CAMILLA CAROLINA ARRIERO RODRIGUES

MARESINA 2 PROTEGE A PELE CONTRA DANOS INFLAMATÓRIOS E OXIDATIVOS CAUSADOS PELA RADIAÇÃO UVB EM CAMUNDONGOS SEM PELO

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciência da Saúde da Universidade Estadual de Londrina

Orientador: Profª. Drª. Rúbia Casagrande

Londrina
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A minha família que sempre me apoiou e esteve ao meu lado nesta trajetória, aos meus pais, irmão, marido e avós que me deram todo suporte para que eu pudesse concluir esse doutorado.

Dedico!

RESUMO

RODRIGUES, Camilla Carolina Arriero. **Maresina 2 protege a pele contra danos inflamatórios e oxidativos causados pela radiação UVB em camundongos sem pelo.** 2024. 100 p. Tese (Doutorado em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2024.

A pele é o maior órgão do corpo humano em termos de extensão e a primeira barreira do corpo aos fatores externos. Dentre estes, destaca-se a exposição à radiação UVB, que pode provocar aumento de radicais livres, inflamação da pele, podendo levar ao desenvolvimento de câncer e envelhecimento precoce. Neste contexto, os mediadores lipídicos encontrados endogenamente são de grande importância, pois possuem ação anti-inflamatória/pró-resolução. Entre os mediadores lipídicos, destaca-se o Maresina 2 (MaR2), um lipídeo pró-resolução derivado do ácido graxo ômega-3 endógeno, o ácido docosahexaenóico (DHA), que demonstrou eliminar radicais livres e inibir a inflamação. Esta pesquisa teve como objetivo avaliar a eficácia do MaR2 intraperitoneal (IP) ou incorporado em uma formulação tópica (FTcMaR2) contra danos cutâneos induzidos pela radiação UVB em camundongos. Para induzir dano cutâneo, camundongos pelados da linhagem HRS/J (CEUA número 148/2016, processo nº 11146.2016.97) foram expostos a uma dose aguda de irradiação UVB de 4,14 J/cm2, e amostras de pele dorsal foram coletadas para avaliação de a resposta inflamatória e o estresse oxidativo por meio de testes de edema cutâneo, atividade/secreção de metaloproteinase-9, capacidade antioxidante global (FRAP), níveis de antioxidantes endógenos glutationa reduzida (GSH) e catalase (CAT), produção de ânion superóxido e hidroperóxidos lipídicos e exame histopatológico avaliação com hematoxilina eosina (H&E), azul de toluidina e tricrômico de Masson. Assim, os animais foram submetidos a tratamentos com MaR2 nas doses de 1, 3 e 10 ng/animal. A dose de 10ng/animal mostrou-se mais eficaz nos parâmetros testados, a mesma dose foi inserida na formulação tópica. Os resultados *in vivo* mostraram que tanto o tratamento IP quanto o FTcMaR2 reduziram a inflamação da pele, reduzindo o edema cutâneo, a atividade da metaloproteinase-9 (MMP-9), a apoptose de queratinócitos, o espessamento epidérmico e o número de mastócitos. e a degradação das fibras de colágeno em comparação aos grupos controle. A mesma comparação foi realizada para determinar o efeito contra o estresse oxidativo induzido pela radiação UVB e constatou-se que tanto MaR2 IP quanto TFcMaR2 mantiveram os níveis de redução férrica (FRAP), atividade de GSH e CAT em níveis básicos, e também diminuíram a produção de LOOH e O_2^-Assim, os resultados sugerem que os tratamentos foram um poderoso aliado na prevenção/resolução da inflamação cutânea e na inibição do stress oxidativo induzido pela radiação UVB.

Palavras-chave: Maresina 2 (MaR2); Lipídio pró-resolução; Antioxidante; Inflamação; Radiação UVB.

ABSTRACT

RODRIGUES, Camilla Carolina Arriero. **Maresin 2 protects the skin against inflammatory and oxidative damage caused by UVB radiation in hairless mice**. 2024. 100 p. Thesis (Doctorate in Health Sciences) – State University of Londrina, Londrina, 2024.

The skin is the largest organ in the human body in terms of extension and the body's first barrier to external factors. Among these, exposure to UVB radiation stands out, which can cause an increase in free radicals, skin inflammation, which can lead to the development of cancer and premature aging. In this context, lipid mediators found endogenously are of great importance, as they have anti-inflammatory/pro-resolution action. Prominent among lipid mediators is Maresin 2 (MaR2), a pro-resolving lipid derived from the endogenous omega-3 fatty acid, docosahexaenoic acid (DHA), which has been shown to scavenge free radicals and inhibit inflammation. This research aimed to evaluate the efficacy of MaR2 intraperitoneal (IP) or incorporated into a topical formulation (TFcMaR2) against skin damage induced by UVB radiation in mice. To induce skin damage, nude mice of the HRS/J strain (CEUA number 148/2016, process number 11146.2016.97) were exposed to an acute dose of UVB irradiation of 4.14 J/cm2, and dorsal skin samples were collected for assessment of the inflammatory response and oxidative stress through skin edema tests, metalloproteinase-9 activity/secretion, global antioxidant capacity (FRAP), levels of endogenous antioxidants reduced glutathione (GSH) and catalase (CAT), anion production superoxide and lipid hydroperoxides and histopathological examination evaluation with hematoxylin eosin (H&E), toluidine blue and Masson's trichrome. Thus, the animals were subjected to treatments with MaR2 at doses of 1, 3 and 10 ng/animal. The dose of 10ng/animal proved to be more effective in the tested parameters, the same dose was inserted in the topical formulation. In vivo results showed that both IP and TFcMaR2 treatment reduced skin inflammation, reducing skin edema, metalloproteinase-9 (MMP-9) activity, keratinocyte apoptosis, epidermal thickening, and mast cell numbers. and the degradation of collagen fibers compared to control groups. The same comparison was carried out to determine the effect against oxidative stress induced by UVB radiation and it was found that both MaR2 IP and TFcMaR2 maintained the levels of ferric reduction (FRAP), GSH and CAT activity at basic levels, and also decreased the production of LOOH and O_2^-. Thus, the results suggest that the treatments were a powerful ally in the prevention/resolution of skin inflammation and in the inhibition of oxidative stress induced by UVB radiation.

Key-words: Maresin 2 (MaR2); TFcMaR2; Lipid pró-resolucion; Antioxidant; Inflammation; UVB radiation.

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LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2’ azino-bis (3-etilbenzotiazolina-6-ácido sulfônico)
ANOVA	Análise de variância
AP-1	Ativador de proteína-1
ARE	Elemento de resposta antioxidante
$CaCl_2$	Cloreto de cálcio
CAT	Catalase
DHA	Ácido docosahexaenoico
DNA	Ácido desoxirribonucleico
DTNB	Ácido 5,5’-ditiobis (2-nitrobenzoico)
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Ensaio imunoenzimático
EPA	Ácido eicosapentaenoico
ERK	Quinase regulada por sinal extracelular
EROS	Espécies Reativas de Oxigênio
FRAP	Poder antioxidante de redução férrica
GPx	Glutationa peroxidase
GSH	Glutationa reduzida
GSSG	Glutationa Oxidada
GST	Glutationa-S-transferase
H&E	Hematoxilina e eosina
H_2O_2	Peróxido de hidrogênio
HCl	Ácido clorídrico
HO-1	Hemeoxigenase-1
HOCl	Ácido hipocloroso
HTAB	Brometo de hexadecil trietil amônio
IL	Interleucina
IP	Intraperitoneal
κB	Fator inibidor de κB
JNK	Quinase c-Jun n-terminal
K_2HPO_4	Fosfato de potássio dibásico
KCl	Cloreto de potássio

KEAP1	Proteína Kelch 1 associada a ECH
KH_2PO_4	Fosfato de potássio monobásico
KOH	Hidróxido de potássio
LOOH	Hidroperóxidos lipídicos
LPO	Peroxidação lipídica
MAPK	Proteína ativada por mitógeno
MaR2	Maresina 2
MMPs	Metaloproteinases da matriz
MPO	Mieloperoxidase
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NBT	*Nitroblue tetrazolium*
NF-κB	Fator nuclear-κB
NO	Óxido nítrico
$NO^{\bullet}$	Radical óxido nítrico
NQO1	NADPH: quinona oxidoredutase 1
Nrf_2	Fator nuclear [derivado eritróide-2] tipo 2
O_2	Oxigênio molecular
$O_2^{\bullet-}$	Radical superóxido
$^1O_2$	Oxigênio singlete
O_3	Ozônio
OH-1	Heme-oxigenase 1
•HO	Radical hidroxil
$ONOO^-$	Peróxido nitrito
PCR	Reação em cadeia da polimerase
RNAm	Ácido ribonucleico mensageiro
RUV	Radiação ultravioleta
SDS	Dodecil sulfato de sódio
SOD	Superóxido dismutase
TCA	Ácido tricloro acético
TGF-β	Fator de transformação do crescimento
TNF-α	Fator de necrose tumoral α
TRIS	Hidroximetil aminometano
UVB	Radiação ultravioleta B
UVB	Radiação ultravioleta B

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1. INTRODUÇÃO

1.1 A Pele

A pele é o maior órgão corpo humano e o único que está exposto diretamente ao ambiente. É a primeira linha de defesa do organismo contra estresses ambientais como patógenos, agentes químicos diferentes e radiação UV (Xu e Fisher, 2005). Como parte do organismo, a pele também controla a troca de água com o meio ambiente e controla a temperatura corporal. O órgão é dividido em três camadas, sendo a epiderme a camada mais externa que está em contato direto com o meio ambiente; a derme a camada intermediária e a hipoderme ou tecido subcutâneo a camada mais profunda. A epiderme é um tecido estratificado, em constante renovação e intenso metabolismo. Ela apresenta espessura que varia de 0,05 mm em áreas como as pálpebras até 1,5 mm em áreas como a sola dos pés e as palmas das mãos (Fonseca, Catini, *et al.,* 2011; Khavkin e Ellis, 2011).

Os queratinócitos compõem 95% das células da epiderme, mas nela também estão presentes outros tipos celulares como as células de Langherans, responsáveis pela resposta imunológica; as células de Merkel responsáveis por captação de estímulos sensoriais e os melanócitos, células responsáveis pela proteção da pele contra a radiação ultravioleta através da produção do pigmento melanina. A camada basal, que é camada mais interna da epiderme, é formada por células tronco que sofrem divisão e diferenciação programada enquanto migram para a superfície, onde chegam como queratinócitos anucleados e preenchidos por citoqueratinas (Fonseca, Catini, *et al.,* 2011; Khavkin e Ellis, 2011).

Os queratinócitos criam uma barreira física de proteção da pele, além de acumularem pigmentos de melanina que são eficazes no bloqueio da penetração da RUV na pele. A melanina também está relacionada com funções importantes na pelecomo a homeostase epidérmica, eliminação de radicais livres e, possivelmente, até mesmo com atividade antimicrobiana (D'Orazio *et al.*, 2013).

A melanina existe em duas formas químicas principais: a eumelanina e a feomelanina. A eumelanina está presente em maior quantidade em indivíduos de pele escura e é muito mais eficiente em bloquear os fótons UV do que a feomelanina,portanto as pessoas de pele clara são quase sempre mais sensíveis a radiação UV e têm risco maior de desenvolver câncer de pele comparada aos indivíduos de pele mais escura (Lassalle *et al.*, 2003; Vincensi *et al.*, 1998).

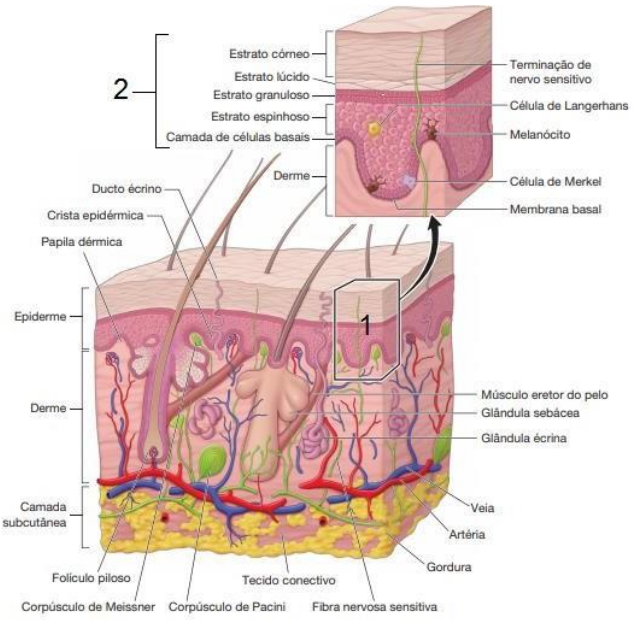

Figura 1 - Corte transversal da pele, delimitação das camadas: epiderme, derme e hipoderme, (1) ampliação da área de epiderme e começo de derme (2) e diferenciação dos estratos da epiderme. Fonte: Menon, Cleary and Lane, 2015.

A derme, que compreende a maior parte da pele, está situada abaixo da epiderme e é composta por um tecido conectivo fibroso, filamentoso e amorfo (Khavkin e Ellis, 2011). Este tecido é composto por fibras de colágeno, responsáveis pela resistência da derme; elastina, responsável pela elasticidade do tecido, e uma matriz composta por polissacarídeos. Os fibroblastos são as células predominantes na derme, mas também podem ser encontrados tipos de células imunológicas, tais como macrófagos, mastócitos, linfócitos e células dendríticas. Além dos vasos sanguíneos e linfáticos, nervos e terminações nervosas, há também os anexos, como glândulas sebáceas, sudoríparas e folículos pilosos, que desempenham um papel fundamental na manutenção da temperatura e das funções sensoriais da pele (Fisher *et al.*, 1997; Khavkin e Ellis, 2011).

O colágeno compõe 70% do peso da derme. As fibras de colágeno estão em constante renovação, sendo degradadas por enzimas como proteinases e metaloproteinases, e substituídas por fibras novas. Além do colágeno, a derme também apresenta fibras de elastina, uma proteína que se destaca pela elasticidade, sendo abundante na pele e em outras estruturas elásticas, como artérias e tendões (Vincensi *et al.*, 1998; D'Orazio *et al.*, 2013; Ozeki *et al.*, 2015; Shimizu, 2017).

A hipoderme é formada por uma camada de gordura subcutânea que funciona como estoque de energia e barreira mecânica. Ademais, a hipoderme também é composta por tecido conectivo e tem como objetivo manter a conexão entre a derme e os músculos e ossos (Shimizu, 2017).

1.2 Pele como via de administração

A pele tem se mostrado uma eficiente via de administração de formulações medicamentosas. O pH da pele se encontra na faixa de 5.5 e 6.5. A aplicação tópica possibilita a administração de medicamentos de forma segura e efetiva, além de evitar efeitos como irritação gastrointestinal, toxicidade sistêmica e metabolismo hepático quando administrado por via sistémica. As formulações precisam estar próximas ao pH da pele, estando abaixo do valor, se tornam uma formulação ácido, que é irritativa a pele. Uma das grandes vantagens da administração tópica de fármacos é a ação direta no sítio alvo ou muito próximo, necessitando assim de uma menor quantidade de substância ativa, e consequentemente menos efeitos secundários (Benson e Watkinson, 2012).

Alguns compostos são indicados para permanecerem na superfície da pele, como por exemplo, os protetores solares ou os cremes barreira, enquanto outras substâncias destinam-se a atingir a epiderme e derme, como por exemplo os analgésicos locais ou os antifúngicos (Ueda *et al.*, 2010).

O transporte de insumos pela pele pode ocorrer por três vias: Intracelular (diretamente através de células), intercelular (em torno das células) ou por anexos da pele (Trommer e Neubert, 2006). A primeira ocorre com a difusão do ativo que passa diretamente pelos corneócitos e pela matriz lipídica intercelular; a segunda o ativo passa entre as células permitindo a difusão de solutos lipofílicos ou apolares através da matriz lipídica contínua (rota proposta para a maioria dos ativos) e a terceira pelos apêndices cutâneos (glândulas ou folículos pilosos) (Allen, l. v. jr.; Popovich, n. g.; Ansel, 2007).

Em média, na superfície cutânea existem 40-70 folículos pilosos e 200-250 ductos sebáceos por metro quadrado de área de pele e por eles a absorção cutânea é muito intensa (Chien e Liu, 1986) entretanto, estes apêndices da pele representam apenas 0,1% da superfície cutânea total, e desta maneira, a absorção percutânea pela via transepidérmica se torna a principal via de permeação de absorção de fármacos (Allen, l. v. jr.; Popovich, n. g.; Ansel, 2007).

