para o ensaio.

Em microplaca de 96 poços, foi adicionado alíquota do sobrenadante das amostras (30 µL). A reação colorimétrica iniciou-se pela adição de 200 µL de uma solução contendo 10% de tampão fosfato 0,05 M (pH 6.0), 0,0167 % de *o*-dianisidina e 0,05% de

H_2O_2 em água deionizada. Após 20 minutos de reação a atividade da MPO das amostras foi determinada em 450 nm (Asys Expert Plus, Biochrom) e comparada com uma curva padrão de neutrófilos de concentração conhecida.

A curva padrão foi preparada na mesma placa com a adição de 100 µL de tampão fosfato 0,05 M e uma solução contendo 200.000 neutrófilos no primeiro poço (A1) com posterior diluição seriada até o décimo primeiro poço (A11). O décimo segundo poço (A12) foi utilizado como branco ao qual foi adicionado apenas 200 µL de água deionizada. Os resultados foram expressos como atividade da MPO (número de neutrófilos/mg de pele).

3.2.6 Determinação da atividade/secreção de proteinases por zimografia em gel de poliacrilamida com dodecil sulfato de sódio (SDS)

Para determinação da atividade da MMP-9 foi utilizado o ensaio de zimografia em gel de poliacrilamida com dodecil sulfato de sódio (SDS-PAGE) (Fonseca *et al.*, 2010, 2011). As metaloproteinases são enzimas importantes em degradar as matrizes extracelulares como o colágeno e podem desenvolver o fotoenvelhecimento cutâneo (Bae *et al.*, 2015; Lima *et al.*, 2016).

Preparou-se um pool das amostras de pele de cada grupo e as mesmas foram armazenadas em eppendorfs. As amostras foram homogeneizadas com auxílio do homogeneizador de tecidos Ultra Turrax® (T18 basic, IKA), na proporção 1:4 em tampão fosfato Tris/HCl 50mM (pH 7,4) com cloreto de cálcio ($CaCl_2$) e 1% de inibidores de proteinases (fenantrolina, fluoreto de fenilmetilsulfonila e N-etilmaleimida).

Os homogenatos foram centrifugados duas vezes a 12.000 *g* por 10 min a 4°C e os sobrenadantes foram submetidos ao ensaio de zimografia. Também foi realizada dosagem de proteínas das amostras pelo método de Lowry (Lowry *et al.*, 1951). Alíquota de 50 µL do sobrenadante foi diluída em 10 µL de tampão Tris/HCl (pH 6,8) contendo 20% de glicerol, 4% de duodecil sulfato de sódio (SDS) e 0,001% de azul de bromofenol. Em seguida estas amostras foram colocadas em banho-maria a 37 ^{o}C durante 8 minutos imediatamente antes de ser aplicada no gel de eletroforese.

A espessura do gel utilizado foi de 1 mm, composto por um gel de separação e um gel de concentração, cuja a constituição está apresentada na Tabela 1.

Tabela 1. Constituintes do gel de separação e do gel de concentração do sistema eletroforético.

Substância	Quantidade (µL)	
	Gel de separação	Gel de concentração
Água miliQ	5870	4060
Tampão Tris/HCl 1 M (pH 8,8) com 0,4% SDS	3750	-
Tampão Tris/HCl 0,5 M (pH 6,8) com 0,4% SDS	-	1670
Acrilamida: bis-acrilamida (30:0,8)	5000	860
Gelatina 10%	375	-
Persulfato de amônio 10%	50	33
Temed 20%	10	6,6

Após a finalização da solução do gel de separação e de concentração, os mesmos foram aplicados no aparato específico do Sistema de eletroforese Mini Vertical (Bio-RAD®).

O interior da cuba de eletroforese foi preenchido com tampão Tris/glicina 190 mM (pH 8.3) contendo 0,1% de SDS. Antes da aplicação das amostras, o gel foi submetido a uma pré-corrida de 10 mA por 15 minutos. Após, foram aplicados 25 µL de cada amostra. Durante a eletroforese a corrente aplicada foi de 10 mA para o gel de concentração e 13 mA para o gel de separação, sendo que a última corrente foi mantida constante por 15 minutos após a saída do corante do gel de separação.

Ao término da eletroforese o gel de poliacrilamida foi lavado por 1 hora com solução de Triton X-100 2% sob constante agitação, posteriormente o gel foi incubado por 16 horas a 37 °C em tampão Tris/HCl 50 mM (pH 7.4) contendo $CaCl_2$ 10 mM e 0,02% de azida sódica. Ao final da incubação o gel foi corado com uma solução contendo 0,25% de azul brilhante, 10% de ácido acético e 50% de metanol em água deionizada. Para visualização das bandas o gel foi descorado com ácido acético 20% (Fonseca *et al.*, 2011). A atividade proteolítica foi analisada por meio da comparação das diferenças de densidades de cor entre as bandas de cada grupo pelo programa ImageJ® (NIH, Bethesda, MD, USA).

3.2.7 Dosagem das citocinas pró-inflamatórias: TNFα, IL-1β e anti-inflamatória: IL-10

A dosagem dos níveis de citocinas pró-inflamatórias foi realizada utilizando-se a técnica de enzima imunoensaio (ELISA) com kits comerciais (eBioscience), baseando-se nas instruções do fabricante e conforme descrito na literatura científica (Verri *et al.*, 2008). As amostras foram coletadas em microtubos contendo 500 μL de solução salina 0,9% estéril e foram trituradas com o homogeneizador de tecidos Tissue-Tearor (Biospec®). Em seguida, foram centrifugadas a 2000 *g* por 15 minutos a 4 °C e o sobrenadante foi retirado para análise.

Para a realização do ensaio, microplacas com 96 poços foram incubadas por toda à noite a 4 °C com anticorpos de captura contra as proteínas de interesse. Após esse tempo de incubação, as placas foram lavadas com PBS com 0,05% de Tween 20 e incubadas por 1 hora à temperatura ambiente com uma solução a 1% de albumina bovina. Após esse bloqueio e lavagem das placas com tampão de lavagem, a curva padrão e as amostras foram adicionadas (50 μL) e incubadas a 4 °C por 16 horas. Posteriormente, as placas foram lavadas com tampão de lavagem e os anticorpos policlonais biotinilados (anticorpo de detecção) foram adicionados (100 μL). Após incubação em temperatura ambiente por 1 hora, as placas foram lavadas com tampão de lavagem e 100 μL de enzima avidina-peroxidase foram adicionados. As placas foram incubadas por 30 minutos à temperatura ambiente, lavadas, e adicionado 100 μL do substrato contendo 0,04% de ortofenilenodiamina (OPD) e 0,04% de H_2O_2 em tampão fosfato, a placa foi mantida no escuro em temperatura ambiente por 15 minutos. A reação enzimática foi interrompida com uma solução de H_3PO_4 1 M e as absorvâncias foram determinadas em 450 nm (Multiskan GO, Thermo Scientific).

Curvas padrões de cada uma das citocinas foram utilizadas para quantificar as citocinas presentes nas amostras e os resultados foram expressos em picogramas (pg) de citocina/mg de pele.

3.2.8 Avaliação do poder antioxidante redutor do ferro (FRAP) na pele

O método de FRAP já foi utilizado para medir o poder redutor do ferro nos tecidos do coração, rim, fígado e cérebro de ratos (Katalinic *et al.*, 2005). O ensaio foi adaptado e utilizado para avaliar o poder antioxidante de redução férrica da pele (Martinez, Pinho-Ribeiro, Steffen, Caviglione, Vignoli, Barbosa, *et al.*, 2015).

As amostras de pele dos animais (aproximadamente 30 mg) foram coletadas em microtubos contendo 500 µL de KCl 1,15%. Para a realização do ensaio, as amostras foram trituradas com auxílio do homogeneizador de tecidos Tissue-Tearor (Biospec®). Em seguida, foram centrifugadas a 1.000 *g* por 10 minutos a 4°C e o sobrenadante foi retirado para a análise. Para a reação, foram adicionados em microplaca contendo 96 poços, 30 µL do sobrenadante e 150 µL de reagente FRAP. O branco foi preparado com 30 µL de KCl a 1,15% e 150 µL do reagente de FRAP. O reagente de FRAP foi preparado adicionando 2,5 mL de uma solução 10 mM de 2,4,6 tripiridil-S-triazina (TPTZ) em HCl 40 mM com 2,5 mL de cloreto de ferro hexahidratado 20mM e 25 mL de tampão acetato 0,3 mM (pH 3.6), e esta solução foi incubada a 37°C por 30 min antes do uso. A microplaca foi lida em espectrofotômetro a 595 nm (EnSpire, Perkin Elmer). Foi realizada o preparo de uma curva padrão utilizando-se diferentes concentrações de trolox (antioxidante análogo solúvel da vitamina E) de 0,5 a 20 nmol. Os resultados foram expressos como nmol equivalente de Trolox/mg de pele (Katalinic *et al.*, 2005).

3.2.9 Avaliação do poder antioxidante pelo ensaio de sequestro do radical 2,2',azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS)

O ensaio baseia-se na capacidade do antioxidante em sequestrar o cátion $ABTS^+$ causando redução da absorbância (Kamal *et al.*, 2015).

Para a reação de ABTS, as amostras foram homegeneizadas em 400 µL de KCl a 1,15% com auxílio do homogeneizador de tecidos Tissue-Tearor (Biospec®) e centrifugadas a 1.000 *g* por 10 min a 4°C, posteriormente o sobrenadante foi utilizado para análise.

A solução de ABTS foi preparada após reagir 7mM da solução de ABTS com 2,45mM de persulfato de potássio resultando no cátion $ABTS^+$. A mistura foi armazenada em frasco âmbar e em geladeira por no mínimo 16 horas antes do uso. Após as 16 horas, a solução de ABTS foi misturada com tampão fosfato até atingir a absorvância de 0,8 em 730 nm. Foi adicionado, na microplaca, 7 µL do sobrenadante e 200 µL da solução de ABTS diluída. O branco era composto apenas do tampão. Após 6 minutos de reação, foi realizada a leitura em 730nm (EnSpire, Perkin Elmer). Uma curva padrão foi preparada com diferentes concentrações de trolox (0,01 a 20 nmol) e os resultados foram expressos em nmol equivalente de trolox/mg de pele (Katalinic *et al.*, 2005; Martinez, Pinho-Ribeiro, Steffen, Caviglione, Vignoli, Barbosa, *et al.*, 2015).

3.2.10 Avaliação dos níveis do antioxidante endógeno (GSH)

A enzima glutationa é importante na homeostasia da pele por eliminar os radicais livres que foram produzidos durante a exposição à radiação UV. O ensaio baseia-se na reação da quebra da ligação do ácido 5',5'-ditio-bis-(2-nitrobenzóico) (DTNB) pelo grupo sulfidrila da glutationa, para tal, é detectado a quantidade do ácido 5-mercapto-2-nitrobenzóico (Srinivasan, Sabitha and Shyamaladevi, 2007).

As amostras de pele dos animais foram coletadas em microtubos e posteriormente diluídas (1:4) em EDTA 0,02 M e trituradas em homogeneizador de tecidos Tissue-Tearor (Biospec®). Ao homogenato foi adicionado ácido tricloroacético (TCA) 50%, com o intuito de precipitar as proteínas do meio reacional, na proporção de 1:0,2 de EDTA e TCA, respectivamente. A mistura foi centrifugada a 2.700 *g* por 10 minutos a 4°C. O sobrenadante foi recentrifugado a 2.700 *g* por 10 minutos a 4°C, e o sobrenadante final foi retirado para análise.

Para o ensaio de quantificação dos níveis de GSH na pele foram adicionados 50 µL do último sobrenadante em microplaca com o meio reacional contendo 100 µL de tampão Tris 0,4 M (pH 8,9) e 5 µL de uma solução de 1,9mg/mL de ácido 5,5'-ditio-bis-(2-nitrobenzóico) (DTNB) em metanol. Após 5 minutos de incubação, a leitura da microplaca foi realizada no espectrofotômetro (EnSpire, Perkin Elmer) em 405 nm. Foi preparada uma curva padrão com 5 a 150 µM de GSH. Os resultados foram expressos em µM de GSH/ mg de pele (Srinivasan, Sabitha and Shyamaladevi, 2007; Martinez, Pinho-Ribeiro, Steffen, Caviglione, Vignoli, Baracat, *et al.*, 2015)

3.2.11 Avaliação da atividade da catalase (CAT)

A CAT é uma heme enzima responsável por reduzir o peróxido de hidrogênio em água e oxigênio. O nível dessa enzima se reduz quando há estresse oxidativo, indicando um possível desequilíbrio entre a produção de radicais livres e a ação antioxidante endógena (Shetty *et al.*, 2015).

As amostras de pele dos animais (aproximadamente 100 mg) foram coletadas em microtubos e diluídas em 500 µL de EDTA 0,02 M. Em seguida, foram trituradas (Tissue-Tearor (Biospec®) e o homogenato foi centrifugado a 2.700 *g* por 10 minutos a 4°C. O sobrenadante foi recentrifugado a 2.700 *g* por 10 minutos a 4°C, e o sobrenadante final foi retirado para análise.

A determinação da atividade da CAT na pele foi realizada em microplaca de 96 poços por meio da adição de 10 µL de amostra, 160 µL de tampão Tris-HCl 1M com EDTA 5 mM pH 8.0, 20 µL de água deionizada e 20 µL de H_2O_2 200 mM. Ao teste foi incluído um branco para cada amostra preparado com 10 µL de amostra, 180 µL de tampão Tris-HCl 1M com EDTA 5 mM pH 8.0 e 20 µL de água deionizada. A velocidade com que o H_2O_2 é reduzido pela ação da CAT foi avaliada por meio da diminuição no valor da absorbância pela diferença entre a leitura inicial e a leitura 30 segundos após a adição do H_2O_2 200 mM. A leitura foi realizada em espectrofotômetro de microplaca (EnSpire, Perkin Elmer) em 240 nm com temperatura mantida em 25 ºC. Os valores de catalase foram expressos como unidade de CAT/mg de pele/minuto.

3.2.12 Avaliação da produção de ânion superóxido ($O_2^{\bullet -}$)

O ânion superóxido é um íon produzido endogenamente durante o processo de respiração celular aeróbia. No entanto, na exposição acentuada a radiação UVB pode apresentar aumento da sua quantidade e consequentemente ativa a enzima superóxido dismutase, a qual converte o ânion superóxido em peróxido de hidrogênio, favorecendo a geração de outro radical que é prejudicial para a manutenção da homeostasia da célula (Cha *et al.*, 2014; Martinez, Pinho-Ribeiro, Steffen, Caviglione, Vignoli, Barbosa, *et al.*, 2015).

A produção de ânion superóxido foi realizada usando o ensaio de redução de *nitroblue tetrazolium* (NBT) (Campanini *et al.*, 2013). As amostras de pele dos animais (aproximadamente 100mg) foram coletadas em microtubos, homogeneizadas em 500 µL de EDTA 0,02 M com homogeneizador de tecidos (Tissue-Tearor (Biospec®) centrifugadas a 2000 *g* por 20 segundos e o sobrenadante foi retirado para a análise.

Para a reação 50 µL do sobrenadante foi incubado em placas de 96 poços por 1 hora. Em seguida o sobrenadante foi cuidadosamente removido e às células fixadas foi adicionado 100 µL de NBT (1 mg/mL). Após 15 minutos, o sobrenadante foi cuidadosamente removido e ao precipitado foram adicionados 20 µL de metanol 100% para fixar, 120 µL de KOH 2 M e 140 µL de dimetilsulfóxido (DMSO) para solubilizar as partículas de formazan (NBT reduzido) presentes dentro das células. A redução do NBT foi determinada espectrofotometricamente em 620 nm e os resultados foram apresentados como densidade óptica (OD)/10 mg de pele.

3.2.13 Avaliação da produção de hidroperóxido (LOOH)

A avaliação da formação de LOOH por quimiluminescência (QL) foi realizada em uma adaptação da técnica descrita por Flecha et al. 1991 (Gonzalez Flecha, Llesuy and Boveris, 1991; Martinez, Pinho-Ribeiro, Steffen, Caviglione, Vignoli, Barbosa, *et al.*, 2015). As amostras de pele dos animais (aproximadamente 100 mg) foram coletadas em microtubos e homogeneizadas em 800 µL de tampão fosfato (pH 7.4) com homogeneizador de tecidos (Tissue-Tearor (Biospec®). Em seguida, foram centrifugadas a 700 *g* por 2 minutos a 4 ºC e 250 µL do sobrenadante foram adicionados a 1730 µL de meio de reação (KCl 120mM, tampão fosfato pH 7,4 30 mM) e 20 µL de tert-butil hidroperóxido 3 mM. Este ensaio foi realizado em contador β marca Beckman® LS 6000 (Fullerton, CA, EUA) em uma faixa de contagem não coincidente com a resposta entre 300 e 620 nm. Todo o experimento foi realizado ao abrigo da luz para evitar a fosforescência dos frascos, a 30 ºC, durante 120 minutos. Os resultados foram medidos em contagem por minuto (cpm) por mg de pele.

3.2.14 Avaliação histopatológica por microscopia óptica

Para análise histopatológica da pele, as amostras foram coletadas 12 horas após UVB, fixadas em paraformaldeído a 4% e desidratadas em banhos de soluções de etanol com concentrações crescentes (70%, 95% e 100%) e diafanizadas com xilol para inclusão de parafina. Foram preparadas secções de 5 µm de amostras de pele e coradas com hematoxilina eosina (H & E). As secções coradas com H & E foram examinadas utilizando microscopia óptica a uma ampliação de 10x e 100x para determinação da espessura epidérmica (Deng *et al.*, 2015) e número de células de queimaduras solares (Schwarz *et al.*, 1995), respectivamente.

3.2.15 Análise estatística dos resultados

Todos os resultados foram analisados estatisticamente por análise de variância (ANOVA) com um fator seguido do teste de comparações múltiplas de Tukey e apresentados pela média ± erro padrão da média (EPM) de mensurações feitas com 6 animais em cada grupo por experimento. As análises foram realizadas usando-se o software GraphPad Prism 6 (GraphPad Software Inc., San Diego, EUA). Os resultados foram

representativos de dois experimentos distintos e foram considerados significativamente diferentes para $p<0,05$.