É necessário ter em consideração o tipo de fármaco a veicular, uma vez que este fator vai estar relacionado com o tipo de transporte. Substâncias hidrofílicas ou com carga penetram com mais dificuldade o estrato córneo, devido à sua natureza lipídica e seu teor baixo de água. Por esta razão, o transporte de substâncias lipofílicas é facilitado, devido à dissolução nos lipídeos intercelulares do estrato córneo (Stahl, Wohlert e Kietzmann, 2011).

1.3 Radiação

A RUV é o agente físico mais presente e a principal causa de danos à pele. A exposição aguda à RUV pode causar diversos efeitos adversos, como edema, queimaduras solares, eritema, inflamação e imunossupressão. Além disso, a exposição crônica pode levar

ao envelhecimento precoce e ao desenvolvimento do câncer de pele (Afaq, Adhami e Mukhtar, 2005; Fonseca, Marquele-Oliveira, *et al.*, 2011; Martinez *et al.*, 2015; Tewari *et al.*, 2013).

Ao longo dos anos, a mudança no estilo de vida da população, o aumento da expectativa de vida e o esgotamento da camada de ozônio provocaram um aumento significativo na quantidade de RUV que as pessoas estão sujeitas, resultando em um aumento na incidência de câncer de pele em indivíduos (Afaq, Adhami e Mukhtar, 2005; Bowden, 2004). De acordo com o INCa (Instituto Nacional de Câncer José Alencar Gomes da Silva), o câncer de pele do tipo não melanoma é o mais frequente no Brasil, representando 31,3% de todos os tumores malignos registados (INCa, 2023), sendo esse um problema significativo associado à mortalidade e à morbidade da população, logo a pesquisa e o desenvolvimento de novas estratégias para a sua prevenção são de extrema importância para a proteção da população (Afaq, Adhami e Mukhtar, 2005).

A luz solar é um espectro eletromagnético contínuo, dividido de acordo com a faixa de comprimento de onda: luz visível, infravermelho e radiação ultravioleta. A radiação ultravioleta é composta por 5% da radiação solar que atinge a Terra e está dividida em três regiões: UVC (100-280 nm), UVB (280-315 nm) e UVA (315-400 nm) A UVC é a mais energética e lesiva para as células, porém é totalmente filtrada pela camada de ozônio, já a UVA e a UVB são capazes de atravessar a camada de ozônio e causar uma série de efeitos lesivos na pele (Svobodova, Walterova e Vostalova, 2006; González, Fernández-Lorente e Gilaberte-Calzada, 2008; Balogh, Pedriali e Kaneko, 2011; Tewari *et al.,* 2013).

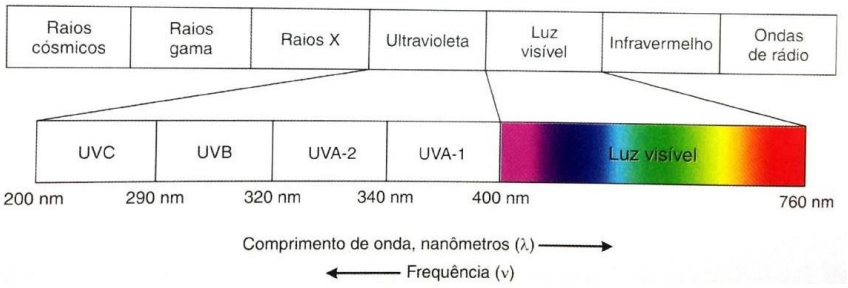

Figura 2 - Espectro eletromagnético. Fonte: Adriano *et al*., 2013.

Quanto menor o comprimento de onda, mais energética é a radiação, e, portanto, maior o grau de severidade das lesões. Assim, os raios UVC são os mais danosos ao ser humano, porém, são absorvidos pela camada de ozônio e normalmente não atingem a terra. Já as radiações UVB e UVA são capazes de penetrar a camada de ozônio e atingir a superfície do planeta, atuando de maneira específica conforme suas características energéticas, causando lesões no organismo com diferentes níveis de gravidade (Svobodova, Walterova e Vostalova, 2006; González, Fernández-Lorente e Gilaberte-Calzada, 2008; Balupillai *et al.,* 2018).

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). Os efeitos da UVA são queimadura solar, envelhecimento precoce da pele e supressão da função imune.

Já a radiação UVB apesar de representar apenas 5% da radiação RUV que atinge a superfície terrestre, é mais energética que a UVA, alcançando a epiderme, afetando diretamente os queratinócitos e a porção superior da derme. Desta forma, além da formação de radicais livres e outras espécies reativas e todos os danos por elas gerados, a UVB causa também alterações diretas no DNA, podendo levar a mutações, responsáveis pela formação de fotoprodutos diméricos, como pirimidinas, que podem causar lesões cutâneas pré-malignas (Figura 3).

A radiação UVB pode também causar manchas, descamação e queimaduras com vermelhidão e bolhas (Nichols e Katiyar, 2010; Shetty *et al.*, 2015; Tewari *et al.*, 2013).

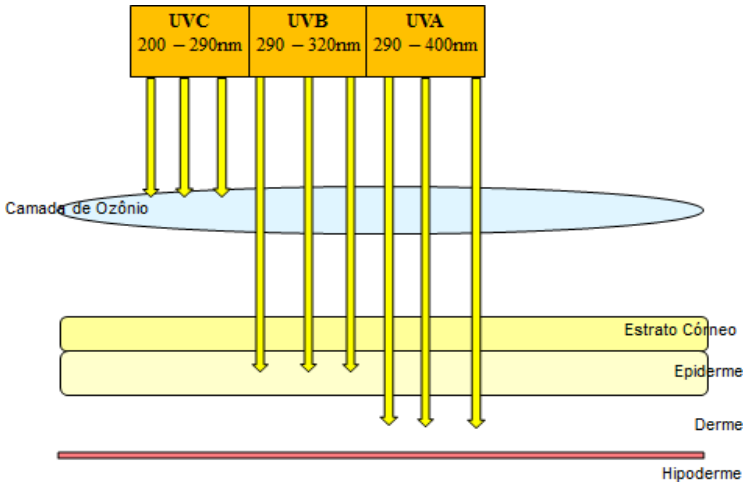

Figura 3 - Absorção da RUV pelas camadas da pele. UVC radiação de menor comprimento de onda sendo bloqueado pela camada de ozônio. UVB absorvida principalmente pela epiderme. UVA absorvida tanto pela epiderme como pela derme. Fonte: próprio autor.

As radiações ao atingirem a pele, são parcialmente refletidas, refratadas e, em parte absorvidas. As moléculas que estão presentes na pele (lipídios, proteínas, ácidos nucléicos) e apresentam duplas ou triplas ligações de carbono podem absorver a radiação UV, sendo então denominadas de cromóforos. 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 (Tewari *et al.*, 2013; Xu e Fisher, 2005).

1.4 Espécies reativas de oxigênio e estresse oxidativo

Um radical livre é qualquer molécula ou átomo que contenha um ou mais elétrons desemparelhados. Os radicais são produzidos por vias metabólicas de forma controlada e executam funções essenciais para homeostase como na respiração aeróbica, na ativação de fagócitos como mecanismo de defesa e podem fornecer controle essencial das vias de sinalização reguladas por redox. No entanto, se produzidos de forma exacerbada estimulados por alguma doença ou xenobióticos podem interagir com vários componentes do tecido, instalando o que chamamos de estresse oxidativo. Tais interações podem causar disfunções agudas e crônicas (Sies e Mehlhorn, 1986).

O mecanismo de ação das espécies reativas de oxigênio (EROs) é por óxido- redução e são gerados de diferentes maneiras. Uma delas ocorre durante a respiração celular aeróbia, na qual o oxigênio sofre redução, resultando na formação de H_2O e formação de intermediários reativos como o radical superóxido ($O_2^{\bullet-}$), o radical hidroxil ($^{\bullet}OH$), o radical óxido nítrico ($NO^{\bullet}$), o radical peroxil ($RO_2^{\bullet}$) e algumas espécies reativas não radicalares como o peróxido de hidrogênico (H_2O_2), ácido hipocloroso (HOCl) e oxigênio singlete ($^1O_2$), que apesar de não possuírem elétron livre, podem facilmente sofrer reações e formar radicais livre (Halliwell, 2015; Kohen e Nyska, 2002; Lin e Beal, 2006).

Em condições normais, a concentração das EROs dentro das células é extremamente baixa, pois o organismo apresenta mecanismos endógenos para manter a concentração de EROs dentro dos limites fisiológicos (Ribeiro *et al.*, 2005). As EROs reagem modificando moléculas por oxidação e desempenham um papel crítico nas vias de sinalização celular, como metabolismo, crescimento, diferenciação e sinalização de morte (Idelchik *et al.*, 2017).

Além disso, a atividade da xantina oxidase, o citocromo P450- oxidase, enzimas envolvidas na via de produção de prostaglandinas e tromboxanos e a NADPH-oxidase da membrana plasmática de células polimorfonucleares, macrófagos e células endoteliais, que produzem uma grande quantidade de EROs em resposta ao estímulo fagocitário, também constituem importantes fontes endógenas geradoras de EROs (Halliwell, 2006).

Quando a produção de EROs ultrapassa a capacidade do sistema antioxidante endógeno em reduzi-las, a concentração de radicais livres aumenta incontrolavelmente, rompendo o equilíbrio oxidante/antioxidante no organismo, instalando o estresse oxidativo (Lobo *et al.*, 2010) que pode ocasionar danos importantes em moléculas como DNA e proteínas, lipídeos bem como causa lesões implicadas na carcinogênese (KANG et al., 1998) e envelhecimento precoce (Ivan *et al.*, 2014; Reeve *et al.*, 2010).

No metabolismo endógeno, existem antioxidantes de defesa que protegem as células contra os efeitos dos radicais livres que podem ser classificados em antioxidantes enzimáticos e não enzimáticos. Entre os enzimáticos destacam-se a superóxido dismutase (SOD) que

decompõe o $O_2^{\bullet-}$ em H_2O_2, catalase (CAT) que converte o H_2O2 em H_2O e O_2, glutationa peroxidade (GPx) que catalisam a degradação de $H2O2$ em glutationa oxidada (GSSG) e água, glutationa redutase (GR). Entre os não enzimáticos incluem-se o α-tocoferol (vitamina E), β-caroteno, ácido ascórbico (viatamina C) e a glutationa reduzida (GSH) (Halliwell e Cross, 1994; Huber, Almeida e Fátima, De, 2008; Reiniers *et al.*, 2014).

1.5 Mecanismos fisiopatológicos da irradiação UVB

A irradiação UVB gera EROs, que causam danos oxidativos às proteínas, lipídios e DNA na pele levando ao envelhecimento (Choi *et al.*, 2013; Karthikeyan *et al.*, 2016). A produção excessiva destas EROs leva a distúrbios também nas vias de sinalização (Balupillai *et al.*, 2018).

Uma das principais respostas celulares contra essa alteração é o estímulo para produção de maior quantidade de antioxidantes através da indução do fator de transcrição Nrf2 (fator nuclear [derivado eritróide-2] tipo 2), que está presente no citoplasma das células (Malhotra *et al.*, 2010). Em condições normais, a proteína Keap1 forma um complexo com Nrf2, regulando sua ação e impedindo sua ida para o núcleo mediada por ubiquitinação e posterior degradação através do sistema proteassomal. Após a exposição à UVB, esse complexo é dissociado e o Nrf2 é deslocado para o núcleo, ligando-se ao elemento de resposta antioxidante (ARE). A interação entre Nrf2 e ARE leva a ativação da transcrição de genes que codificam proteínas com ações antioxidantes/detoxificantes como a hemeoxigenase-1 (HO-1), NADPH quinona oxidoredutase 1 (NQO1), GR e GPx, CAT, SOD. Neste contexto, o Nrf2 tem apresentado efeito protetor por meio da regulação de moléculas com ações benéficas, garantindo a sobrevivência da célula através da eliminação de substâncias oxidantes (Kobayashi *et al.*, 2004; Kobayashi e Yamamoto, 2005; Lee e Chau, 2002; Motohashi e Yakamoto, 2007).

Acredita-se que a modulação da atividade de Nrf2 por qualquer composto natural ou sintético é uma nova estratégia terapêutica para o tratamento de doenças (Abed *et al.*, 2015; Lu *et al.*, 2016; O'Connell e Hayes, 2015; Yu e Kensler, 2005; Zhuang *et al.*, 2014). Com a inibição da atividade de Nrf2, aumenta os danos cutâneos induzidos pela radiação UVB, devido ao aumento da produção de mediadores inflamatórios e metaloproteinase de matriz do tipo 9 (MMP-9) (Kumar *et al.*, 2011).

A irradiação ultravioleta ativa o fator de crescimento e os receptores na superfície dos queratinócitos (KC) e fibroblastos (FB). Os receptores ativados estimulam as cascatas de transdução de sinal que induzem o fator de transcrição AP-1, que estimula a transcrição dos genes da metaloproteinase de matriz (MMP), a MMP-9 (gelatinase), entre outras (Quan *et al.*, 2009). As metaloproteinases da matriz são secretadas pelos queratinócitos e fibroblastos e

também pelos grânulos terciário dos neutrófilos e quebram o colágeno e outras proteínas que compõem a matriz extracelular dérmica, além de interferirem na transcrição do gene do colágeno (Lee *et al.*, 2008). O reparo imperfeito do dano dérmico prejudica a integridade funcional e estrutural da matriz extracelular. A exposição repetida ao sol provoca acúmulo de danos dérmicos que eventualmente resultam em enrugamento característico da pele fotodanificada (John e Tuszynski, 2001).

Em resumo, a exposição à radiação UVB induz a formação de espécies reativas de oxigênio (EROs). O aumento de EROs causa o desequilíbrio entre moléculas pró e antioxidantes, gerando estresse oxidativo, que por sua vez, danifica lipídios, proteínas e DNA, este dano ao DNA pode ser pelas espécies reativas ou pelo dano direto da radiação ao DNA, podendo ocasionar erro na divisão celular. As EROs causam a ativação de fatores de transcrição como Nrf2, JNK e NF-kB. Esses fatores de transcrição se ligarão às suas sequências específicas no DNA, O elemento responsivo antioxidante (ARE), AP-1 e NF-kB, respectivamente. Estes fatores produzem mediadores inflamatórios como COX-2, prostaglandinas e interleucinas, porém também estimulam a produção de enzimas e proteínas antioxidantes como catalase e superóxio dismutase. A inflamação causa edema, eritema além de aumentar ainda mais a formação de EROs. A alteração induzida por EROs em lipídios e proteínas leva a uma sinalização celular anormal potencialmente promovendo a carcinogênese. O estresse oxidativo causa a síntese e liberação de metaloproteinases de matriz (MMPs) que degradam o colágeno, um biomarcador do envelhecimento da pele (Figura 4) (Dunaway *et al*., 2018).

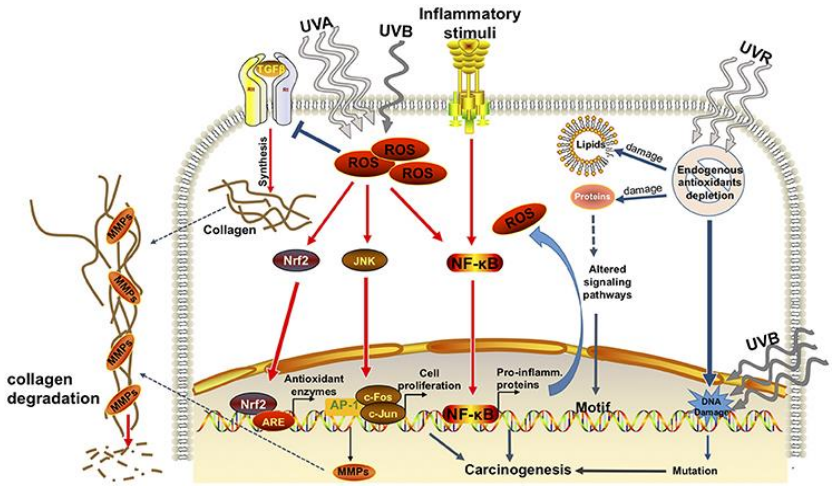

Figura 4 – Esquema da formação de EROs, a partir da radiação UVB, e consequências desta exposição nos lipídios, proteínas e DNA. Fonte: Dunaway *et al*., 2018.