4. RESULTADOS E DISCUSSÃO – ARTIGO

4.1 The 7(S) Maresin 1 inhibits UVB irradiation-induced inflammation and oxidative stress in the skin of hairless mice

Photochemical & Photobiological Sciences

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ABSTRACT

The skin is the largest organ of the human body and it is the first barrier of the body with the external environment. Among environment factors, UVB radiation exposure stands out as an inducer of free radicals and skin inflammation, which in turn induce cancer and premature aging. Our organism has endogenous mechanisms to actively reduce and resolute inflammation. 7(S) Maresin 1 (MaR1) is a pro-resolution lipid mediator with anti-inflammatory effects, however, it remains to be determined if MaR1 can effectively inhibit UVB skin inflammation. The treatment with MaR1 reduced UVB-induced skin inflammation, edema, neutrophil recruitment (myeloperoxidase activity), cytokines production, matrix metalloproteinase-9 activity, keratinocyte apoptosis and epidermal thickening in hairless mice. MaR1 also inhibited UVB-irradiation-induced decrease of reduced glutathione (GSH) levels, catalase activity, ferric reducing ability and ABTS radical reducing capacity, and lipid hydroperoxide and superoxide anion production. Therefore, the present data demonstrate that treatment with MaR1 effectively inhibits UVB-induced skin inflammation and oxidative stress, which suggests this endogenous pro-resolution lipid mediator as a promising pharmacological tool in controlling UVB deleterious effects related to inflammation.

Keywords: 7(S) Maresin 1; Antioxidant; Inflammation ; UVB radiation.

Introduction

The skin plays a key role as a barrier in innate immunity, in thermoregulation, protection against dehydration and release of substances as melanin that is important against the deleterious effects of ultraviolet radiation (UVR). The skin also presents endogenous antioxidant including reduced glutathione (GSH) and catalase that protect from oxidative stress [1], [2]. Despite prominent effect of endogenous protection systems, excessive exposure to UVR can deplete endogenous antioxidants, making the skin susceptible to reactive oxygen species (ROS) [3], [6].

The UVR can be divided into UVA (320 to 400nm), UVB (290 to 320nm) and UVC (200 to 290nm). The UVR reaching the surface of the earth is made of up to 95% of UVA and only 5% by UVB [7]. The radiation reaching the skin is in part reflected, refracted and absorbed. The molecule that absorbs this radiation is known as the chromophore. When the chromophore absorbs a photon, the molecule leaves the idle state and becomes excited and thus is capable of reacting with molecules in the biological medium, leading to formation of photoproducts, or even to cellular biochemical modifications leading ultimately to tissue change [8]. Both UVA and UVB radiation induce the formation of photoproducts and cause skin damage. Nevertheless, despite the lower percentage of UVB compared to UVA in UVR, UVB is more relevant to photocarcinogenesis than UVA. This is related to the UVB direct damage to DNA, RNA, proteins and other components [9].

Understanding the activity of novel compounds with protective activity against the deleterious effects of UVR is a promising strategy for the development of novel therapies [10]. Taking into account the UVB-induced deleterious mechanisms, novel therapies should diminish UVB-induced skin inflammation and oxidative stress. Besides, it has become clear that in addition to inflammation and oxidative stress, there are active mechanisms to resolute inflammatory processes. However, to our knowledge, there is no therapeutic approach that

has applied inflammation resolution molecules to inhibit UVB deleterious effects. Lipid pro-resolution mediators include four classes derived from cell membrane arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid: lipoxins, protectins, resolvins and maresins (MaR1).

MaR1 is the latest discovered pro-resolution lipid molecule. And (MaR1) it has been recently identified as a potent lipid mediator generated endogenously by macrophage enzymes. Docosahexaenoic acid undergoes metabolizia by 14-lipoxygenase producing 14-hydroperoxy docosahexaenoic acid, which in turn produces an intermediate epoxide 13S, 14S-epoxide that after hydrolysis results in bioactive MaR1 [11], [30]. The current understand is that endogenous MaR1 production occurs in the resolution phase of the inflammatory process.

In face in a model of dust extract-induced inflammation, treatment with MaR1 lowered the production of IL-6 and IL-8 by epithelial bronchial cells by a mechanism related to the block of protein activation kinase C (PKC) [13]. Treatment with MaR1 also reduced 2,4,6-trinitrobenzene sulfonic acid (TNBS)- and dextran sulfate sodium (DSS)-induced inflammatory bowel disease by reducing the production of cytokines such as TNFα, IL-1β, IL-6 and IFNγ depending on the stage of the disease, and induction of macrophages with a M2 anti-inflammatory profile due to inhibition of NFκB activation [14]. In addition to inflammation prevented in models involving adaptive immunity mechanisms, MaR1 also reduced LPS-induced innate immune pulmonary inflammation by inhibiting the production of cytokines such as TNFα, IL-1β, IL-6 and the chemokine KC, MCP-5, MIP-1α and MIP-1γ. The decrease of cytokine production resulted in lowering of adhesion molecule expression including ICAM-1, P-selection and CD24. Thus, it results in decreased LPS-induced lung myeloperoxidase activity and neutrophil recruitment [15]. MaR1 also enhances the phagocytosis of apoptotic neutrophils by macrophages, a process known as efferocytosis. Therefore, MaR1 limits the influx of inflammatory cells and induces nonphlogistic macrophages [11], [12]. In addition to these anti-inflammatory and pro-resolution effects, it

also presents analgesic effect by inhibiting TRPV1-induced neuronal currents and inhibits in vivo inflammatory- and chemotherapy-induced pain [16]. The prominent regulatory properties of MaR1 in inflammation have raised the question on whether this endogenous lipid could function as an exogenous anti-inflammatory agent. In the present study, the anti-inflammatory effect of MaR1 was investigated in a model of UVB-induced skin inflammation and oxidative stress in hairless mice

Materials and Methods

Materials

Brilliant blue R, reduced glutathione (GSH), hexadecyltrimethylammonium bromide (HTAB), N-ethylmaleimide, *o*-dianisidine dihydrochloride, phenylmethanesulfonyl fluoride, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), (2,4,6-Tris(2-pyridyl)-*s*-triazine) (TPTZ), nitroblue tetrazolium (NBT) and bisacrylamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). 7- (S) maresina 1 from Cayman Chemical (Ann Arbor, Michigan, USA). Tert-butyl hydroperoxide from Acros (Pittsburgh, PA, USA). Xylene cyanol and Tris were obtained from Amresco (Solon, OH, USA). ELISA kits for determination of cytokine were obtained from eBioscience (San Diego, CA, USA). Acrylamide and sodium dodecyl sulfate (SDS) (Superscrip III). All other reagents used were from pharmaceutical grade.

Animals. The experiments were performed in sex matched hairless mice (HRS/J), weighing 20-30 g, they were obtained from the University Hospital of Londrina State University. Mice had free access to water and food at a temperature of 23 ºC ± 2 and a 12 h light and 12 h

dark cycles. The Animal Ethics Committee (CEUA process number 1447.2015.10) of the Londrina State University approved all procedures of this study. All efforts were made in order to minimize the number of animals used and their suffering.

Experimental protocol. Hairless mice were randomly assigned to the following groups: non-irradiated control group, irradiated control group (saline), and irradiated treated groups. Mice were treated intraperitoneally with 7- (S) maresina 1 (MaR1: 0.1, 1,0 and 10 ng/kg, diluted in sterile saline) 1 h before and 7 h after the beginning of UVB irradiation. The doses of 7-(S) maresina 1 used in these assays were selected based on therapeutic effects reported previously [11], [14], [17]. Samples of skin were collected 2 h, 4 h or 12 h after the UVB exposure. Each parameter was evaluated at a specific time, which was previously determined as suitable to detect significant differences between negative and positive control groups, therefore, being suitable for determination of possible treatment effect [18]–[20]. All experiments were performed twice with 6 mice per group per experiment.

Irradiation. The UVB source used in the experiments to induce oxidative stress was a Philips TL/12 RS 40W (Medical-Holand) which emits a continuous spectrum between 270 and 400 nm with a peak emission at 313 nm. The lamp was mounted 20 cm above the place where the mice were placed on, resulting in an irradiation of 0.384 mW/cm^2 as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with sensor of UV (SED005) and UVB (SED240). The radiation dose for induction of inflammation and oxidative stress was 4.14 J/cm [1], [21], [22]. The mice were terminally anesthetized with 3% isoflurante 12 h (Figs. 3, 4, 5A, 5B and 5C), 2 h (Figs. 5D and 6B) or 4 h (Figs 6A and 7) after the UVB exposure, and the full dorsal skin was removed and stored at -80°C to further analysis. The sample collected for verification of cutaneous edema was weighed just at the moment it was removed. For that, it was not frozen [5], [23].

Skin edema. Constant areas (5 mm diameter) of dorsal skin were removed from euthanized mice with the aid of a mold and weighed [24], [25] . The skin edema analysis was obtained by comparing the weight of irradiated groups with non-irradiated group, and the results were expressed in mg of skin.

Myeloperoxidase (MPO) activity. The UVB-induced leukocyte migration to the skin was evaluated using the MPO colorimetric assay as described previously [25]. Samples of dorsal skin were homogenized in K_2HPO_4 buffer 0.05 M (pH 6.0) containing 0.5% HTAB using a Tissue-Tearor (Biospec). The homogenates were centrifuged at 16,100 *g* for 2 min at 4°C. The supernatant was removed to assay. Briefly, 30 µL of the resulting supernatant were mixed with 200 µL of 0.05 M K_2HPO_4 buffer (pH 6.0), containing 0.0167% *o*-dianisidine dihydrochloride and 0.05% hydrogen peroxide. The absorbance was determined at 450 nm (Asys Expert Plus, Biochrom). The MPO activity of samples was compared to a standard curve of neutrophils. The results are presented as MPO activities (number of neutrophils per mg of skin).

Matrix metalloproteinase-9 activity. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) substrate-embedded enzymography was used to detect enzymes with gelatinase activity. Assays were carried out as they were described previously [20], [25]. The dorsal skin of hairless mice (1:4, w/w dilution) was homogenized (T 18 basic, IKA) in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.01 M $CaCl_2$ and 1% protease inhibitor cocktail. Whole homogenates were centrifuged (12,000 *g*, 10 min, 4 °C) twice. The Lowry method was used to measure protein levels in skin homogenates. Aliquots of 50 µL were mixed with 10 µL of 0.1 M Tris-HCl (pH 7.4) containing 20% glycerol, 4% SDS and 0.005% xylene cyanol, and 25 µL of the mixture (40 µg of protein) were taken for electrophoresis in a gel containing 10% acrylamide and 0.025% gelatin. After electrophoresis, the gels were incubated for 1 h with

2.5% Triton X-100 under constant shaking, incubated overnight at 37°C in 0.05 M Tris-HCl (pH 7.4), 0.01 M $CaCl_2$ and 0.02% sodium azide, and stained on the following day with Brilliant blue R. After destaining in 20% acetic acid, the zone of enzyme activity was analyzed by comparing the groups in the ImageJ Program (NIH, Bethesda, MD, USA).

Reduced glutathione (GSH) assay. GSH levels were determined as described previously. [19], [20], [24]. The samples of skin were homogenized in 0.02 M EDTA using a Tissue-Tearor (Biospec 985370). Whole homogenates were treated with 50% trichloroacetic acid and they were centrifuged twice (2,700 *g*, 10 min, 4°C). The reaction mixture contained 50 µL of sample, 100 µL of 0.4 M Tris and 5 µL DTNB (1.9 mg/mL in methanol). The absorbance was read at 405 nm. The standard curve was prepared with GSH (5-150 µM) and the results are presented as µM of GSH per mg of skin.

Catalase assay. The catalase activity was evaluated by measuring the decay in the concentration of hydrogen peroxide (H_2O_2) and the generation of oxygen [18], [19]. Skin of hairless mice was homogenized in 500 µL of 0.02 M EDTA using a Tissue-Tearor (Biospec 985370), and it was centrifuged twice (2,700 *g*, 10 min, 4°C). The reaction mixture contained 10 µL of sample, 160 µL of buffer Tris-HCl 1 M with EDTA 5 mM (pH 8.0), 20 µL of deionized water and 20 µL of H_2O_2 200 mM. Measurement of catalase activity was estimated through the difference between the initial reading and the reading conducted 30 seconds after the addition of H_2O_2 at 240 nm in a microplate reader at 25ºC. The catalase values were expressed as unit of catalase/mg of skin/minute.

ABTS assay. The ability of reducing the ABTS radical was measured by the decreasing of absorbance at 730 nm [18], [19], [26]. Skin of hairless mice was homogenized in 400 µL of

KCl (1.15%) using a Tissue-Tearor (Biospec 985370) and it was centrifuged (1,000 *g*, 10 min, 4°C), and the supernatant was employed to measure the antioxidant capacity of skin. The solution of ABTS was prepared with 7 mM of ABTS and 2.45 mM of potassium persulfate diluted with phosphate buffer pH 7.4 to an absorbance of 0.7-0.8 in 730 nm. The supernatant (7 μL) was mixed on ABTS solution and after 6 min the absorbance was determined at 730 nm in a microplate reader. A curve of trolox (0.01-20 nmol) was prepared and the results are presented as nmol trolox equivalent per mg of skin.

FRAP assay. The reducing ability of skin sample was determined by FRAP assay [18], [19], [26]. The samples of skin were homogenized in 400 μL of KCl (1.15%) using a Tissue-Tearor (Biospec 985370), centrifuged (1,000 *g*, 10 min, 4°C), and the supernatant was employed to measure the antioxidant capacity of skin. The supernatant (30 μL) was mixed with the FRAP reagent (0.3 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM hydrochloride acid, and 20 mM ferric chloride). The absorbance was determined at 595 nm in a microplate reader. A curve of trolox (0.01-20 nmol) was prepared and the results are presented as nmol trolox equivalent per mg of skin.

Lipid peroxidation (LPO). LPO was measured by tert-butyl lipid hydroperoxides (LOOH)-initiated chemiluminescence [18], [19]. This test is based on the premise that there is an increase in chemiluminescence that is associated with oxidative stress leading to the consumption of the antioxidant defenses from the formation of hydroperoxides. Samples of skin were homogenized in 800 μL of phosphate buffer (pH 7.4) using a Tissue-Tearor (Biospec 985370), centrifuged (700 *g*, 2 min, 4°C). Then, 250 μL the supernatant was diluted in 1730 μL reaction medium (120 mM KCl, 30 mM phosphate buffer, pH 7.4) and mixed with 20 μL of 3 mM tert-butyl hydroperoxide. The reading was conducted in a β-counter Beckman®LS 6000SC in a non-coincident counting for 30 s with a response range between

300 and 620 nm. The vials were kept in the dark up to the moment of the assay, and determinations were obtained in dark in order to avoid vial phosphorescence activated by light. The experiment was conducted at 30°C for 120 min. The results were measured in counts per min (cpm) per mg of skin.

Superoxide anion production. The measurement of superoxide anion ($O_2^{\bullet-}$) production in the skin was performed using the nitroblue tetrazolium assay (NBT) as it was described previously [18], [19], [25]. Samples of skin were homogenized in 500 µL of 0.02 M EDTA using a Tissue-Tearor (Biospec 985370) and centrifuged (2000 *g*, 20 seconds, 4°C). Then, 50 µL of the supernatant were incubated in 96-well plate for 1 h. The non-adherent/non-precipitated supernatant was carefully removed, 100 µL of NBT (1 mg/ml) was added to each well and incubated over 15 min. NBT reaction medium was then carefully removed followed by fixation in methanol 100%. Formazan particles were dissolved by adding 120 µL of KOH 2M and 140 µL of dimethylsulfoxide. Reduction of NBT to formazan was measured at 600 nm using a microplate spectrophotometer reader. For that reason, the results are presented as optical density (OD) per 10 mg of skin.

Cytokine measurement. The skin samples were homogenized in 500 µL of saline solution using Tissue-Tearor (Biospec 985370). The homogenates were centrifuged (2,000 *g*, 15 min, 4 °C) and stored at -70ºC. Supernatants were used to measure the cytokine levels by an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience) [24]. Absorbance was determined at 450 nm in a microplate spectrophotometer reader and the results are expressed as picograms (pg) of each cytokine/mg of skin.

Histopathological analysis: For skin histopathological analysis, samples were collected 12 hours after UVB, fixed in 4% paraformaldehyde and dehydrated in a graded series of ethanol solutions for paraffin embedding. Five µm sections of skin samples were prepared and stained with hematoxylin e eosin (H&E). H&E stained sections were examined using light microscopy at 10x and 100x magnification for determination of epidermal thickness [27] and the number of sunburns cells [28], respectively.

Statistical analysis. The bars in the figures indicate the mean values ± standard error of the mean (SEM) of 6 mice per group per experiment and they are representative of two separate experiments. Data were statistically analyzed by one-way ANOVA followed by Tukey's *t* test. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Results were considered significantly different when $p < 0.05$.

Results

Treatment with MaR1 reduces UVB radiation-induced skin edema and MPO activity. The anti-inflammatory action of MaR1 was first assessed by the edema assay and MPO activity (neutrophil marker). UVB radiation induced significant increasing in skin edema and MPO activity in unthreading irradiated mice. Skin edema (Fig 1A) and MPO activity (1B) were inhibited by MaR1 treatment only at the dose of 10,0 ng/Kg .

Treatment with MaR1 reduces UVB radiation-induced apoptosis of Keratinocytes. After UVB radiation keratinocytes express a wide variety of cytokines and chemokines and alter their morphology by displaying condesian chromatin and eosinophil cytoplasm. Thus, these cells enter into apoptosis to eliminate damaged DNA [34], [35]. We investigated how was the

behavior of the apoptosis of keratinocytes in the treatment with different MaR1 doses (Fig. 2 A, B, C and D). The results showed that doses of 1 and 10 ng/Kg MaR1 inhibited sunburn cells (Fig. 2 F) and no effect was observed with 0.1ng/Kg of MaR1 and vehicle.

Treatment with MaR1 reduces UVB radiation-induced epidermal thickness. UVB radiation is absorbed by the epidermis and the acute skin response to its exposure includes erythema, edema and pigmentation, followed by epidermal thickening [29]. The results showed that 0.1, 1 and 10 ng/Kg MaR1 reduced epidermal thickening (Fig. 3).

Treatment with MaR1 inhibits UVB radiation-induced skin inflammation by reducing cytokine production. The inflammatory cytokine IL-1β and TNF-α and IL-10 are produced after excessive exposure to UVB radiation [30]. Based on this fact, we investigated whether the MaR1 treatment modulates cytokine production after UVB exposition. The results showed that MaR1 at the dose of 10 ng/Kg inhibited the increases of inflammatory cytokines IL-1β (Fig. 4A), TNF-α (Fig. 4B) and IL-10 (Fig. 4C).