O processo oxidativo induzido pela radiação UVB afeta macromoléculas, incluindo lipídios. Os radicais livres em excesso agem sobre a membrana biológica das células e a

disponibilidade de ácidos graxos poli-insaturados regula o processo de peroxidação lipídica (LPO). A LPO leva a uma desorganização da membrana celular com liberação de ácido graxo como o ácido araquidônico a partir de fosfolipídios, o que desencadeia aumento da fosfolipase A2 e da cicloxigenase-2 (COX-2), resultando em níveis aumentados de prostaglandinas, incluindo a prostaglandina E2 (PGE2) e inflamação na pele, os produtos primários da LPO incluem os hidroperóxidos lipídicos (LOOH) e sua decomposição da origem ao radical peroxil ($ROO^{\bullet}$) muito reativo e o responsável por propagar a LPO para as outras células (Halliday, 2005a).

A radiação UVB leva a uma inflamação aguda, e suas principais características são marcadas pela dilatação vascular e aumento no fluxo sanguíneo causando eritema e calor, extravasamento e deposição extravascular de fluido plasmático e proteínas a chamada exsudação causando edema, e a migração e acúmulo de leucócitos no local da injúria (Rittié e Fisher, 2002). A exsudação indica um aumento na permeabilidade normal dos pequenos vasos sanguíneos em uma área de injúria e, portanto, uma reação inflamatória, este evento é um marco na inflamação aguda (Coleman, 2010).

O mecanismo mais comum de extravasamento vascular é pela contração das células endoteliais resultando em espaços interendoteliais aumentados, e é deflagrado por histamina, bradicinina e muitos outros mediadores químicos, e chamamos de resposta transitória imediata, pois ocorre rapidamente após a exposição ao mediador. Em algumas formas de injúria, como a radiação UV, o extravasamento vascular se inicia entre 2 e 12 horas após a injúria e pode durar horas ou mesmo dias. A reação se reduz quando a inflamação aguda é bem-sucedida na eliminação dos agentes agressores (Coleman, 2010).

Com o excesso de radiação UVB, os queratinócitos entram em apoptose para tentar eliminar o DNA danificado e este processo é proporcional ao tempo de exposição, com isso, as células alteram sua morfologia apresentando cromatina condensada, citoplasma eosinofílico e são denominadas como células de sunburn (Lim e Hyun, 2007).

Outro evento ligado a exposição excessiva a radiação UVB é chamada de hiperqueratose, que ocorre devido ao acúmulo de queratinócitos na epiderme. Horas após o estímulo da radiação UV cessar, os sinais de resposta a danos dos queratinócitos diminuem e ocorre a sua hiperproliferação e subsequente espessamento da epiderme. O aumento da espessura da epiderme tem como objetivo diminuir a penetração da radiação UVB na pele (Kumar *et al.*, 2015).

Considerando os mecanismos fisiopatológicos abordados, a administração de antioxidantes por via tópica tem se mostrado uma alternativa simples e efetiva para a proteção da pele contra os danos oxidativos e inflamatórios causados pela radiação UVB (Campanini *et al.*, 2014; Casagrande *et al.*, 2007).

1.6 Resposta inflamatória induzida pela radiação UVB

O processo inflamatório *provocado* pela exposição à radiação UVB *é provocado pela* estimulação dos queratinócitos*,* que *atuam de forma ativa na* imunidade inata. Os queratinócitos estimulados produzem e liberam citocinas que *levam* ao processo inflamatório*,* como a IL-1, *o* fator de necrose tumoral alfa (TNF-α), *a* IL-6 e *outros componentes* da família das quimiocinas (Bangert, Brunner e Stingl, 2011).

Além dos queratinócitos*,* outros tipos de células também produzem citocinas quando *estimuladas* pela RUV, como as células de Langerhans e os mastócitos (Durán-Aniotz *et al.*, 2013). Além disso, a exposição à RUV *estimula a* secreção de citocinas anti-inflamatórias. Essas citocinas *têm* como *objetivo* regular negativamente a resposta imune, como a IL-10*,* produzida principalmente por macrófagos infiltrativos na pele lesionada pela radiação UVB. A IL-10 é considerada um mediador primário da supressão induzida local e *sistêmica* por UVB. A diminuição *da* imunidade celular observada após a exposição da pele à radiação UVB pode *causar* alterações na capacidade do organismo de responder a uma variedade de micro-organismos virais e intracelulares (Clydesdale, Dandie e Muller, 2001). Além da IL-10, *é possível* citar também a produção da citocina anti-inflamatória TGF-*β,* que *tem* propriedade regulatória e está envolvida em *diversos* tipos de *câncer*, inclusive no melanoma (Durán-Aniotz *et al.*, 2013).

Os queratinócitos protegem a epiderme contra a *proliferação* de células potencialmente cancerosas que podem surgir após a exposição *prolongada* à RUV. As respostas dos queratinócitos ao estresse genotóxico *são: a* morte celular programada, *a* resposta inflamatória para *eliminar as* células danificadas e *a* substituição das células malignas por hiperproliferação. Além disso, o estímulo *à* RUV *estimula* a sinalização MAPK (proteínas quinases ativadas por mitógenos) nos queratinócitos, *um* processo *crucial* no seu sistema de vigilância (Bayerl *et al.*, 1995). Assim, quando a dose de RUV excede o limiar de resposta dos queratinócitos, ou seja, se o dano causado pela RUV nessas células for irreparável, elas ativam a apoptose e morrem. *Os* queratinócitos apoptóticos podem ser identificados *microscópicamente devido ao seu núcleo picnótico* (Bayerl *et al.*, 1995). Outra consequência é o aumento da espessura da epiderme*,* que ocorre devido ao acúmulo de queratinócitos. Após *a interrupção do* estímulo da RUV, os sinais de resposta *aos* danos dos queratinócitos *tendem a diminuir, o que resulta na* hiperproliferação e*, consequentemente, no* espessamento da epiderme. O aumento da espessura da epiderme tem como objetivo diminuir a penetração da RUV na pele (Scott *et al.,* 2012; D'Orazio *et al.*, 2013).

A radiação UVB induz a produção de mediadores inflamatórios que promovem a atração de diferentes tipos celulares a epiderme (Witko-Sarsat *et al.*, 2000). Os neutrófilos constituem a primeira linha de defesa imunológica a migrar para o local da lesão ou infecção

(Seki *et al.*, 2010). Esses fagócitos são responsáveis pela produção adicional de ânion superóxido (O_2^-) via ativação da enzima nicotinamida adenina dinucleotídeo fosfato (NADPH) oxidase 2, chamado de*burst* respiratório. Concomitantemente, ocorre a produção de H_2O_2, ambos fazem parte do sistema antimicrobiano dependente de oxigênio dos leucócitos fagocíticos (Garcia *et al.*, 1999; Robinson, Ohira e Badwey, 2004).

Uma das subunidades da NADPH oxidase é a $gp91^{phox}$, que catalisa a transferência de elétrons do NADPH para o O_2 e está diretamente envolvida na produção de $O_2^{\bullet-}$ induzida pela radiação UVB. O $O_2^{\bullet-}$ é uma espécie reativa intermediária utilizada como substrato para geração de espécies mais reativas como o radical hidroxila ($^{\bullet}OH$), H_2O_2, oxigênio singlete ($^1O_2$) e peróxido nitrito ($ONOO^-$) (Garcia *et al.*, 1999; Robinson; Ohira; Badwey, 2004; Anrather, Racchumi e Iadecola, 2006). A produção de EROs pela $gp91^{phox}$ é crucial para o estabelecimento do estresse oxidativo (Anrather, Racchumi e Iadecola, 2006).

Além disso, a exposição à RUV leva a ativação do fator de transcrição nuclear (NF-κB). NF-κB regula a expressão de genes pró-inflamatórios, anti-apoptóticos e a resposta imune modulatória. A ativação desse fator induz a expressão de $gp91^{phox}$ que, por sua vez leva a uma maior produção radical $O_2^{\bullet-}$ via NADPH oxidase aumentando a produção de EROs, existindo assim um ciclo de feedback positivo importante para o aumento da produção de EROs (Anrather; Racchumi; Iadecola, 2006).

Os neutrófilos podem produzir também metaloproteinases (MMPs) (John e Tuszynski, 2001). As metaloproteinases de matriz (MMPs) constituem uma famíliacomposta por mais de 25 enzimas proteolíticas que atuam sobre os componentes damatriz extracelular (MEC) e são secretadas em uma variedade de processos fisiológicos e patológicos envolvendo a pele (Toi, Ishigaki e Tominaga, 1998; Sternlicht e Werb, 2001; Nakopoulou *et al.*, 2002; Ribeiro *et al.,* 2008).

Além dos neutrófilos, outros diferentes tipos de células da pele podem produzir MMPs quando estimulados pela RUV, tais como os queratinócitos, os fibroblastos, os macrófagos, as células endoteliais, os mastócitos e os eosinófilos (Staniforth *et al.*, 2012).

As MMPs são categorizadas de acordo com a sua estrutura e os tipos de proteínas da MEC que elas são capazes de degradar. As MMPs da classe das gelatinases, como as MMPs dos tipos 2 (MMP-2) e 9 (MMP-9) são capazes de degradar o colágeno IV, principal componente da lâmina basal, sendo relevante na aquisição do fenótipo invasivo das neoplasias malignas. A regulação dessas proteases ocorre através da expressão dos inibidores teciduais das MMPs (TIMPs) durante a remodelação tecidual fisiológica. Os TIMPs contribuem para a manutenção do equilíbrio metabólico e estrutural da MEC, o desequilíbrio entreMMPs e seus inibidores teciduais (TIMPs) parece ser essencial para a degradação da MEC (Ribeiro *et al.*, 2008).

A integridade estrutural e a função da derme dependem da sua MEC, composta principalmente de colágeno tipo I e III, em menor quantidade (Pittayapruek *et al.*, 2016). O remodelamento da MEC é feito pelas MMPs e seus inibidores (TIMP). AMMP-1, MMP-3 e MMP-9 são altamente induzidas por RUV em queratinócitos residentes e fibroblastos. Uma das causas do fotoenvelhecimento seria a hidróliseda MEC dérmica induzida pela RUV e iniciada pela MMP-1, que tem atividade colagenolítica. O dano extenso a matriz colágena da pele é uma das características do fotoenvelhecimento. O aumento da expressão de MMP ao longo de anos ou décadas por exposição crônica ou esporádica à RUV contribui para o dano do colágeno observado no fotoenvelhecimento (Brennan, 2003).

O fotoenvelhecimento pode demorar anos para se desenvolver,diferentemente do eritema e da imunossupressão cutânea que ocorrem nas primeiras 24h e nos primeiros 10 dias após a exposição à RUV, respectivamente (Yarosh, 2004). A pele fotoenvelhecida é caracterizada por desorganização edegradação de fibras de colágeno, perdendo sua força e elasticidade, o que acarretaem mudanças na aparência da pele como o aparecimento de rugas e pigmentação irregular (Baron and Suggs, 2014).

As MMPs desempenham papéis importantes na degradação de tecidos durante processos patológicos, como artrite e fotoenvelhecimento, além de estarem associadas ao potencial invasivo e metastático de tumores. As MMPs são induzidas por vários estímulos extracelulares como a RUV, radiação infravermelha, fatores de crescimento, citocinas e promotores tumorais (Zaid *et al.*, 2007). Além disso, fatoresativados pela RUV como os fatores como AP-1 e NF-κB também induzem a secreção de MMP- 9 e MMP-2 (Vicentini *et al.*, 2011).

Por fim, o processo inflamatório agudo desencadeado pela exposição à RUV acarreta eventos celulares específicos, incluindo aumento da permeabilidade doendotélio e epitélio, infiltração de leucócitos polimorfonucleares, macrófagosinflamatórios e linfócitos no local da lesão que consequentemente levam ao edema de tecido (Coleman, 2010)A exposição à RUV também leva a umaumento do número de mastócitos na pele. Ativados pela radiação UVB os mastócitos degranulam e liberam histamina. A histamina é a principal amina biogênica dos mastócitos e está relacionada com a imunossupressão sistêmica induzida pela radiação UVB (Hart *et al.,* 1998).

1.7 Mediador lipídico anti-inflamatório/pró-resolução MaR2

O processo inflamatório é a primeira resposta do sistema imunológico a uma infecção ou lesão tecidual e tem como objetivo proteger o hospedeiro. Este processo deve ser controlado de forma autolimitada, pois respostas inflamatórias excessivas ou inadequadas podem causar uma série de doenças humanas agudas e crônicas (Serhan *et al.*, 2012).

Neste contexto, alternativas terapêuticas para o controle do processo inflamatório e estresse oxidativo induzidos pela radiação UVB devem ter como alvo seus mecanismos

fisiopatológicos, uma vez que o sistema antioxidante que protege a pele contra os danos oxidativos após a exposição crônica ou excessiva à radiação se torna deficiente (Casagrande *et al.,* 2006; Vicentini *et al*., 2011; Martinez *et al*., 2018; Saito *et al*., 2018).

Foram descobertas novas famílias de mediadores lipídicos, chamados mediadores lipídicos pró-resolução especializado (SPM) são biossintetizados durante a fase da resolução da inflamação aguda por macrófagos, são derivados dos ácidos ômega - 6 (ácido aracdonico) que dão origem as lipoxinas, e dos ácidos ômega - 3 (ácido eicosapentaenóico - EPA e ácido docosapentaenóico - DHA) que dão origem as resolvinas do tipo E, além das resolvinas do tipo D, protectinas e maresinas respectivamente (Deng *et al.,* 2014). Os macrófagos desempenham um papel central na resolução, cicatrização de feridas e reparo tecidual (Deng *et al.*, 2014).

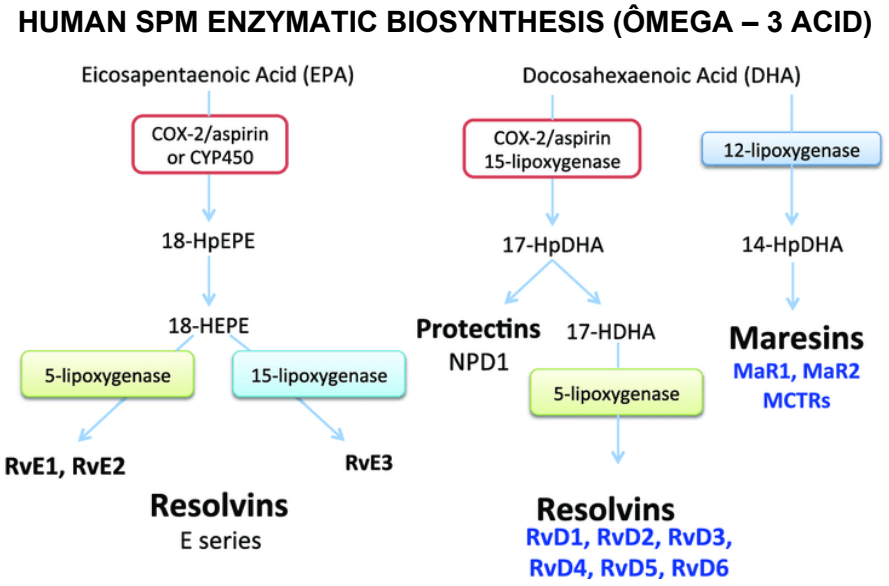

Figura 5 – Esquema de formação dos SPM a partir do ácido ômega – 3**.**
Fonte: Serhan, Chiang e Dalli, 2018.

As maresinas (MaR), como já citado acima são mediadores inflamatórios, uma família biossintetizada por macrófagos a partir do DHA através da 12-lipoxigenase humana (12-LOX), que dão origem a maresina 1 (MaR1) e maresina 2 (MaR2) (Marcon *et al*., 2013; Deng *et al*., 2014). A cascata para a biossíntese de MaR2 começa com a conversão de DHA no intermediário 13S, 14S-epoxi-maresina, por 12-LOX. Posteriormente, esse intermediário é convertido em MaR2 através de uma epóxido hidrolase solúvel, enquanto a MaR1 utiliza uma epóxido hidrolase (Serhan *et al.,* 2009; Deng *et al.,* 2014; Fattori *et al.,* 2022) (Figura 6). A síntese orgânica total de MaR2 também é descrita por diferentes grupos, indicando que métodos sintéticos podem ser aplicados para dar origem a MaR2 (Rodriguez e Spur, 2015;

Fattori *et al.,* 2022). O tratamento intraperitoneal com MaR2 reduz o recrutamento de neutrófilos peritoneais em um modelo de peritonite induzida por zimosan em camundongos (Deng *et al*., 2014).