Treatment with MaR1 prevents UVB radiation-induced skin MMP-9 activity. After exposure to UVB radiation occurs a significant increasing in the secretion/activity of gelatinases in the skin of mice. In this study, MMP-9 activity in the skin was induced by UVB exposition and it was inhibited by MaR1 treatment at dose of 10,0 ng/Kg MaR1 (Fig. 5 B).

Treatment with MaR1 prevents UVB radiation-induced oxidation. Firstly, we analyzed FRAP and ABTS radical scavenging assays to evaluate the effect of MaR1 in antioxidant capacity of the skin. In both assays, MaR1 inhibited the antioxidant depletion only at the dose of 10,0 ng/Kg , maintaining the antioxidant capacity close to the baseline (Fig. 6A and 6B).

Next, we evaluated the effects of MaR1 on two crucial ROS elimination systems: reduced glutathione (GSH) (Fig. 6C) and catalase (CAT) (Fig. 6D). In line with the FRAP and ABTS results, MaR1 protected mice skin from UVB-induced GSH and CAT depletion at the dose of 10,0 ng/kg.

Treatment with MaR1 reduces UVB radiation-induced hydroperoxides (LOOH) and superoxide anion ($O_2^{\bullet -}$) production. To complete the study of antioxidant activity of MaR1, we evaluated the production of LOOH and $O_2^{\bullet -}$. The $O_2^{\bullet -}$ reacts with H_2O_2 generating hydroxy radical that induces lipid peroxidation (LPO) [31]. In these assays, MaR1 inhibited LPO at the dose of 1,0 and 10,0 ng/kg, while no inhibition was observed at the other tested dose (0,1ng/Kg) (Fig. 7A). Lining up with the LPO data, MaR1 at the dose of 10,0 ng/kg inhibited UVB irradiation-induced $O_2^{\bullet -}$ production (Fig. 7B). Thus, it is plausible that MaR1 inhibits $O_2^{\bullet -}$ production and all the deleterious effects that follow this early event.

Discussion

Maresin (MaR1) is a pro-resolution lipid mediator derived from docosahexaenoic acid. Importantly, despite the current common sense that pro-resolution lipid mediators actively reduce inflammation, it remained to be investigated whether MaR1 would represent a therapeutic strategy to reduce UVB-induced skin inflammation and oxidative stress. The present study shows, to our knowledge, for the first time that MaR1 or a pro-resolution lipid mediator inhibits UVB-induced skin inflammation.

The present study starts by showing that MaR1 consistently reduced signs of inflammation and inflammatory parameters essential in the pathophysiology of UVB-induced skin inflammation. MaR1 inhibited UVB-induced skin edema, epidermis thickening,

neutrophil/macrophage recruitment (myeloperoxidase activity) and the number of sunburn cells in a dose-dependent manner. These results are in line with the MaR1 block of neutrophil chemotaxis as well as MaR1 induction of neutrophil apoptosis followed by efferocytosis by macrophages [11]. UVB also induces the apoptosis of keratinocytes, which can be observed as sunburn cells. Therefore, consistent and concurrent data reveal the inhibition of UVB-induced skin inflammation by MaR1.

After exposure to UV irradiation, keratinocytes express a variety of cytokines and chemokines. They are considered the major cells that initiate inflammatory responses [14] consisting of vascular permeability, edema and recruitment inflammatory cells (neutrophils) [32], [33]. The pro-inflammatory cytokines (TNF-α and IL-1) stimulate metalloproteinase expression in keratinocytes as well as fibroblasts in the skin, especially to MMP-9 that degrades collagen causing the skin photoaging [34], [35]. We demonstrated that treatment with MaR1 inhibited the production of TNF-α, IL-1β and the activity of MMP-9. In agreement, our work, previous studies showed that the MaR1 regulates pro-inflammatory cytokine TNF-α, through AP-1, inhibit the secretion of inflammatory cytokines such as IL-1β and IL-6 and consequently inhibits the enzyme MMP [36], [37]. According to Lo *et al.*, (2000) lipids mediators decrease LPS-induced phosphorylation and activation of mitogen-activated protein kinase. Thus, a variety of intracellular signaling steps are partly inhibited, reducing the production of pro-inflammatory molecules inhibiting COX-2 expression and activation of NF-kB. The MaR1 inhibition of TNF-α and IL-1β production might also have accounted to the reduction of myeloperoxidase activity since these cytokines are chemoattractant to neutrophils [50]. MaR1 also inhibited UVB-induced IL-10 production. IL-10 is an anti-inflammatory cytokine that is co-released with pro-inflammatory cytokines to limit the inflammatory reaction [51]. The concomitant inhibition of pro and anti-inflammatory cytokine production by MaR1 suggests that that blocks the release pathway of these cytokines.

Evidence demonstrates that MaR1 inhibits cytokine production in other models, Chatterjee *et al.*, (2014) demonstrated that MaR1 attenuates TNF- α induced of NAPH-

oxidases in vascular smooth muscle and endothelial cells. For instance Marcon *et al.*, (2013) demonstrated that the treatment with MaR1 reduced cytokines proinflammatory levels in murine models of colitis and Nordgren *et al.*, (2013) demonstrated that MaR1, given prior to or during organic dust extract treatment, reduces releasing of pro-inflammatory cytokines (TNF-α, IL-6, and CXCL1) in mouse lung slice. Further Gong *et al.*, (2014) showed that high-dose of MaR1 mitigated LPS-induced lung injury in mice by inhibiting neutrophil adhesions and decreasing the levels of pro-inflammatory cytokines.

The molecules that are present in the skin (lipid, protein and nucleic acid) can absorb ultraviolet radiation [29] and they can react with molecular oxygen (O_2) causing excess ROS through depletion of antioxidants and induction de cytokines [8], [40], [41]. UVB also has the ability induces H_2O_2 directly and indirectly though $O_2^{\bullet-}$ production [6].

Another important factor caused by UVB radiation is the hydroperoxide production (LOOH), [42] which may result in increasing production of inflammatory mediators in the skin such as phospholipase A2 and cyclooxygenase 2 (COX-2) [43]. UVB radiation is absorbed mainly in the epidermis, affecting the keratinocytes. The defense mechanisms present on the skin involve GSH chelates metal ions forming inert complexes preventing the oxidation process and CAT maintains skin integrity by neutralizing H_2O_2 into water and O_2, thus, preventing the generation of hydroxyl radicals ($^{\bullet}OH$).

Pro-resolution lipid mediators induce antioxidant effects *in vitro* as evidenced by two recent studies showing RvD1 [44] and RvE1 [45] reduce ROS production in macrophages. However, to our knowledge, no study has examined the effects of MaR1 on ROS generation in the skin. Here we have demonstrated that MaR1 inhibited UVB-induced depletion of GSH and reduction of CAT activity as well as reduced UVB-induced $O_2^{\bullet-}$ production. In the present study, MaR1 also maintained the skin ability to reduce the ions Fe^{+3} to Fe^{+2} and the ability to reduce the $ABTS^{+}$ radical. There is evidence that lipid mediators inhibit the production of LOOH, [46]–[48], which corroborate with the results this study, where treatment with MaR1

inhibited UVB-induced formation of hydroperoxide. Adding the beneficial effects of the use of MaR1 in damage caused by UVB, it inhibits sunburn cells keratinocytes.

In conclusion, the systemic administration of MaR1 protected the skin from the deleterious effects of UVB irradiation. We demonstrated that MaR1 inhibited the oxidative stress by maintaining GSH and CAT levels FRAP and ABTS activity, and inhibited superoxide anion production and lipid peroxidation. These antioxidant effects might be related to the pro-resolution/anti-inflammatory effects of MaR1, which inhibited UVB-induced skin edema, thickening, sunburn cell counts and myeloperoxidase activity, which were dependent on inhibiting cytokine production and MMP-9 activity. Importantly, MaR1 presented a dose-dependent effect.

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Figures

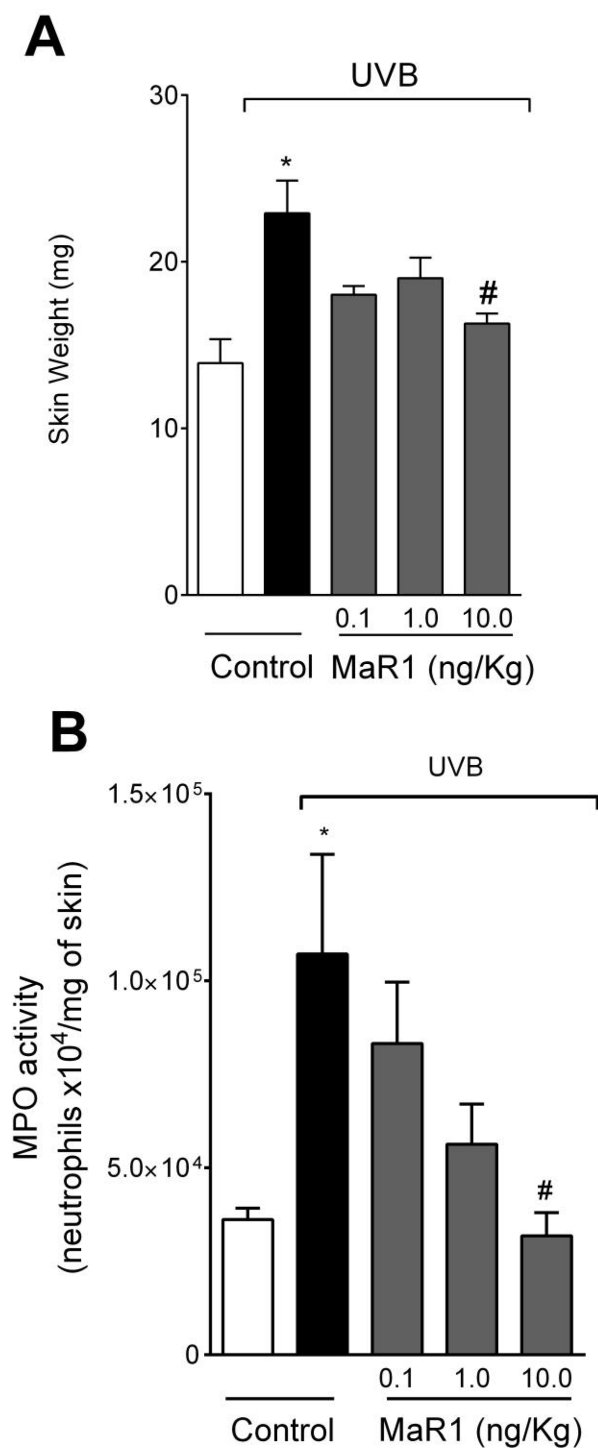


Fig. 1. MaR1 reduces UVB irradiation-induced skin edema and MPO activity. The skin edema (A) and MPO activity (B) were determined in samples collected 12 h after the end of radiation. Bars represent means ± SEM of 6 mice per group and are representative of two separate experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey´s test. * $p < 0.05$ compared to the non-irradiated control and # $p < 0.05$ compared to the irradiated control group (vehicle).

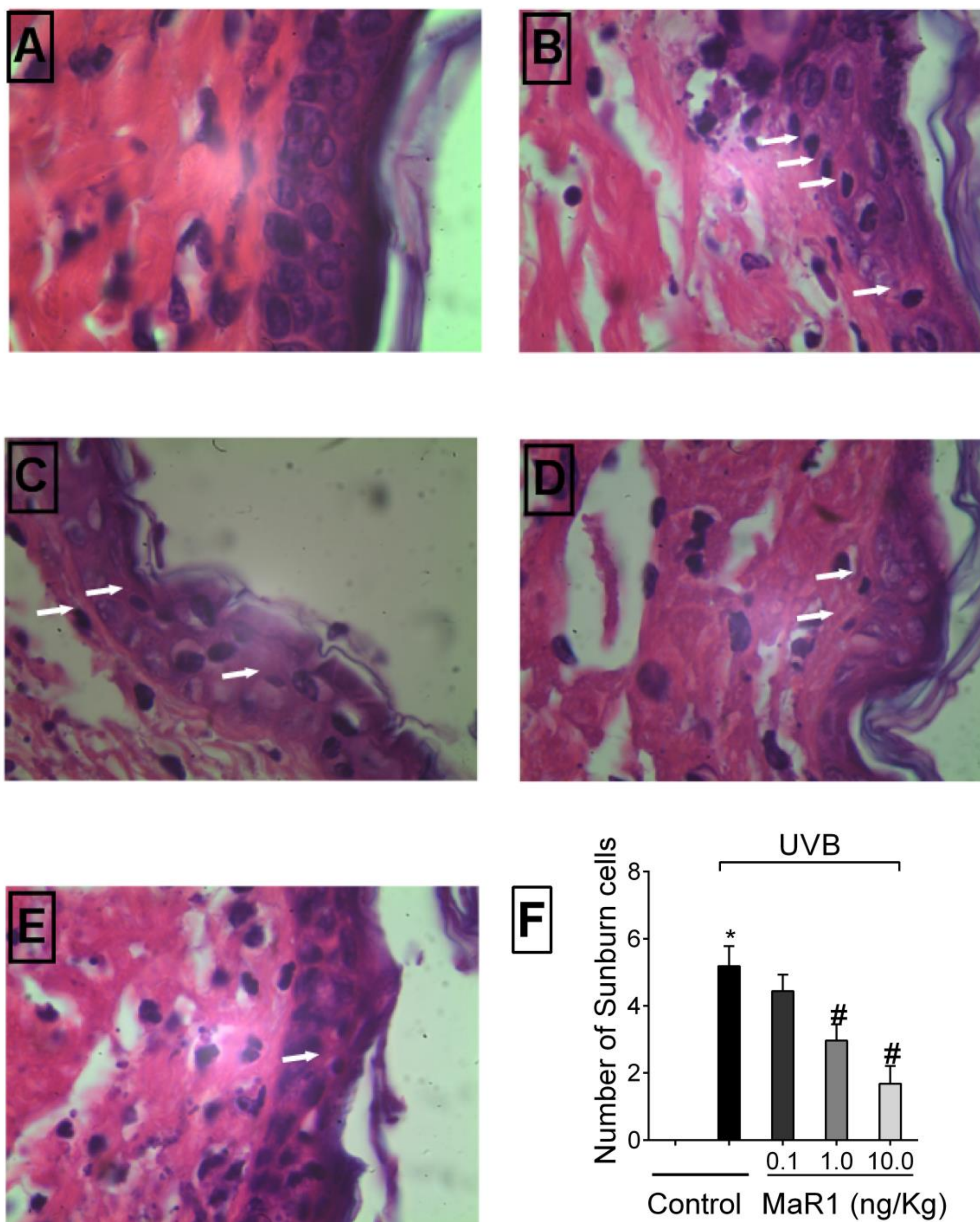


Fig. 2. MaR1 reduces UVB radiation-induced sunburns cells of Keratinocytes. The sunburns cells were evaluated in skin samples collected 12 h after radiation. Representative histological images of (A) negative control (B) irradiation control (C) 0.1 ng/Kg MaR1 (D) 1.0 ng/Kg MaR1 (E) 10.0 ng/Kg MaR1. (F) Bars represent means ± SEM of 6 mice per group and are representative of two separate experiments. Statistical analysis was perfomed by one-way ANOVA followed by Tukey´s test. *$p < 0.05$ compared to the non-irradiated control and # $p < 0.05$ compared to the irradiated control group (vehicle).