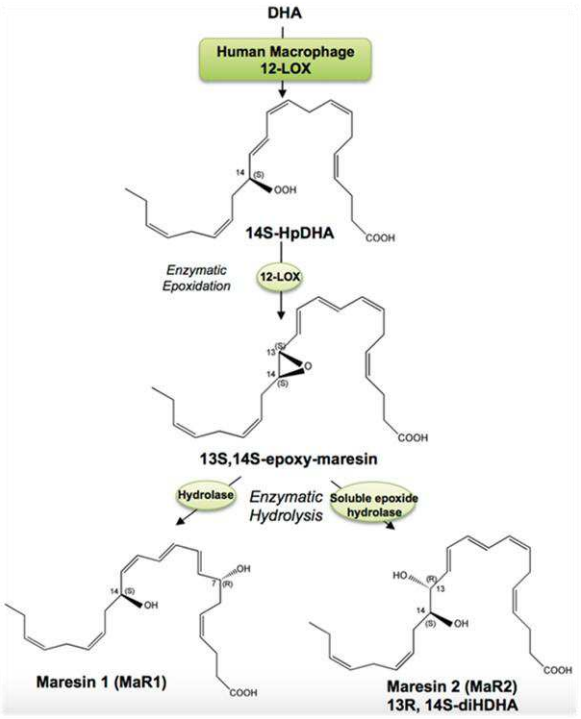

Figura 6 – Biossíntese da MaR 1 e MaR2. Fonte: Serhan, Chiang e Dalli, 2018.

Ambas as MaRs consistem em uma cadeia de carbono com 22 carbonos de comprimento, um grupo carboxila, dois grupos hidroxila e seis ligações duplas; no entanto, a colocação dos grupos hidroxila e das ligações duplas são diferentes (Figura 7). Além dos efeitos pró-resolução, a MaR1 estimula a regeneração e reduz a dor (Serhan *et al.,* 2009; Olsen *et al*., 2022; Sugimoto *et a*l., 2022).

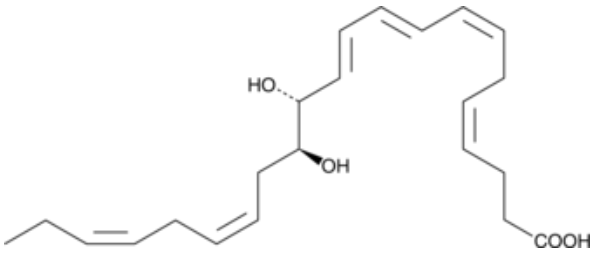

Figura 7 – Estrutura química da MaR2 ($C_{22}H_{32}O_4$).

A identificação e elucidação estrutural dessas novas famílias de mediadores abriu a possibilidade de diversas ações em vários processos, incluindo dor, inflamação, regeneração

tecidual, distúrbios neuroprotetores-neurodegenerativos, cicatrização de feridas, estresse oxidativo e outros (Serhan *et al.,* 2015).

Em contraste com a MaR1, no qual extensos estudos documentaram os seus papéis na resolução da inflamação e do estresse oxidativo (Li *et al*., 2016; Tang *et al.,* 2017; Yang *et al.,* 2019; Cezar *et al.,* 2019; Fattori *et al.,* 2019; Lv e Jin, 2019; Saito-Sasaki, Sawada e Nakamura, 2022;), além disso, a MaR1 está presente no tecido linfóide humano (baço e gânglios linfáticos) e no soro humano, indicando um possível papel no sistema imunológico (Colas *et al*., 2014), pouco se sabe ainda sobre os papéis da MaR2.

Já foi demonstrado que o tratamento com MaR2 ou 13R, 14S- dihidroxi DHA tem resultados como a redução do recrutamento de neutrófilos em modelo de peritonite induzido por zimosan e aumento da atividade fagocítica de macrófagos (Deng *et al*., 2014), modulam a função das células caliciformes conjuntivais no tratamento de doenças inflamatórias da superfície ocular (Olsen *et al*., 2022), promove reparo da mucosa e tem propriedades terapêuticas quando encapsulado em nanopartículas termoestáveis (Miranda *et al.,* 2023), redução da inflamação e estresse oxidativos nas vias áereas de camundongos alérgicos (YU, 2022a), diminuição da inflamação em tecido adiposo (Sugimoto *et al*., 2022), poder analgésico em camundongos, inibindo o recrutamento de neutrófilos e monócitos, a ativação do neurônio nociceptor TRPV1 e TRPA1 e a liberação de CGRP (Fattori *et al.,* 2022).

Vale ressaltar que há estudos sobre a MaR 2 e seu efeito anti-inflamatório e pró-resolução em outro modelo de doença como citado acima, porém não há estudos sobre a ação deste lipídeo pró-resolução no modelo de estresse oxidativo cutâneo e inflamatório induzidos pela radiação UVB, até o momento, existem apenas relatos com a MaR 1 (Cezar *et al.,* 2019).

2. OBJETIVOS

2.1 Objetivo geral

Avaliar o potencial terapêutico da MaR2 intraperitoneal e inserida em formulação tópica na inflamação e estresse oxidativo induzidas pela radiação UVB em camundongos sem pelo.

2.2 Objetivos Específicos

Avaliar a eficácia e mecanismo de ação *in vivo* da MaR2 quando administrada via intraperitoneal (IP) e em formulação de uso tópico na redução do estresse oxidativo e inflamação cutânea induzidos pela radiação UVB com enfoque em:

- Edema de pele;
- Avaliação do poder antioxidante da pele por meio dos ensaios de ABTS (2,2' azino-bis (3-etilbenzotiazolina-6-ácido sulfônico) e FRAP (Poder antioxidante de redução férrica);
- Atividade/secreção de metaloproteinase-9;
- Níveis do antioxidante endógeno GSH e atividade da CAT;
- Produção de hidroperóxidos e de ânion superóxido;
- Avaliação histológica da pele para determinar:
    - Espessura e número de queratinócitos apoptóticos na epiderme
    - Número de mastócitos na derme
    - Densidade das fibras de colágeno da derme.

3. MATERIAIS

3.1. Reagentes

Maresina 2 foi obtida da Cayman Chemical (Ann Arbor, Michigan, USA). Ácido clorídrico, hidróxido de sódio e fosfato de potássio da Merck (grau analítico, Darmstadt, Hessen, Alemanha); terc-butil hidroperoxido, azul brilhante R, glutationa reduzida (GSH), brometo de hexadeciltrimetil amônio (HTAB), *o*-dianisidina, nitroblue tetrazolium (NBT), bisacrilamida foram obtidos da Sigma-Aldrich (St Louis, MO,USA). Hidroximetil aminometano (Tris) da Amresco (Solon, OH, USA). Acrilamida, dodecil sulfato de sódio (SDS). Acrilamida, dodecil sulfato de sódio (SDS), todos os outros reagentes utilizados foram de grau analítico.

3.2 Equipamentos

Agitador mecânico, Fisatom®; Balança analítica, HR-120, A&d®, precisão de4 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 Tissue-Tearor (Biospec 985370); Lâmpada ultravioleta fluorescente PHILIPS TL/12 40W RS-UVB,, Enspire, Perkin Elmer®; Leitorde microplaca 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- Protean® Tetra System, Bio-RAD®, Amplitherm®; Ultra-som, TSO, Thornton®.

4. MÉTODOS

4.1 Animais experimentais

Foram utilizados camundongos sem pelo da linhagem HRS/J, de ambos os sexos, adultos e com peso 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 (registro Of. CIRC. CEUA sob o n° 148/2016, processo nº 11146.2016.97).

4.2 Sistema e fonte de radiação UVB

A fonte de luz utilizada nos experimentos para indução de estresse oxidativo e processo inflamatório agudo nos camundongos foi uma lâmpada UVB fluorescente modelo PHILIPS TL/12 40W RS (MEDICAL-HOLANDA). A lâmpada emite radiação na faixa de 270 a 400nm com pico máximo de emissão em torno de 313 nm. A dose de radiação utilizada para indução da inflamação e do estresse oxidativo foi de 4,14 J/cm^2 (Campanini *et al.*, 2013). A lâmpada foi instalada em uma caixa retangular de madeira com capacidade para 6 caixas de polipropileno padrão de 30x19,5x13 cm, desenvolvida especificamente para esse fim. Os camundongos foram distribuídos aleatoriamente e sobre eles foi colocada uma tela que garante a exposição total do dorso dos animais a luz UVB. Durante toda a irradiação foi realizado o rodízio das caixas para assegurar igual dose de irradiação nos animais, pois pode ocorrer variações de emissão de luz ao longo da lâmpada.

4.3 Protocolo experimental de avaliação da eficácia da MaR 2 em camundongos

4.3.1 Tratamento dos camundongos via intraperitonial (IP)

Os animais foram alocados aleatoriamente em seis grupos contendo cincoanimais cada grupo, sendo:

- Grupo 1: Controle não irradiado e tratado com salina
- Grupo 2: Controle irradiado e tratado com salina
- Grupo 3: Irradiado e tratado com a dose MaR 2 - 1 ng/animal
- Grupo 4: Irradiado e tratado com a dose MaR 2 - 3 ng/animal
- Grupo 5: Irradiado e tratado com a dose MaR 2 - 10 ng/animal

Os animais foram tratados com 200µL de diferentes concentrações de MaR 2 (1, 3 e 10 ng/animal) via intraperitoneal (IP), 10 min antes do início da irradiação (Cezar *et al.,* 2019) Os animais dos grupos controles receberam tratamento apenas com o veículo (salina) utilizado na diluição do fármaco. As doses de MaR2 usadas nos tratamentos foram selecionadas com base nos efeitos terapêuticos de estudos previamente publicados (Cezar *et al.,* 2019) e nos experimentos de dose resposta realizados nesse trabalho.

4.3.2 Tratamento com formulação tópica contendo MaR2

As formulações tópicas foram preparadas sem a adição dos lipídios. Posteriormente foram divididas em duas porções, sendo uma reservada para controle (sem a adição do fármaco) e a outra acrescida dos lipídios MaR2.

Os animais foram tratados com formulação tópica contendo MaR2 ou tratados com a formulação controle (sem MaR2). As formulações foram aplicadas em todo o dorso dos animais com o auxílio de um pincel, cerca de 0,5g de formulação, 10 min antes da irradiação. A concentração de MaR2 utilizada na formulação foi de 10ng/0,5g de formulação. A dose foi escolhida de acordo com os resultados obtidos nos testes preliminares desse estudo, com o tratamento via IP e outros estudos realizados pelo nosso laboratório (Cezar *et al*., 2019).

Os animais foram alocados aleatoriamente em três grupos contendo seis animais cada grupo, sendo:

- Grupo 1: Controle não irradiado e não tratado;
- Grupo 2: Controle irradiado e tratado com a formulação controle (sem MaR2);
- Grupo 3: Irradiado e tratado com a formulação tópica de MaR2 (TFcMaR2);

4.3.3 Preparo da formulação tópica

A comparação entre os resultados obtidos pelo tratamento via IP e tópica dos camundongos é importante para determinar se o tratamento local (via tópica) é tão eficaz quanto o tratamento sistêmico (IP).

As formulações foram preparadas e divididas em duas porções, sendo uma reservada para controle (sem adição da MaR2) e outra acrescida de 10ng de MaR2 por 0,5g de formulação.

As porcentagens dos componentes utilizados para o preparo da emulsão constam na Tabela 1. A fase A (lipossolúvel) e a fase B (hidrossolúvel) foram aquecidas separadamente até a temperatura de 70 ºC. Após a fusão dos sólidos e quando as duas fases atingiram a temperatura determinada, verteu-se a fase B sobre a fase A com agitação constante até a formação de uma emulsão e resfriamento completo. Quando a emulsão atingiu a temperatura ambiente adicionou-se a fase C. Após 24 horas do preparo da formulação foi incorporada a MaR2 10ng/0,5g de formulação. A formulação foi usada para o tratamento dos animais imediatamente após a adição da MaR2.

Tabela 1: Componentes da formulação contendo MaR2

	Matérias-primas	**%**
A	Polawax®[1] (emulsionante e doador de viscosidade)	2%
B	Água	qsp* 100%
	Dispersão de Aristoflex®[2] 5%	20%
C	Triglicerídeos do ácido cáprico e caprílico (emoliente)	5%
	Propilenoglicol (umectante e solubilizante)	6%
	Phenonip®[3] (conservante)	0,8%

[1]Polawax®: Base auto emulsionante não-iônica (álcool cetoestearílico + monoestearato de sorbitol polioxietileno 20 0E);
[2]Aristoflex®: Co-polímero do ácido sulfônico acriloildimetiltaurato e vinilpirrolidona neutralizado (formador de gel aniônico);
[3]Phenonip®: mistura de parabenos e fenoxietanol;
*Quantidade suficiente para preparação.
Fonte: próprio Autor

4.3.4 Avaliação da estabilidade físico-químico

4.3.4.1 Análise visual

As formulações foram analisadas quanto à sua consistência, cor e odor e separação de fases após 24 horas do seu preparo (Shatalebi e Rafiei, 2014).

4.3.4.2 Determinação do pH

O pH das formulações foi determinado por meio de uma dispersão aquosa 1:10 (p/p). Desta forma, pesou-se 1g de cada formulação e adicionou-se 9g de água deionizada. Após completar a homogeneização em placa com agitação magnética, o pH das amostras foi medido em pHmetro digital. As leituras foram realizadas em duplicatas (Anchisi *et al.*, 2001; Georgetti *et al.*, 2008).

4.3.4.2 Teste de Centrifugação

O teste de centrifugação permite informar e comparar as propriedades de estabilidade de diferentes emulsões. Este teste, além disso, permite verificar a estabilidade avaliando se houve ou não a separação de fases da formulação, ou seja, permite analisar se houve instabilidade físico-química (AZZINI, 1999). Cada formulação foi pesada na quantidade de 2g em tubos de ensaios para centrífuga, de formato cônico e graduado. As amostras foram submetidas a 3600rpm durante 30 minutos, à temperatura ambiente observando-se a possível ocorrência de separação de fases (Georgetti *et al.,* 2008).

4.4 Coleta das amostras de pele

Os animais foram terminalmente anestesiados com 3% de isoflurano e as peles coletadas 12h após o final da irradiação. Para avaliação de outros parâmetros,os animais foram anestesiados (3% de isoflurano), decapitados e as peles coletadas 2h após o final da irradiação.

A coleta da pele dos animais para análise foi realizada em diferentes tempos, de acordo com protocolos experimentais pré-estabelecidos. Para as análises de edema, dosagem de GSH, FRAP, ABTS, atividade/secreção da MMP-9 e histologias (capacidade em reduzir apoptose dos queratinócitos, capacidade em reduzir o espessamento da epiderme, número de mastócitos e degradação das fibras de colágeno as peles dos animais foram coletadas 12 horas após a o fim da irradiação. Já para as análises de atividade da catalase, produção de ânion superóxido (NBT) e hidroperóxidos lipídicos as peles foram coletadas 2 horas após a irradiação (Figura 8) (Campanini *et al.*, 2014; Martinez *et al.*, 2015).

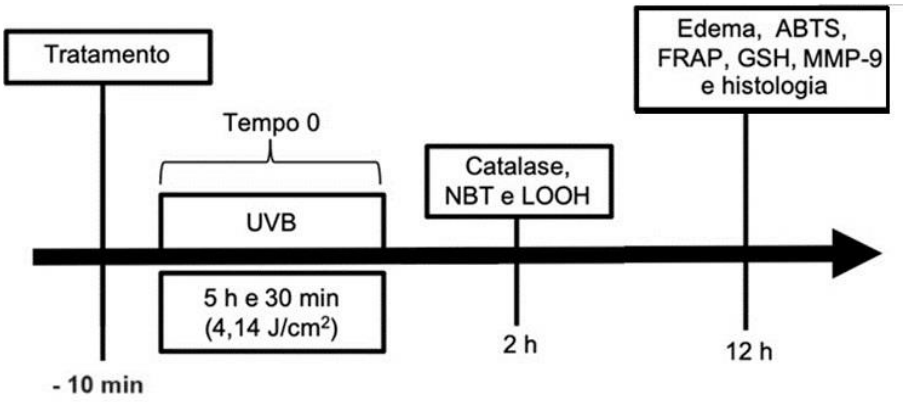

Figura 8 - Fluxograma do tempo de tratamento IP, tópico e tempos de coleta das peles dos animais de acordo com os protocolos experimentais pré-estabelecidos para avaliação da eficácia da MaR2 administrada via IP e tópica. Os animais foram submetidos à radiação UVB por um período de 5 horas e 30 min (4,14. J/cm^2). Nos animais foi realizado um tratamento com MaR2 10min antes do início da irradiação nas doses de 1, 3, 10ng/animal e também com tratamento tópico com formulação controle e formulação contendo MaR2 na dose de 10ng/0,5g. Os animais foram eutanasiados e as amostras de pele foram coletadas em diferentes tempos: 2 horas e 12 horas após o fim da irradiação UVB de acordo com o teste a ser realizado. Fonte: próprio autor.