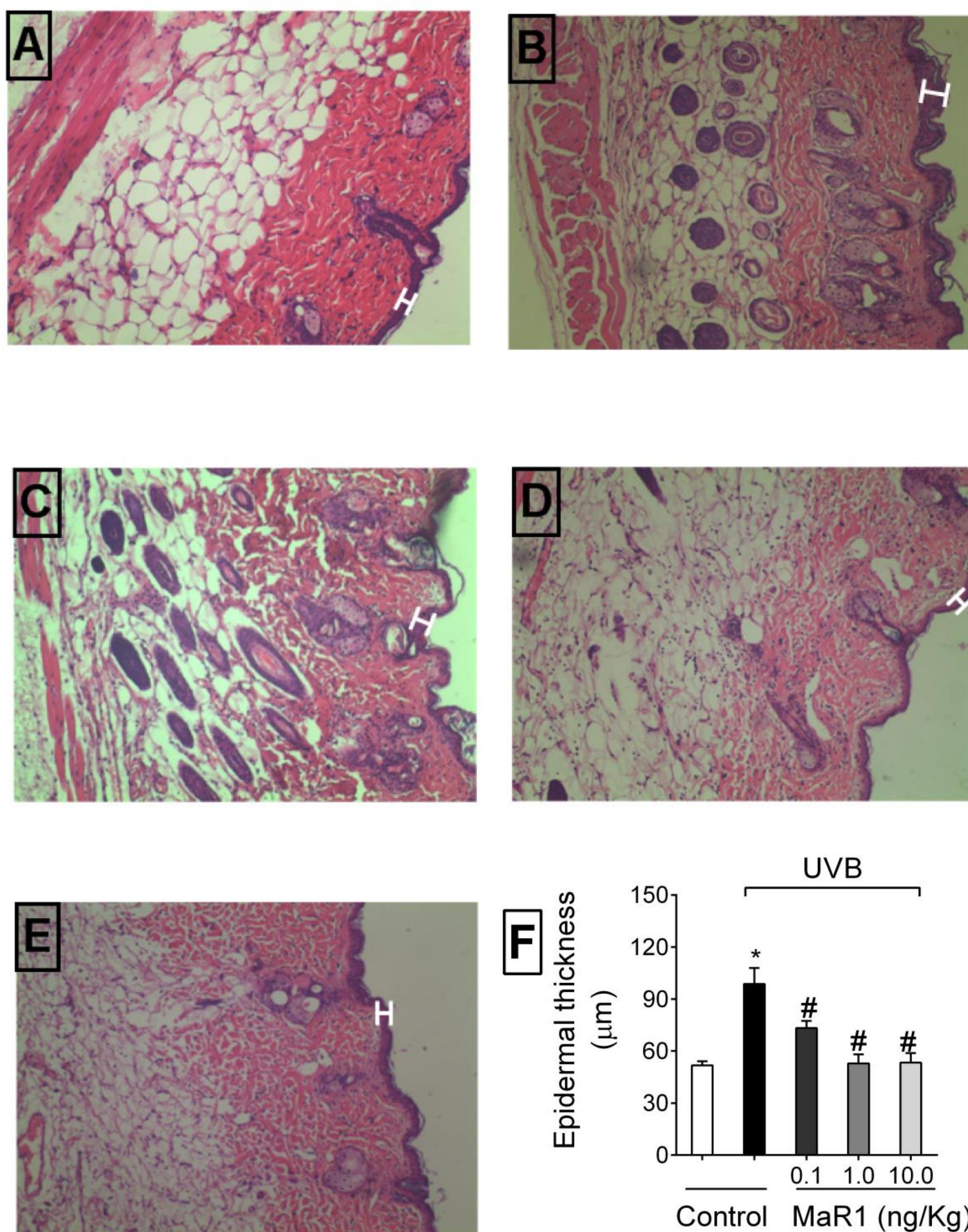


Fig. 3. MaR1 reduces UVB radiation-induced sunburns cells of Keratinocytes. The sunburns cells were evaluated in skin samples collected 12 h after radiation. Representative histological images of (A) negative control (B) irradiation control (C) 0.1 ng/Kg MaR1 (D) 1.0 ng/Kg MaR1 (E) 10.0 ng/Kg MaR1. (F) Bars represent means ± SEM of 6 mice per group and are representative of two separate experiments. Statistical analysis was perfomed by one-way ANOVA followed by Tukey´s test. *$p < 0.05$ compared to the non-irradiated control and # $p < 0.05$ compared to the irradiated control group (vehicle).

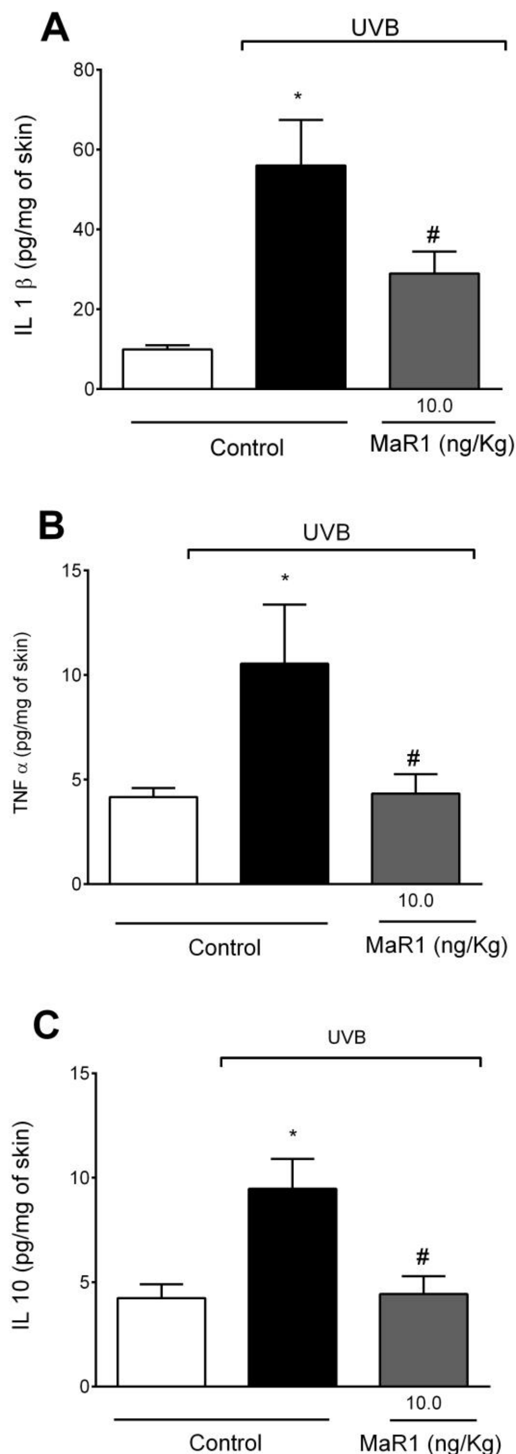
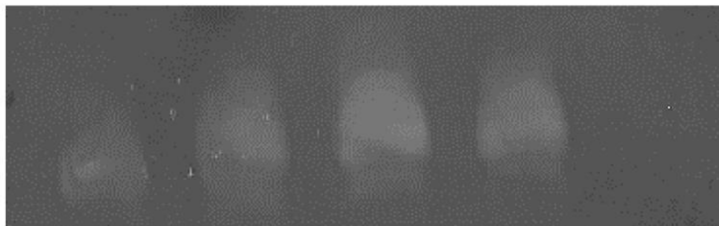


Fig. 4. MaR1 inhibits UVB irradiation-induced cytokine production. The levels of inflammatory cytokines IL-1β (A), TNF-α (B), and Il-10 (C) were determined by skin samples collected 4 h after the end of exposure to radiation. Bars represent means ± SEM of 6 mice per group and are representative of two separate experiments. Statistical analysis was perfomed by one-way ANOVA followed by Tukey´s test. * $p < 0.05$ compared to the non-irradiated control and # $p < 0.05$ compared to the irradiated control group (vehicle).

A



B

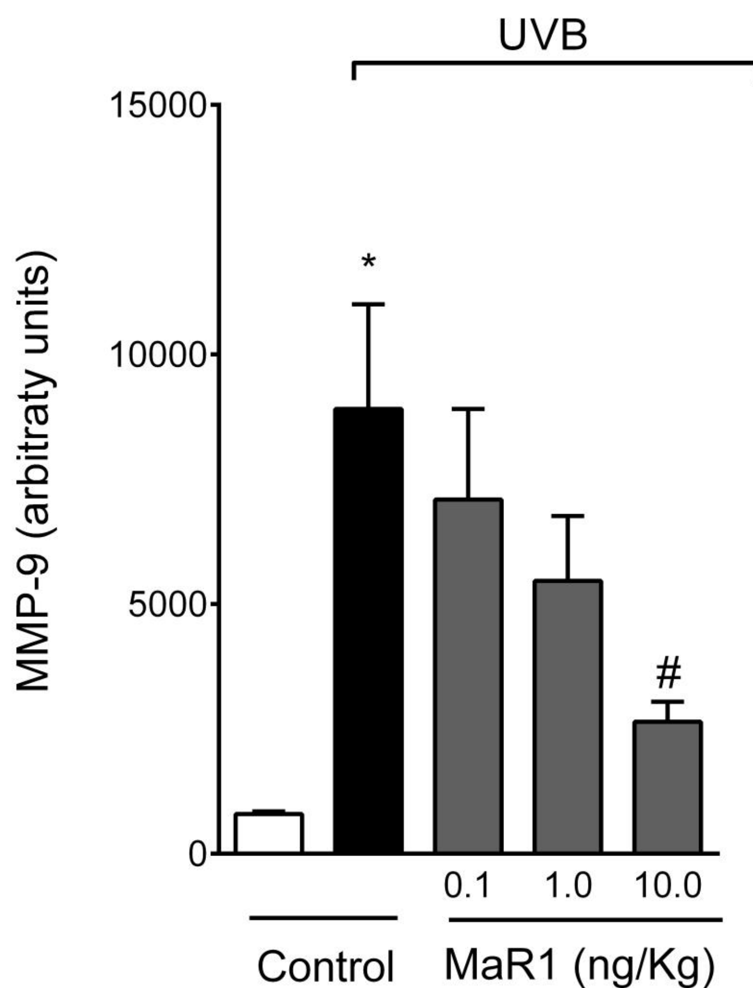


Fig. 5. MaR1 inhibits UVB irradiation-induced increase of MMP-9 activity. The MMP-9 activity was determined in samples collected 12 h after the end of radiation. (A) Image of gelatin zymography. (B) Bars represent means ± SEM of 6 mice per group and are representative of two separate experiments. Statistical analysis was perfomed by one-way ANOVA followed by Tukey´s test. * $p < 0.05$ compared to the non-irradiated control and # $p < 0.05$ compared to the irradiated control group (vehicle).