4.5 Avaliação do edema de pele

No processo inflamatório agudo, desencadeado pela exposição à radiação UV, ocorre a liberação de mediadores químicos que aumentam a permeabilidade do endotélio vascular, levando a formação de edema como resultado do extravasamento defluidos e proteínas para pele (Dusting e Macdonald, 1995).

Para avaliação do edema de pele associado ao processo inflamatório foi feita a coleta das amostras de pele do dorso dos animais com o auxílio de um molde com área fixa (5mm diâmetro). O edema foi expresso pela variação do peso de pele entre os diferentes grupos controles e tratados (Ivan *et al.*, 2014).

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

As metaloproteinases são importantes na degradação das matrizes extracelulares como o colágeno e podem desenvolver o fotoenvelhecimento cutâneo (Bae *et al.*, 2015; Lima *et al.*, 2016).

Usou-se para a determinação da atividade da MMP-9 o ensaio de zimografia em gel de poliacrilamida com dodecil sulfato de sódio (SDS-PAGE) (Fonseca, Catini, *et al.*, 2011). Este método é amplamente utilizado para a detecção de proteases, consistindo em análise qualitativa da atividade por meio da degradação da gelatina adicionada ao gel de eletroforese

(Lim e Hyun, 2007).

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, Catini, *et al.*, 2011). A zimografia é um método amplamente utilizado para a detecção de proteases, consistindo em análise qualitativa da atividade por meio da degradação da gelatina adicionada ao gel de eletroforese (Lim e Hyun, 2007).

As amostras de pele dos animais foram coletadas em microtubos e para este teste foi feito um *pool* das amostras de cada grupo de animais. Primeiramente, as peles dos animais foram diluídas (1:4) e trituradas (T18 basic, IKA) em tampão Tris/HCl 50 mM (pH 7,4) contendo cloreto de cálcio ($CaCl_2$) 10 mM e 1% de inibidores de proteinases (fenantrolina, fluoreto de fenilmetilsulfonila e N-etilmaleimida). O homogenato foi duplamente centrifugado a 12.000 g por 10 minutos a 4 °C e o sobrenadante utilizado no ensaio. Também foi realizada dosagem de proteínas das amostras pelo método de Lowry (LOWRY *et al.*, 1951). Alíquota de 50 µL do sobrenadante das amostras foi diluída em 10 µL de tampão Tris/HCl (pH 6,8) contendo 20% de glicerol, 4% de dodecil sulfato de sódio (SDS) e 0,001% de azul de bromofenol. Em seguida estas amostras foram colocadas em banho-maria a 37 ºC 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, preparado conforme tabela 2.

Tabela 2. Constituintes do gel de separação e do gel de concentração**.**

Substâncias	**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

Fonte: Próprio Autor

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 CaCl2 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.,* 2011a).

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).

4.7 Determinação da atividade doadora de elétrons ao radical ABTS+

Os ensaios usando o radical ABTS estão baseados na habilidade dos antioxidantes em sequestrar o radical de longa vida ABTS. Neste ensaio o ABTS é oxidado pelo radical peroxil ou outros oxidantes para seu radical cátion, $ABTS^{+}$, que é intensamente colorido. A capacidade antioxidante é medida pela capacidade do composto em teste em descolorir a solução reagindo diretamente com o radical $ABTS^{+}$. O método foi realizado de acordo com Sánchez-Gonzalez *et al.* (2005), com algumas modificações. A solução ABTS foi preparada em meio aquoso e ocátion ABTS foi obtido após a reação de 7 mM da solução de ABTS com 2,45 mM de persulfato de potássio. A mistura foi armazenada em frasco âmbar e em geladeira por no mínimo 16 horas antes do uso. A solução ABTS foi diluída com tampão fosfato 20 mM (pH 7,4) até uma atingir absorbância de 0,7 em 730 nm. Para preparar a curva de dose-resposta, uma alíquota de 50 µL da amostra diluida foi adicionada ao meio reacional contendo 4 mL da solução de ABTS diluída, de modo que as concentrações de finais no meio reacional em cada tubo foram: 1,01; 2,02; 4,04; 8,08; 16,16 e 32,31 µg/mL. A mudança de absorbância foi mensurada após 6 minutos de incubaçãoà temperatura ambiente a 730 nm com um espectrofotômetro Helios alfa Thermo Spectronic® (Sánchez-González, I., Jiménez-Escrig, A., Saura-Calixto, 2005a).

4.8 Determinação do poder antioxidante baseado na capacidade de redução do ferro (FRAP)

A reação de FRAP mede a redução férrica de 2,4,6 tripiridil-S-triazina (TPTZ) para um

produto colorido. O reagente de FRAP foi preparado como segue: 2,5mL de uma solução de TPTZ 10 mM em ácido clorídrico (HCl) 40 mM foram adicionados a 2,5mL de cloreto férrico hexahidratado ($FeCl_3.6H_2O$) 20mM e 25mL de tampão acetato 0,3mM (pH 3,6). Essa solução foi incubada a 37 °C por 30min antes do uso. Para a avaliação da capacidade antioxidante, 900 µL do reagente de FRAP preparado previamente foram adicionados de 90 µL de água deionizada e 10 µL do padrão de Trolox ou 10 µL da amostra diluída. As amostras foram então incubadas a 37°C por 30 minutos e o aumentoda absorbância foi mensurado a 595 nm com um espectrofotômetro Helios alfa ThermoSpectronic®. Para comparação do poder redutor dos flavonóides uma curva analítica com diferentes concentrações de Trolox. Os resultados foram expressos em µm de equivalente de Trolox por grama de extrato. Todas as medidas foram feitas em triplicata (Sánchez-González, I., Jiménez-Escrig, A., Saura-Calixto, 2005b).

4.9 Avaliação dos níveis do antioxidante endógeno glutationa reduzida(GSH)

O GSH desempenha um papel importante na proteção de células da pele contra o dano oxidativo. O dano induzido pela radiação UVB é medido pelo aumentoda geração de EROs que resultam no estresse oxidativo que, por sua vez, levam a depleção de antioxidantes endógenos. A perda de viabilidade celular mediada pela radiação UVB está associada a uma diminuição acentuada do conteúdo de GSH quepode predispor a célula a uma defesa menor contra o estresse oxidativo (Halliwell, 2009a).

As amostras de pele (aproximadamente 0,1g) foram diluídas (1:4) em EDTA 0,02M e trituradas utilizando o Tissue-Tearor (Biospec 985370). Ao homogenato foi adicionado ácido tricloroacético (TCA) 50% na proporção de 1:0,2 de EDTA e TCA, respectivamente. Em seguida, a mistura foi centrifugada a 2.700 *g* por 10 minutos a 4°C. O sobrenadante foi retirado e recentrifugado a 2.700 *g* por mais 15 minutos a 4°C. O sobrenadante final foi retirado para análise. Para o ensaio, foram utilizados 50 µl do sobrenadante da amostra, 100 µl de tampão Tris 0,4 M pH 8,9 e 5µl de umasolução de 1,9 mg/mL de ácido 5,5'-ditio-bis-(2-nitrobenzóico) (DTNB) em metanol.A absorvância foi determinada em espectrofotômetro (EnSpire, Perkin Elmer) após 5minutos de incubação, em 405 nm. A curva padrão foi preparada com 0 a 150 µM deGSH. Os resultados foram expressos em µM de GSH/mg de pele (Srinivasan, Sabitha e Shyamaladevi, 2007).

4.10 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., 2015a).

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 20µL de H_2O_2 200mM. Ao teste foi incluído um branco para cada amostra preparado com 10µL de amostra, 180µL de tampão Tris-HCl 1M com EDTA 5mM pH 8.0 e 20µL 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 200mM. A leitura foi realizada em espectrofotômetro de microplaca (EnSpire, Perkin Elmer) em 240nm com temperatura mantida em 25ºC. Os valores de CAT foram expressos como unidade de CAT/mg de pele/minuto (Shetty et al., 2015a).

4.11 Avaliação da produção de ânion superóxido

A produção do ânion superóxido foi avaliada pela sua capacidade de redução de *nitroblue tetrazolium* (NBT) (Campanini *et al.*, 2013). As amostras de pele (cerca de 0,1 g) foram homogeneizadas com o Tissue-Tearor (Biospec 985370) em 500 µL EDTA 0,02M e centrifugadas (2000g por 20 segundos a 4°C). Para a reação, o sobrenadante (50 µL) foi incubado em microplaca por 1 hora para fixação das células. Em seguida, o sobrenadante foi removido e NBT foi adicionado (1 mg/mL) às células fixadas. Após 15 min, o NBT foi cuidadosamente removido e ao precipitado foram adicionados 20 µLde metanol 100% para fixação. O composto formado pela redução do NBT pelo ânion superóxido (Formazan) foi solubilizado com 120 µL de KOH 2M e 140 µL de dimetilsufóxido (DMSO). A redução do NBT para formazan foi medida em espectrofotômetro de microplacas (EnSpire, Perkin Elmer) a 620 nm e os resultados foram apresentados como densidade óptica (OD)/10 mg de pele (Campanini *et al.*, 2013).

4.12 Avaliação da produção de hidroperóxido (LOOH)

As amostras de pele coletada foram trituradas em 800 µL de tampão fosfato (PBS) pH 7,4 contendo NaCl 137 mM, KCl 2,7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1,8 mM, com auxílio do homogeneizador de tecidos Tissue-Tearor (Biospec 985370). Em seguida, centrifugadas a 700 x*g* a 4°C por 2 minutos. Foi usado 70 µL do sobrenadante para a reação com 420 µL de tampão de reação (KH_2PO_4 20 mM e NaCl 0,9%, pH 7,4), 10 µL de terc-butil e 10 µL de

luminol. Cada amostra foi analisadapor 3.600 segundos no equipamento Glomax® 20/20 (Madison, Wisconsin, EUA), resultando na mesma quantidade de pontos de quimioluminescência. Os resultados foram expressos em unidades de luz relativa por mg de pele (Melo *et al.*, 2021).

4.13 Avaliação histopatológica por microscopia óptica

Para análise histopatológica, as amostras de pele do dorso dos animais foram coletadas 12 horas após irradiação e armazenadas em formol 10%. Subsequentemente, as amostras de pele foram fixadas em paraformaldeído 4% e desidratadas em banhos de soluções de etanol em concentrações crescentes (70%, 95% e 100%). Em seguida, foram diafanizadas com xilol e incluídas em parafina. Foram preparadas secções de 5 µm que foram coradas com hematoxilina eosina (H&E), azul toluidina e tricrômico de masson.

As secções coradas com H&E foram examinadas utilizando microscopia óptica a uma ampliação de 400 vezes (objetiva de 40) para determinação da espessura epidérmica (DENG et al., 2015) e uma amplificação de 1000 vezes (objetiva de 100) para contagem do número de queratinócitos apoptóticos (Schwarz *et al.*, 1995). Para contagem de mastócitos, as secções foram coradas com azul de toluidina e a análise feita em microscopia óptica a uma ampliação de 400 vezes (objetiva de 40). As análises foram feitas com o software Infinity Analyze (Lumenera® Software). Já as secções coradas com tricrômico de masson foram examinadas utilizando microscopia óptica a uma ampliação de 100 vezes (objetiva de 10) para visualizar alterações nas fibras de colágeno através da análise da intensidade da coloração azul nas áreas dérmicas da pele exposta a radiação UVB com auxílio do software Image J (NIH) (Song et al., 2016a).

4.14 Análise estatística dos resultados

Os resultados foram analisados por análise de variância 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 e representativos de 2 experimentos separados. As análises estatísticas foram realizadas usando o software GraphPad Prism 8 (GraphPad Software Inc., San Diego, EUA). Os resultados foram considerados significativamente diferentes para $p<0,05$.

5. RESULTADOS E DISCUSSÕES – ARTIGOS CIENTÍFICOS

5.1 Evaluation of the intraperitoneal effect of Maresin 2 (MaR2) in reducing inflammation and oxidative stress induced by UVB radiation in hairless mice.

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ABSTRACT

Exposure to ultraviolet radiation (UVR) causes damage to the body and the skin is the organ that is anatomically most susceptible to this exposure. UVR induces skin and inflammatory damage that can lead to premature aging and even result in skin cancer caused by increased production of free radicals and inflammatory processes. In this context, Maresin2 (MaR2), an anti-inflammatory/pro-resolution lipid mediator derived from macrophages, having docosahexaenoic acids (DHA) as a precursor, would be an alternative. To date, there are no studies on the possible contributions and mechanisms of action of MaR2 in reducing inflammation and oxidative stress induced by UVB radiation through in vivo experimental tests. Intraperitoneal (IP) treatment was performed at doses of 1, 3 and 10ng/animal of MaR2, 10 minutes before irradiation, where only the dose of 10ng/animal showed a reduction in oxidative damage by improving the global antioxidant capacity in the iron-reducing antioxidant power (FRAP) and scavenging of the ABTS radical (2,2' azinobis (3-ethylbenzothiazoline)-6-sulfoni acid), levels of the endogenous antioxidant GSH and catalase activity (CAT), in addition to reducing the production of superoxide anion (O_2^-) and lipid hydroperoxides (LOOH), it has also been shown to be a potent anti-inflammatory and pro-resolution lipid being effective in reducing skin edema and in the activity of the enzyme metalloproteinase type 9 (MMP-9), in reducing the degradation of collagen fibers, in epidermal thickness, in the apoptosis of keratinocytes and in the reduction of mast cells, indicating the mediator lipid as a potential strategy to control/prevent inflammatory diseases caused by exposure to UVB radiation.

Keywords: Maresin 2, Topical formulation, Inflammation, Hairless.

Highlights

- Maresin 2 protected the skin from the deleterious effects of UVB irradiation.
- Maresin 2 inhibited UVB-induced edema.
- Maresin 2 suppressed the secretion of MMP-9 induced by UVB-irradiation.
- Maresin 2 inhibited UVB-induced skin oxidative stress.

Abbreviations

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
CAT	Catalase
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
FRAP	Ferric reducing antioxidant power
GSH	Reduced glutathione
$HO^{\bullet}$	Hydroxyl radical
HO-1	Heme oxygenase 1
H_2O_2	Hydrogen peroxide
LPO	Lipid peroxidation
LOOH	Lipid hydroperoxides
KC	Keratinocytes
MaR1	Maresin 1
MaR2	Maresin 2
NBT	Nitroblue tetrazolium
Nrf2	Nuclear factor erythroid 2-related factor 2
$O_2^{\bullet-}$	Superoxide anion
ROS	Reactive oxygen species
SBC	Sunburn cells
SOD	Superoxide dismutase
TPTZ	2,4,6-tris(2-pyridyl)-*s*-triazine
UV	Ultraviolet
UVB	Ultravioleta tipo B

1. INTRODUCION

In recent years there has been a significant increase in the population's concern about health and this is partly due to greater access and demand for information on the subject [1]. Allied to this fact, changes in society have been occurring due to changes in lifestyle habits, as well as the aging of the population, increasing the incidence of chronic, degenerative, inflammatory diseases, decreased action of the immune system, cellular aging, and carcinogenesis [1].

The skin is the largest organ in the human body and the body's main protective barrier against external aggressors. The skin is constantly exposed to oxidative stress induced by reactive oxygen species (ROS), which are generated both by endogenous stimuli, such as by-products of aerobic or cellular oxidation due to inflammation response, and by external pro-oxidant stimuli, such as ultraviolet radiation (UVR) [2,3].

Excessive exposure to UV can trigger harmful effects resulting from the intense inflammatory reaction and increase in free radicals [4–7], this acute exposure causes a series of adverse effects such as edema, sunburn, erythema, inflammation and immunosuppression, in addition, chronic exposure can lead to premature aging and the development of skin cancer.

The effects of UVB radiation on the skin can be divided into immediate response, with the appearance of erythema, edema and leukocyte infiltration, in addition to a decrease in the levels of endogenous antioxidants such as reduced glutathione (GSH), which is a tripeptide sensitive to oxidative stress mediated by UVB radiation [8–12] and late with damage to elastic fibers, collagen and glycosaminoglycans of the extracellular matrix of the dermis, losing elasticity and contributing to greater damage.

Considering the consequences of skin exposure to UVB radiation mentioned above, it is observed that inflammation and oxidative stress are closely linked, as the excessive production of ROS activates several signaling pathways, including the production of inflammatory mediators such as nitric oxide (NO), the prostaglandins PGE2 and the cytokines IL-1α (interleukin-1α) and TNF (tumor necrosis factor) that trigger the inflammatory process [13].

These, in turn, recruit inflammatory cells to the site, which ends up resulting in an additional production of free radicals as a defense mechanism, making this process a vicious cycle that generates significant tissue damage. Against the harmful effects of radiation caused by ROS, our body has a system of low molecular weight antioxidants (ascorbate, glutathione, tocopherol and ubiquinol) and enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase), but exposure to UV radiation leads to depletion of this system [14].

In this context, therapeutic alternatives for controlling the inflammatory process and

oxidative stress induced by UVB radiation must target its pathophysiological mechanisms, since the antioxidant system that protects the skin against oxidative damage after chronic or excessive exposure to radiation becomes disabled [4,5,15,16].

The literature has previously established the potential benefits of dietary omega-3 fish oils, which are high in polyunsaturated fatty acids like docosahexaenoic acid (DHA), for the treatment of inflammatory diseases [17].

Through human 12-lipoxygenase (12-LOX), DHA is the precursor of a novel family of lipid mediators derived from macrophages known as maresins [18]. The first member of this family to be discovered, Maresin 1 (MaR1), demonstrated strong pro-resolution and anti-inflammatory properties, suppressing neutrophil-induced in vivo inflammation and stimulating macrophage phagocytic activity [18].

Little is known about the roles of MaR2. In contrast to MaR1, which has been the subject of extensive studies that have documented its roles in resolving inflammation and oxidative stress, MaR1 is also present in human serum and lymphoid tissue, including the spleen and lymph nodes, suggesting a potential role in the immune system [19]. Treatment with MaR2 or 13R, 14S-dihydroxy DHA has already been shown to produce effects like decreased neutrophil recruitment in a model of zymosan-induced peritonitis and increased macrophage phagocytic activity [20], as well as modifying conjunctival goblet cell function in the management of ocular surface inflammatory diseases [21], promotes mucosal repair and has therapeutic properties when encapsulated in thermostable nanoparticles [22].

The discovery of these new families of mediators and their structural clarification have allowed the exploration of a wide range of potential applications in processes such as wound healing, inflammatory pain, infection, tissue regeneration and neuroprotective-neurodegenerative diseases [23].

2. MATERIALS AND METHODS

2.1 Chemicals

Maresin 2, brilliant blue R, reduced glutathione (GSH), 2,2'- azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,4,6-tripyridyl-S-triazine (TPTZ), Trolox, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT) and bisacrylamide were obtained from Sigma-Aldrich (St Louis, MO, USA). Tert-butyl hydroperoxide from Acros (Pittsburgh, PA, USA); Hydroxymethyl aminomethane (Tris) from Amresco (Solon, OH, USA). Acrylamide, sodium dodecyl sulfate (SDS) was obtained from Invitrogen. Isoflurane 5% from Abbott (Abbott Park, IL, USA). Materials for formulation was obtained from Galena (Campinas, SP, Brazil). All other reagents used were of pharmaceutical grade.

2.2 Animals

The experiments were carried out on hairless mice (HRS/J) weighing between 20 and 30 g and obtained from the University Hospital of the State University of Londrina (UEL), Paraná, Brazil. The mice were maintained with free access to water and food throughout the experiment with a 12/12-hour light/dark cycle and controlled temperature (23 ± 2°C). The Animal Ethics Committee of the State University of Londrina approved all procedures used in this study (CEUA process number 11146.2016.97). All methods were performed following relevant guidelines and regulations. All experiments were performed with a minimum number of animals and minimal suffering. Euthanasia at the end of the experiments involved terminal anesthesia procedures with 5% isoflurane (Abbott Park, IL, USA) and anesthesia with 5% isoflurane after decapitation. Rats were continuously monitored for welfare-related assessment before, during, and after experiments [5].

2.3 Irradiation

The UVB source to induce pathological changes in the skin was a Philips TL/12 RS 40W (Medical-Holand) lamp emitting a continuous spectrum at 313 nm. In the apparatus, the lamp was positioned 20 cm above the area where the mice were placed, resulting in an irradiation of 0.209 mW/cm2 as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with sensor of UV (SED005) and UVB (SED240). The irradiation dose was 4.14J/cm2 [5,24]. Experiments using this UVB irradiation model have been validated in previous studies using the same irradiation dosage as described above [5,24–26].

2.4 Experimental protocol

First, HRS/J mice (a hairless strain) were randomly engineered to different groups with 6 mice each: control, irradiated, saline-treated and irradiated group treated with 1ng of MaR2,

irradiated group treated with 3ng of MaR2, irradiated group treated with 10ng of MaR2. The mice were treated intraperitoneally (IP) 10 minutes before the start of UVB irradiation. The mice were anesthetized with 3% isoflurane after 2 hours or 12 hours after irradiation, depending on the assay. Dorsal skin samples were collected followed by storage at −80°C prior to analysis. Exceptions were skin edema where samples were weighed immediately after collection and samples for histology that were stored in 10% formaldehyde. In a previous study with Mar1 doses of 10ng/animal provided protective, pro-resolution anti-inflammatory effects [26]. To test whether MaR2 would present this same effect, we used concentrations of 1ng, 3ng, and 10ng/animal/treatment to allow an observation of the best dose-response in a skin irradiation model. After the first step, we use a MaR2 10ng for experiments involving catalase activity, superoxide anion, and hydroperoxide production.

3. MAR2 TOPICAL EFFECTIVENESS EVALUATION (TFcMaR2)

3.1 Skin Edema

Dorsal skin biopsy was carefully removed from euthanized mice and weighed using a precision scale [27]. All samples presented a constant diameter of 5mm. Results are expressed in mg of skin tissue obtained from the weight of each sample.

3.2 ABTS assay

This assay is based on the inhibition of the absorbance of the radical ABTS. Approximately 30 mg of hairless mice skin was homogenized in 500 µL of KCl (1.15%) using a Tissue-Tearor (Biospec 985370), centrifuged at 1000xg for 10min at 4°C and the supernatant was used measuring the antioxidant capacity of skin. The solution of ABTS was prepared with 7mM of ABTS and 2.45mM of potassium persulfate diluted with phosphate buffer pH 7.4 to an absorbance of 0,7 – 0,8 in 730nm was prepared. The supernatant (7µL) was mixed on ABTS solution and after 6 min the absorbance was determined in 730 nm microplate reader (EnSpire, Perkin Elmer) [6,28]. Previously, a curve of trolox (0,01 a 20nmol) was prepared and the results were expressed as equivalent nmol of Trolox/mg skin.

3.3 FRAP assay.

This assay was used to evaluate the antioxidant power of ferric reduction of skin 7 [29]. The sample of hairless mice skin was homogenized in 500µL KCl (1.15%) using a Tissue-Tearor (Biospec 985370), centrifuged at 1000xg for 10 min at 4°C and the supernatant was employed for measurement the antioxidant capacity of skin. The reaction consisted in adding the supernatant (30µL) to the FRAP reagent prepared with 0.3 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM hydrochloride acid and 20 mM ferric chloride. The FRAP reagent was warmed up to 37°C for 30 min. The absorbance was determined at 595 nm (EnSpire, Perkin

Elmer). Previously, a curve of trolox (0.5 – 20nmol) was prepared and the results were expressed as equivalent nmol of Trolox/mg skin [6,28].

3.4 GSH assay

Followed the method described elsewhere [30–32]. Briefly, skin sample was homogenized in 0.02 M EDTA using an Ultra Turrax® (T18 basic, IKA) at a ratio 1:4 w/w dilution. Whole homogenates were treated with 50% trichloroacetic acid and centrifuged twice at 2700xg for 10 min at 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 determined after 5 min incubation at 405 nm (Enspire, Perkin Elmer®). The standard curve was prepared with GSH 5-150 µM. The results are presented as µM of GSH per mg of skin [30,33].

3.5 Catalase assay

Was evaluated by measuring the decay in the concentration of hydrogen peroxide (H_2O_2) and the generation of oxygen [24,34] sample was homogenized in 500µL of 0.02 M EDTA using Ultra Turrax® - (T18 basic, IKA), and centrifuged twice at 2700xg for 10min at 4°C. The reaction mixture contained 10µL sample, 160µL buffer Tris-HCl 1 M with EDTA 5mM pH 8.0, 20L of deionized water and 20µL H_2O_2 200mM. Measurement of CAT activity was estimated through the difference between the initial reading and the reading performed 30 seconds after the addition of $H2O_2$ at 240nm in a microplate reader (EnSpire, Perkin Elmer) at 25°C. The CAT values were expressed as unit of CAT/mg of skin minute.

3.6 Lipid hydroperoxides (LOOH) assay

This assay was performed based on the measure of the quimioluminescence (QL) initiated by the tert-butyl hydroperoxide [35]. Skin samples were homogenized in 800 µL of Phosphate Buffered Saline (PBS) containing NaCl 137 mM, KCl 2.7 mM, Na2HPO4 10 mM, KH2PO4 1.8 mM pH 7.4 and centrifuged at 2.000 xg, for 2 minutes. The supernatant (70 µL) was mixture with 420 µL reaction buffer (20 mM KH2PO4 with 0.9% NaCl pH 7.4), added 10µL tert-butyl and 10 µL luminol. Each sample was analyzed for 3600 seconds resulting the same quantity of points of chemioluminescence. Analysis of the reaction buffer without the sample was performed to check if there was interference in the chemiluminescence reading using the Glomax 20/20 (Madison, Wisconsin, USA). Results were expressed in relative light unit per mg of skin.

3.7 Evaluation of the production of superoxide anion ($O_2^{\bullet-}$)

The measurement of $O_2^{\bullet-}$ production in the skin was performed using the nitroblue tetrazolium assay (NBT) [36]. The samples of hairless mice skin were homogenized in 500µL of 0,02M EDTA using a Tissue-Tearor (Biospec 985370), centrifuged at 2000g for 20 seconds

at 4°C, the supernatant (50 µL) was incubated in microplate for 1 h. The non-adherent/nonprecipitated supernatant was carefully removed, 100µL of NBT (1 mg/mL) was added to each well and incubated over 15 min. NBT reaction medium was the carefully removed followed by fixation in methanol. Formazan particles were dissolved by adding 120µL of KOH 2M and 140µL of dimethylsulfoxide. Reduction of NBT to formazan was measured at 600 nm using a microplate spectrophotometer reader (EnSpire, Perkin Elmer) and the results are presented as optical density (OD) per 10mg of skin.

3.8 Analyses of skin proteinase substrate-embedded enzymography

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) substrateembedded enzymography was used to detect enzymes with gelatinase activity. Assays were carried out as previously described [16]. The dorsal skin of hairless mice (1:4, w/w dilution) was homogenized (Ultra Turrax® - T18 basic, IKA) in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.01M CaCl2 and 1% protease inhibitor cocktail. Whole homogenates were centrifuged twice at 12000xg for 10min at 4°C. The Lowry method was used to measure protein levels in skin homogenates [33]. Aliquots of 50µL of samples were mixed with 10µL of 0.1 M Tris-HCl (pH 7.4) containing 20% glycerol, 4% SDS and 0.005% xylene cyanol. For electrophoresis, 25µL of the mixture was used 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 in 0.05 M Tris-HCl (pH 7.4), 0.01M $CaCl_2$ and 0.02% sodium azide at 37°C, and stained the following day with brilliant blue R. After distaining in 20% acetic acid, zone of enzyme activity was analyzed by comparing the groups in the ImageJ software package (NIH, Bethesda, MD, USA).

3.9 Skin histologic evaluation assay

Dorsal skin samples were collected in formaldehyde 10%, fixed in paraformaldehyde 4%, dehydrated in ascending ethanol concentrations, cleared inxylene, embedded in paraffin and sectioned to a thickness of 5µm. The sections were stained with Hematoxylin and Eosin (HE), Toluidine Blue and Masson's Trichrome stains. The sections stained with HE was examined using light microscopy at 40x magnification for determination of epidermal thickness [37] and a 100x magnification for counting the number of sunburn cells [38]. For mast cell count, the sections were stained with Toluidine Blue and analyzed under light microscopy at 400x magnification. Both analyses were done with the software Infinity Analyze (Lumenera R Software).

The sections stained with Masson’s Trichrome were examined using light microscopy at a magnification of 100x. Density of collagen fibers was analyzing by the intensity of the blue

coloration in the dermal areas of the skin. UVB unexposed and exposed controls were compared to CVE treated group with the aid of Image J software (NIH) [39].

3.10 Statistical analysis

Results were analyzed by GraphPad Prism® software package, version 9 and expressed as means ± standard error of the mean (SEM). In vitro data represent triplicate analysis per experiment and are representative of two separate experiments. In vivo results are presented of 6 mice per group per experiment and are representative of two separated experiments. The differences were evaluated by ANOVA followed by Tukey´s test. Results were considered significantly different when $p < 0.05$.

4. RESULTS

4.1 MaR2 reduces UVB irradiation-induced skin edema.

The anti-inflammatory effects of MaR2 were first assessed by the edema assay. Skin edema was inhibited by 10ng/mouse MaR2 treatment compared with the irradiated group (Figure 1). Skin edema is a parameter associated with the inflammatory process induced by UV radiation [16] because it causes increased permeability of the vascular endothelium [40].

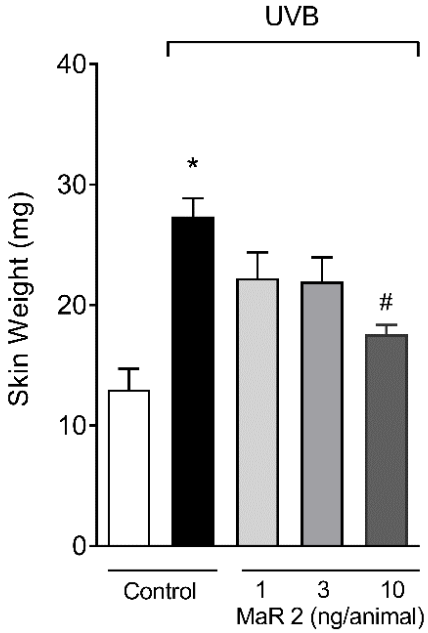

Figure 1 - MaR2 reduces UVB radiation-induced skin edema. Mice were treated intraperitoneally with 1, 3 or 10ng of MaR2 10 minutes before the beginning of UVB irradiation. The skin edema was determined in samples collected 12 hours after the end of irradiation. Bars represent means ± SEM of six mice per group per experiment and are representative of two separate experiments. [*p<0.05 compared to the non-irradiated control group; #p<0.05 compared to the irradiated control group]**.**

4.2 MaR2 maintains antioxidant levels even after UVB irradiation.

Subsequently, we examined the impact of MaR2 on maintaining antioxidant levels by analyzing four parameters: The ability to sequester ABTS+ (Figure 2A), the ability to reduce iron (FRAP; Figure 2B), the ability to maintain reduced levels of glutathione (GSH) (Figure 2C), and the activity of the catalase enzyme (Figure 2D). Lower concentrations of MaR2 (1 and 3ng/animal) were not effective in preventing depletion of antioxidant levels after UVB stimulation. The dose of 10ng/animal of MaR2 maintained levels like those simulated. As expected, antioxidant levels in the irradiated control group were lower than those in the control group. On the other hand, the MaR2 10ng/mouse group was able to guarantee FRAP levels, the ability to sequester ABTS, GSH and catalase activity.