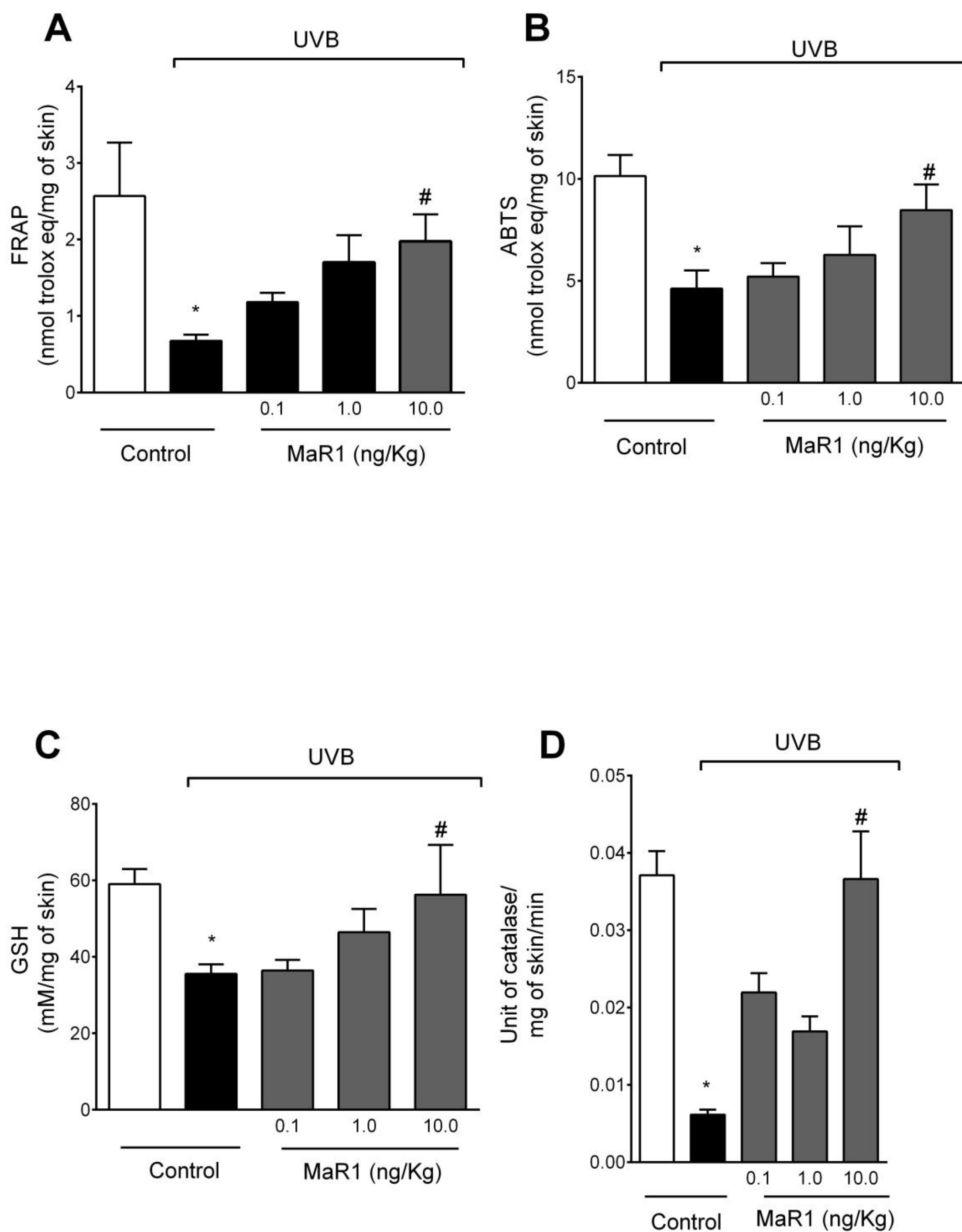


Fig. 6. MaR1 inhibits UVB irradiation-induced decrease of skin antioxidant capacity. The antioxidant capacity was determined by FRAP (A), ABTS (B), GSH (C) assays in samples collected 12 h after radiation. The CAT assays (D) was determined in samples collected 2 h after of radiation. Bars represent means ± SEM of 6 mice per group and are representative of two separate experiments. Statistical analysis was perfomed by one-way ANOVA followed by Tukey´s test. * $p < 0.05$ compared to the non-irradiated control and # $p < 0.05$ compared to the irradiated control group (vehicle).

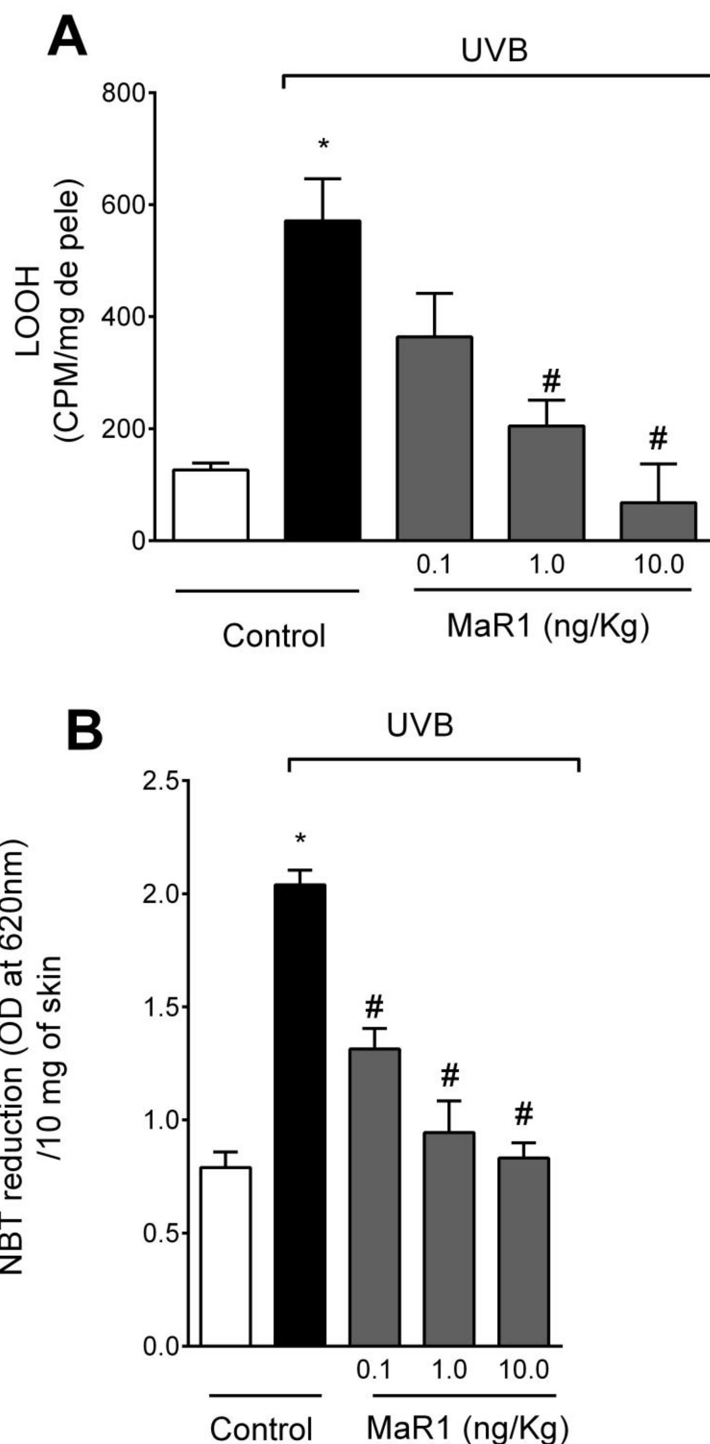


Fig. 7. MaR1 inhibits UVB irradiation-induced lipid peroxidation and superoxide anion generation.. The t-butyl LOOH-initiated chemiluminescence (QL) (A) and nitroblue tetrazolium (NBT) reduction (B) were determined in samples collected 4 h and 2 h after radiation, respectively. Bars represent means ± SEM of 6 mice per goup and are representative of two separate experiments. Statistical analysis was perfomed by one-way ANOVA followed by Tukey´s test. * $p < 0.05$ compared to the non-irradiated control and # $p < 0.05$ compared to the irradiated control group (vehicle).

5. CONSIDERAÇÕES GERAIS

A exposição à radiação ultravioleta induz profundas mudanças biológicas na pele, pois afeta os antioxidantes endógenos que protegem as células contra os efeitos deletérios causados pelas espécies reativas de oxigênio. Os efeitos podem ser agudos como o eritema, inflamação local e até mesmo crônico como o fotoenvelhecimento. Por isso, é necessário o estudo de novas substâncias antioxidantes com o intuito de prevenir a pele contra a ação da radiação UV. Diante desse quadro é promissor desenvolver terapias para o controle da inflamação e estresse oxidativo induzidos pela radiação UVB, tendo como alvo seus mecanismos fisiopatológicos. Estas terapias podem ser consolidadas baseando-se na redução ou resolução da inflamação tratando o processo desencadeado pela radiação UVB com mediadores lipídicos anti-inflamatórios/pró-resolução como a 7(S) Maresina 1. De maneira geral, os mediadores lipídicos inibem a produção de citocinas, recrutamento de células pró-inflamatórias, aumentam o recrutamento de células anti-inflamatórias, inibem a ativação do NFκB, inibem a expressão/atividade da NADPH oxidase e aumentam a expressão do Nrf2 e OH-1.

Os resultados *in vivo* mostraram que o lipídio MaR1 protegeu a pele contra a inflamação e contra os danos oxidativos induzidos pela radiação UVB comparado com o grupo controle radiado que não recebeu tratamento. O efeito antioxidante deste lipídio foi relacionado à manutenção do poder redutor do ferro, da capacidade em reduzir o radical ABTS, dos níveis de GSH, atividade de catalase e produção de ânion superóxido. Somam-se a isso, a MaR1 também foi capaz de inibir o, recrutamento de neutrófilos, atividade da MMP-9, produção de hidroperóxidos lipídicos e citocinas inflamatórias (IL-1 e TNF-α) e anti-inflamatória (IL-10).

Considerando os parâmetros oxidativos e inflamatórios avaliados e o efeito da MaR1 em reduzir esses sinais, sugere-se a MaR1 como uma nova alternativa terapêutica para o tratamento dos danos causados pela radiação UVB na pele.

6. REFERÊNCIAS

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7. ANEXO

Informações sobre submissão do artigo a revista Photochemical and Photobiological Science

Find out how to prepare your article and present your research clearly, ensuring that all the relevant information is included.

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