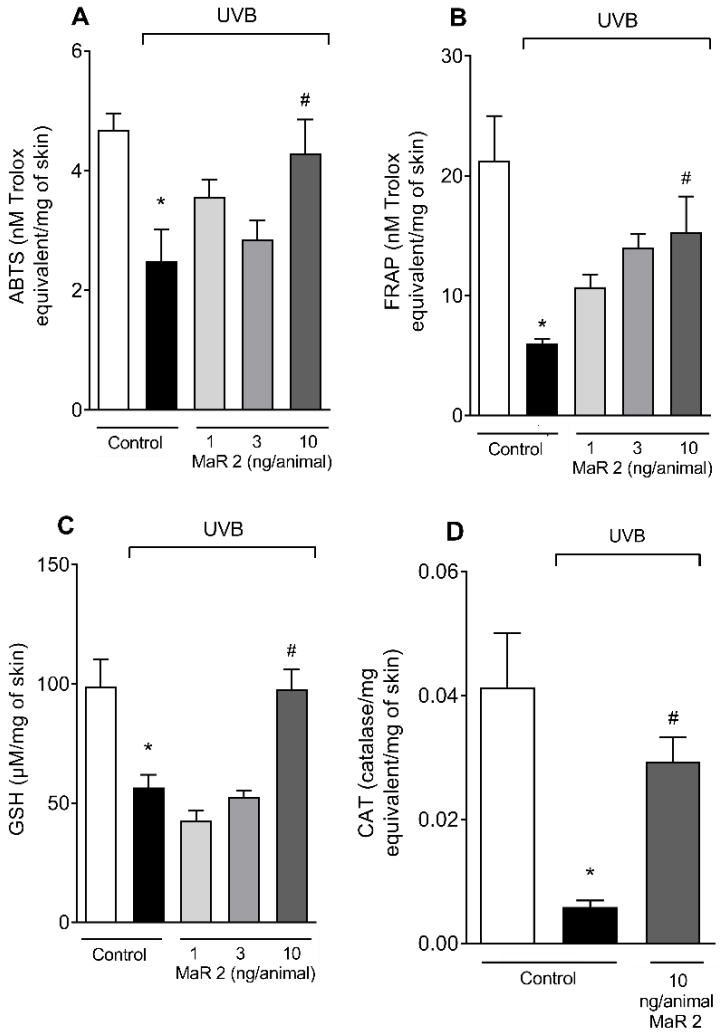

Figure 2 – MaR2 inhibits the reduction of the antioxidant capacity of the skin induced by UVB-radiation. The antioxidant capacity was determined by ABTS (A), FRAP (B) and GSH (C) tests on samples collected after 12h and for CAT (D) on samples collected after 2h of the end of irradiation. The bars represent mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [* p <0.05 compared to the non-irradiated control group; #p <0.05 compared to the irradiated control group].

In order to conclude the investigation of antioxidant activity, we assessed the

production of hydroperoxide (LOOH; Figure 3A) and superoxide anion (O_2^-) (Figure 3B). Nonetheless, due to the pre-determined dose in four other tests and to minimize the use of animals, we utilized only three groups for these tests, namely the control, irradiated control, and MaR2 10ng/mouse. In both tests, UVB irradiation resulted in an increase in the production of LOOH and O_2^- in the irradiated control group, as substantiated by previous studies [5,16,41]. However, the group treated with 10ng/animal of MaR2 was able to reduce the production of these radicals (Figure 3).

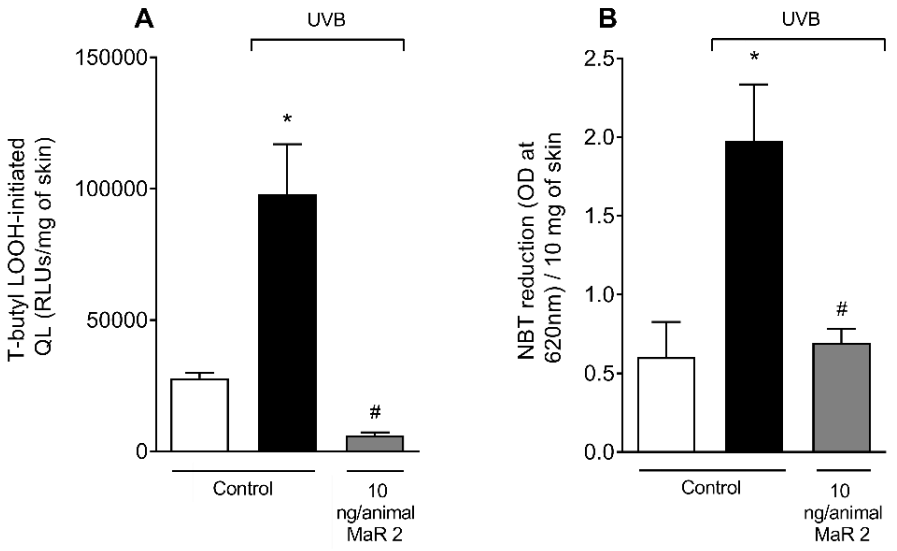

Figure 3 - The effect of MaR2 on UVB radiation-induced lipid peroxidation and superoxide anion production, A and B, respectively, were determined in samples collected 2 hours after UVB exposure, using a t-butyl LOOH-initiated chemiluminescence method. The bars represent mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [* p <0.05 compared to the non-irradiated control group; #p <0.05 compared to the irradiated control group].

4.3 MaR2 inhibits UVB irradiation-induced MMP-9 activity and collagen fibers degradation.

MMP-9 is an enzyme secreted predominantly by neutrophils, mast cells, and macrophages that has proteolytic activity against the main component of the basement membrane, type IV collagen [42,43]. MMP-9-induced damage to the skin's collagen matrix is one of the hallmarks of photoaging and non-melanoma skin cancer [44].

After exposure to UVB irradiation, there was a significant increase in MMP-9 secretion/activity from skin cells in hairless mice. In this study, treatment with MaR2 at a dose of 10ng/mice suppressed MMP-9 secretion (Figure 4). These data evidence that MaR2 reduces dermal connective tissue damage.

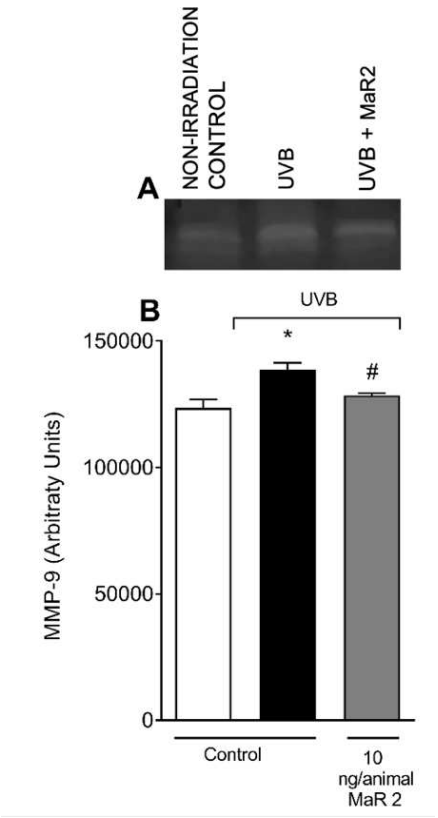

Figure 4 - MaR2 inhibits the increased activity of MMP-9 induced by UVB radiation. The MMP-9 activity was determined in samples collected 12 hours after the end of the irradiation. (A). Representative image of the gelatin zymography. (B) Bars represent mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [* $p < 0.01$ compared to non-irradiated control group; #$p < 0.05$ compared to the irradiated control group].

The deleterious effect of MMP-9 enzyme activity on collagen fibers, we measured collagen density in sections of skin tissue stained with Masson's trichome [45]. It is worth mentioning that the administration of MaR2 at a dose of 10ng/mouse resulted in a reduction in the degradation of skin collagen, evidenced by the preservation of the blue color in Masson's trichrome staining compared to the irradiated group (Figure 5). The results of the enzyme activity assay (MMP-9) and tissue staining (Masson's trichrome) are in agreement.

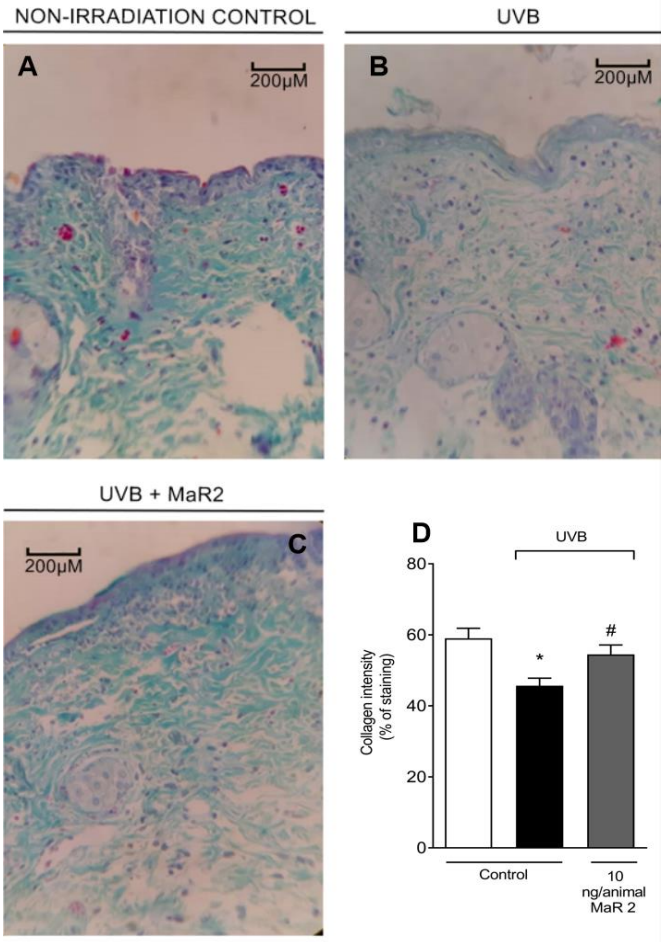

Figure 5 – MaR2 reduces the degradation of collagen fibers induced by UVB radiation. The intensity of collagen fibers was measured using Masson's Trichrome stain on skin samples collected 12 hours after the end of irradiation. Representative images of the groups: non-irradiated control (A), UVB (B), and irradiated treated with 10ng/mouse of MaR2 (C) (10x magnification). Quantitative analysis of collagen degradation of experimental groups is presented as percentage of staining in panel (D). Bars represent means ± SEM of six mice per group per experiment and are representative of two separate experiments. *p<0.05 compared to the non-radiated control group (white bar); #p <0.05 compared to the radiated control group (black bar).

4.4 MaR2 decreases mast cell recruitment, the development of sunburn cells, and epidermal thickness triggered upon UVB-irradiation.

UVB-irradiation increased mast cell counts in the dermis in the irradiated control group (Figure 6B) MaR2 suppressed the increase of dermal mast cells (Figure 6D). Keratinocytes in the process of apoptosis are known as sunburn cells and present condensed nucleus and eosinophilic cytoplasm (SAIJA et al., 2000) as indicated by arrows in Figure 7 (A, B e C). MaR2 inhibited sunburn cell formation (Figure 7D). Moreover, UVB-irradiation caused an increase of epidermal thickness in irradiated control (8B) not evident in group MaR2 (8C) and control non-irradiation (8A).

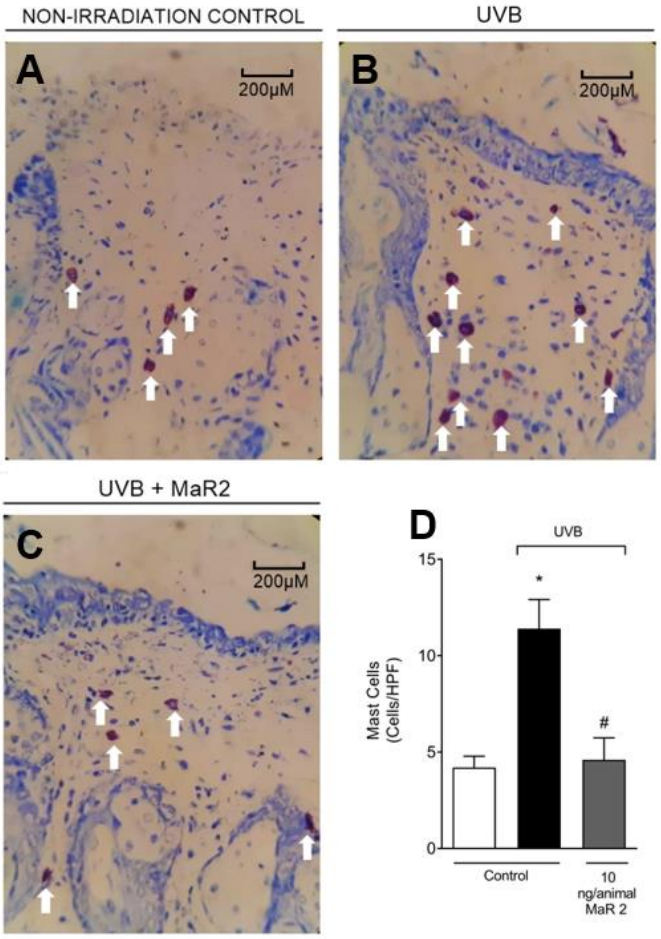

Figure 6 - MaR2 reduces the proliferation of mast cells induced by UVB radiation. Samples were collected 12 hours after the end of irradiation. Representative images of the groups: non-irradiated control (A), UVB (B), and irradiated treated with 10ng/mouse of MaR2 (C) (40x magnification). Quantitative analysis of mast cell recruitment from experimental groups is presented as percentage of staining in panel (D). Bars represent means ± SEM of six animals per group per experiment and are representative of two separate experiments. [*$p<0.05$ compared to the non-radiated control group (white bar); #$p<0.05$ compared to the radiated control group (black bar)].

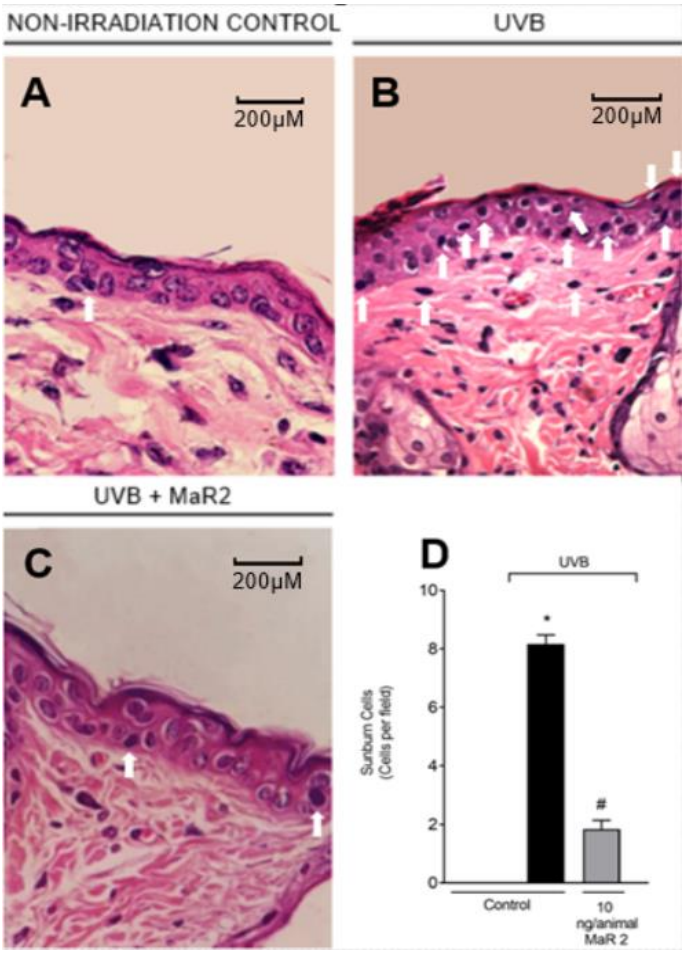

Figure 7 – MaR2 decreases the cell count of sunburn induced by UVB radiation. Sunburn cell counts were assessed using slices of hematoxylin and eosin-stained skin samples collected 12 hours after the end of irradiation. Representative images of the groups: non-irradiated control (A), UVB (B), and irradiated treated with 10ng/mouse of MaR2 (C) (100x magnification). Quantitative analysis of sunburn cells from experimental groups is presented as percentage of staining in panel (D). Bars are means ± SEM of six mice per group per experiment. [* $p<0.05$ compared to placebo and #$p < 0.05$ compared to irradiated control group, group (white bar); #$p < 0.05$ compared to irradiated control groups (black bars)].

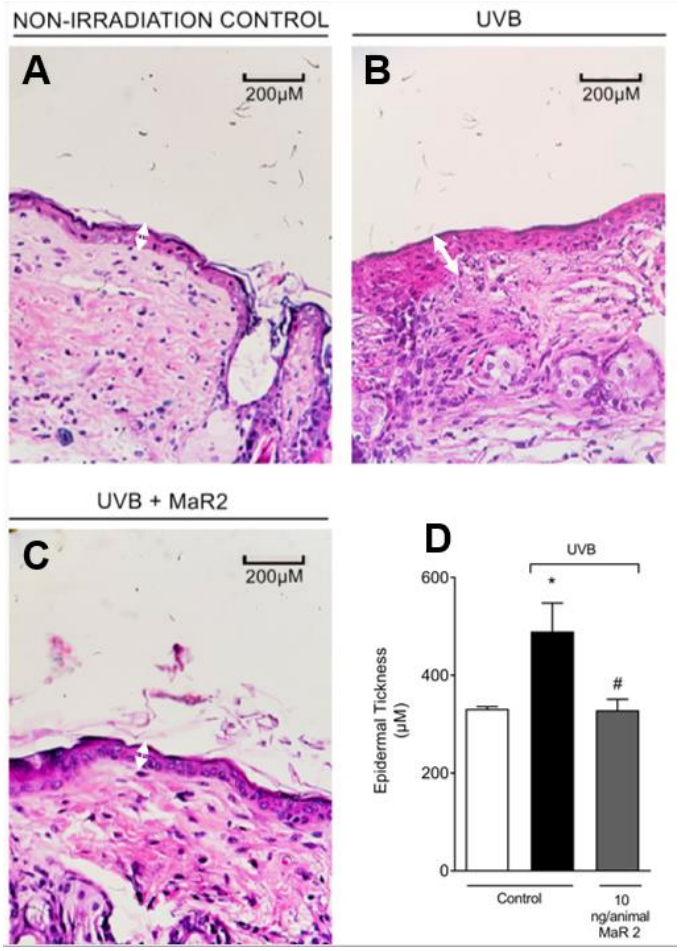

Figure 8 - MaR2 reduces the thickness of the epidermis. Epidermal thickness was evaluated by staining with hematoxylin and eosin (H&E) in skin samples collected 12 hours after the end of irradiation. Representative images of the groups: non-irradiated control (A), UVB (B), and irradiated treated with 10ng/mouse of MaR2 (C) (40x magnification). Quantitative analysis of the epidermal thickness of the experimental groups is presented as percentage of staining in panel (D). Bars are representative of two separate experiments and represent means ± SEM of six mice per group per experiment. [*$p < 0.05$ compared to the non-irradiated control group (white bar); #$p < 0.05$ compared to irradiated control groups (black bars)].

5. DISCUSSION

Currently, a series of studies have revealed that maresin 1 (MaR1) [41] and resolvin [16], endogenously produced from omega-3 fatty acid and another pro-resolution mediator called lipoxin [5,46], derived from omega-6, can contribute to the reduction of inflammation and oxidative stress in models of UVB irradiation. To date, little is known about the effects of MaR2 on the resolution of inflammation and oxidative stress and it has not yet been evaluated in this type of model induced by UVB irradiation.

Maresin-2 (MaR2) is a DHA derivative produced by macrophages and the newest identified member of the maresin family [47,48]. In human macrophages, the cascade for

MaR2 biosynthesis begins with the conversion of DHA to the 13S intermediate, 14S-epoxy-maresin, by 12-LOX. Subsequently, this intermediate is converted into MaR2 through a soluble epoxide hydrolase, while MaR1 uses an epoxide hydrolase [47]. The total organic synthesis of MaR2 is also described by different groups, showing that synthetic methods can be applied to give rise to MaR2 [48].

In this study, we standardized the concentration of MaR2 and used the time described previously [41]. No antioxidant and anti-inflammatory results were observed at concentrations of 1 and 3ng/animal of MaR2 (unpublished data). Treatment with MaR2 10ng/animal, administered for 10 minutes before UVB irradiation, it improves photooxidative and photo-inflammatory damage.

In fact, UVB radiation can cause acute events, and its main characteristics are increased vascular permeability induced by inflammatory mediators, which results in exudation and vascular dilation, increased blood flow, erythema and heat, emigration and accumulation of neutrophils in the lesion [49]. In the present study, MaR2 was shown to be able to reduce skin edema compared to the non-irradiated control, which was also demonstrated by MaR1 in the same model [41].

In addition to being indicators of inflammation, neutrophils are also responsible for the secretion of enzymes that produce highly reactive ROS, causing tissue and structural damage, and release metalloproteinase type 9 (MMP-9), which degrades collagen fibers and leads to photoaging. Furthermore, the AP-1 pathway can also secrete MMP-9, UVB activates keratinocyte (KC) and fibroblast (FB) growth factor and surface receptors that stimulate signal transduction cascades that produce the AP-1 transcription factor, which stimulates MMP-9 gene transcription [50]. Our study revealed that MaR2 10ng protects the skin, reducing the secretion/activity of MMP-9, as demonstrated in zymographic and histological examinations with Masson's trichrome staining. Furthermore, the number of collagen fibers is like the non-irradiated group. Another maresin from the same group also had the same results [41].

Under normal conditions, living organisms have an effective antioxidant system against ROS, composed of non-enzymatic antioxidant substances, such as (glutathione) and enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX) and glutathione reductase (GSH) [51]. CAT is the antioxidant most sensitive to ultraviolet radiation. This enzyme catalyzes the conversion of H_2O_2 into oxygen and water, preventing the formation of free hydroxyl radicals (•HO) [52].

When CAT activity is reduced, H_2O_2 accumulates in the cell and damages several structures, including the enzyme itself [53].

MaR2 was able to block the loss of cell viability associated with the decrease in endogenous antioxidant defenses that were reflected in this study by measuring the levels of catalase (CAT) and reduced glutathione (GSH). Where MaR2 treatment increased CAT and

GSH levels, results supported by previous studies [41,54]. Yu 2022, study also demonstrated the antioxidant effect of MaR2, which reduces oxidative stress and allergic inflammation in the airways of mice, inhibiting the activation of the NLRP3 inflammasome, the Th2-type immune response [55].

Other tests that confirmed this antioxidant effect were ABTS and FRAP, which reflect the general antioxidant capacity of the skin. In the present study, treatment with 10ng/animal of MaR2 was able to reduce the Fe+ ion in the FRAP method and donate electrons to the ABTS cation, reducing the number of free electrons produced by UVB radiation.

Therefore, UVB radiation on the skin increases the number of free radicals, such as superoxide anion (O_2^-) and hydroxyl radical (•HO), hydrogen peroxide (H_2O_2), or through lipid peroxides (LOOH). Specifically, LOOH exerts its effect through two mechanisms: first, it refers to the alteration of the architecture and structure of lipid membranes, and second, through the propagation of new ROS, which can react with DNA and proteins [35]. One strategy to prevent the formation of LOOH is the reduction of peroxide to a less reactive compound, exerted by the glutathione peroxidase (GPx) class of enzymes present in cells [56]. Another strategy is to use exogenous substances with antioxidant effects, preventing the formation of hydroperoxides and their reactive intermediates [24,57].

Another significant finding that supports the assertion that MaR2 treatment is a potent agent in preventing and resolving oxidative stress is the reduction in O_2^- production and LOOH UVB-induced. Three recent studies have shown that pro-resolving lipid mediators reduced oxidative stress in vivo, as evidenced by two recent studies showing that LXA4 [5], RvD1 [16] and MaR1 [41] reduce UVB-induced ROS production.

Because of UVB radiation, the nucleus of keratinocyte cells absorbs energy, resulting in the formation of chromophores, which in turn leads to mutations in the molecular structure of DNA [58]. To prevent the replication of this mutated molecule, additional components are activated, thereby initiating the cellular repair process. These include the p53 protein, which inhibits the mitotic cycle and initiates the transcription of DNA repair genes [59]. When DNA damage cannot be repaired, the process of apoptosis ensues, triggering proteins, caspases, and other related mechanisms [60]. Keratinocytes that undergo apoptosis exhibit a distinct morphology, with a condensed nucleus that is more abundant in the basal layer and lower third, commonly referred to as sunburn cells [61,62]. In response to UVB irradiation, there is an increase in the proliferation and hyperplasia of epidermal cells, which serves as a protective mechanism for the deeper layers [63].

The detection of damage to the skin caused by UVB radiation is carried out through the activity of cells that undergo apoptosis [64] and mast cells, which secrete inflammatory mediators and recruit other leukocytes, such as neutrophils [50]. Furthermore, the epidermal thickness serves as a quantitative indicator to evaluate inflammation. In our model of UVB

irradiation, it has been observed that the dose-response of MaR2 plays a significant role in reducing the number of burned cells, epidermal thickness, and mast cell count. Similar studies have demonstrated that the use of resolvin D1 (RvD1) [16] and MaR1 [41] attenuated acute skin damage in a UVB-induced skin injury model in hairless mice.

In summary, the present study demonstrated for the first time that treatment with MaR2 effectively inhibits oxidative and inflammatory damage induced by UVB irradiation in hairless mice [65]. MaR2 inhibited the inflammatory cell counts in the skin (sunburn cells and mast cells). Those results showed that MaR2 protected the skin from UVB irradiation-induced tissue alterations such as collagen degradation. Therefore, the present results suggest MaR2 as a potential therapy to control UVB-induced skin inflammation and oxidative stress related alterations.

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5.2 Evaluation of the topical effect of the formulation containing maresin 2 (TFcMaR2) in reducing inflammation and oxidative stress induced by UVB radiation in hairless mice.

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Evaluation of the topical effect of the formulation containing maresin 2 (TFcMaR2) in reducing inflammation and oxidative stress induced by UVB radiation in hairless mice.

ABSTRACT

The skin is the body's main protective barrier against external aggressors. Among the external factors, exposure to UVB radiation stands out, which is one of the main causes of skin damage. Acute exposure to UVB radiation causes a series of adverse effects on the skin such as edema, sunburn, erythema, inflammation and immunosuppression. In this context, the use of anti-inflammatory/pro-resolution lipid mediators such as Maresin 2 (MaR2) derived from macrophages, with docosahexaenoic acid (DHA) as a precursor, stands out. It would be a promising alternative to enrich the system and endogenous protective processes altered by irradiation. The objective of this work was to investigate the effectiveness of TFcMaR2 at a dose of 10ng/animal in reducing oxidative and inflammatory damage. MaR2 was inserted into a stable emulsion and was effective in reducing skin edema and the activity of the enzyme metalloproteinase type 9 (MMP-9), in histological parameters it was also statistically effective in reducing the degradation of collagen fibers, epidermal thickness, apoptosis of reduction of keratinocytes and mast cells, as well as to prevent depletion of endogenous antioxidants such as GSH and CAT, improve overall antioxidant capacity in iron-reducing antioxidant power (FRAP) and ABTS radical scavenging tests, and ultimately help to reduce the production of superoxide anion (O_2^-) and lipid mediating hydroperoxides (LOOH). Pro-resolving lipid mediator is a potential strategy to control/prevent oxidative and inflammatory diseases caused by exposure to UVB radiation in hairless mice.

Keywords: Maresin 2, UVB-irradiation, inflammation, oxidative stress, pro-resolution;

Highlights

- TFcMaR2 protected the skin from the deleterious effects of UVB irradiation.
- TFcMaR2 inhibited UVB-induced edema.
- TFcMaR2 suppressed the secretion of MMP-9 induced by UVB-irradiation.
- TFcMaR2 inhibited UVB-induced skin oxidative stress.

Abbreviations

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
CAT	Catalase
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
FRAP	Ferric reducing antioxidant power
GSH	Reduced glutathione
HO$^{\bullet}$	Hydroxyl radical
HO-1	Heme oxygenase 1
H_2O_2	Hydrogen peroxide
LPO	Lipid peroxidation
LOOH	Lipid hydroperoxides
KC	Keratinocytes
MaR1	Maresin 1
MaR2	Maresin 2
NBT	Nitroblue tetrazolium
Nrf2	Nuclear factor erythroid 2-related factor 2
O_2	Superoxide anion
ROS	Reactive oxygen species
SBC	Sunburn cells
SOD	Superoxide dismutase
TPTZ	2,4,6-tris(2-pyridyl)-*s*-triazine
TFcMaR2	Topical formulation containing MaR2
UV	Ultraviolet
UVB	Ultravioleta tipo B

1. INTRODUÇÃO

The skin has the function of covering our body and forming a barrier to protect the body against external aggressors. The skin is constantly exposed to external pro-oxidant stimuli, such as ultraviolet radiation (UVR) [1,2]. Excessive exposure to UV can trigger harmful effects resulting from the intense inflammatory reaction and increase in free radicals [3-5] this acute exposure causes a series of effects adverse effects such as edema, sunburn, erythema, inflammation and immunosuppression, in addition, chronic exposure can lead to premature aging and the development of skin cancer.

The UVB radiation spectrum is mainly responsible for the contractual effects of sunlight, as it reaches the epidermal layer, directly affecting the DNA of keratinocytes, which results in the production of mutagenic photoproducts [6,7] In addition to causing extensive damage to the Directly to the skin, UVB radiation can also induce damage through indirect mechanisms that involve the production of reactive oxygen species (ROS) and an intense inflammatory response, making it important for photocarcinogenesis [5,8].

Irradiation is a process that appears as a response to infectious agents or the stimulus that provoked it, with the aim of eliminating and repairing the tissue damage caused. Generally, it is an adequate and controlled response, with the release of mediators to restore the site, reestablishing homeostasis. When this response is exacerbated, the damage can be irreversible, favoring the development of several chronic diseases. In order to control the progress of the inflammatory process, local mediators are produced to resolve inflammation, called pro-resolution lipid mediators [9,10].

Epidemiological studies indicate that sunscreens are not fully effective in preventing ultraviolet radiation (UVR)-induced skin damage [11]. Therefore, it is necessary to use protectors or blockers combined with antioxidant and anti-inflammatory sources in order to prevent damage caused by radiation to the skin [12].

The literature demonstrates that dietary omega-3 fish oils contain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA), which may be beneficial for inflammatory disease [13-15].

DHA is the precursor of a new family of lipid mediators, derived from macrophages, called maresins, which are produced by human 12-lipoxygenase (12-LOX) [15]. This species of maresin was the first to be identified. It has shown anti-inflammatory and pro-resolution action, inhibiting inflammation by neutrophils and stimulating phagocytic macrophages [13-16].

Maresin-2 (MaR2) is a DHA-derived specialized pro-resolving lipid mediator (SPM) produced by macrophages and the newest identified member of the maresin family (Deng et al., 2014). In human macrophages, the cascade for MaR2 biosynthesis begins with the conversion of DHA to the 13S intermediate, 14S-epoxy-maresin by 12-LOX. Subsequently,

this intermediate is converted into MaR2 through a soluble epoxide hydrolase, while MaR1 uses an epoxide hydrolase [14,17]. Total organic synthesis of MaR2 is also described by different groups, indicating that synthetic methods can be applied to give rise to MaR2 [17,18]. Intraperitoneal treatment with MaR2 reduces peritoneal neutrophil recruitment in a mouse model of zymosan-induced peritonitis [14]. Although several studies have reported the anti-inflammatory effects of MaR1 [19-21] few studies showed the anti-inflammatory and pro-resolution effect of MaR2 is known [17,22-27], importantly, administration of exogenous MaR2 promoted mucosal repair after dextran sulfate sodium-induced colitis or biopsy-induced colonic mucosal injury [28] but no previous study has demonstrated the anti-inflammatory or pro-resolution effects of topical MaR2 in a UVB-induced skin injury model in hairless mice [29].

2. MATERIALS AND METHODS

2.1 Experimental animals

The animals used were hairless mice of the HRS/J lineage, female, adult and weighing 20 to 30 g, kept in the animal house of the Hospital Universitario de Londrina-PR, in a temperature-controlled room, with a 12-hour light/dark cycle and with water and feed supply. The use of animals was approved and the experiments were carried out following the rules of the Ethics Committee on the use of Animals (CEUA) of the State University of Londrina, project approved by CEUA protocol no. 11146.2016.97.

2.2 System and source of UVB radiation

The light source used in the experiments to induce oxidative stress was a fluorescent UVB lamp model PHILIPS TL/12 40W RS (Medical Netherlands). The lamp emits radiation in the λ range of 270 to 400 nm with a maximum emission peak at 313 nm [30].

The lamp is installed in a wooden compartment designed to induce inflammation. The animals were placed in plastic boxes and covered with a plastic screen to ensure full exposure of the dorsal region. The animals were 20 cm from the irradiation source and moved freely in the box [31].

2.3 Preparation of the formulation containing MaR2

The formulation was prepared using the non-ionic self-emulsifying wax Polawax® (cetostearyl alcohol and polyoxyethylene sorbitol monostearate 20 OE) and a hydrophilic colloid, Aristoflex® (AVC - Co-Polymer of Sulfonic Acid Acryloyldimethyltaurate and Neutralized Vinylpyrrolidone (former of anionic gel).

The emulsion was produced by the phase inversion method at 75°C. The oily phase consisted of 2% Polawax® (w/w) and the aqueous phase consisted of 20% of a 5% dispersion of Aristoflex® (w/w). Afterwards, the emollient capric and caprylic acid triglyceride (5%), a

preservative solution (Phenonip®) at 0.4% and 6% propylene glycol (humectant) were incorporated. The formulations were prepared and divided into two portions, one reserved for control (without addition of Mar2) and the other added 10ng of MaR2/0.5g of formulation. MaR2 was added to the formulation after 24 hours of preparation. The formulation was used to treat the animals immediately after the addition of MaR2.

2.4 Physical chemical characterization

After preparation of the formulation, the formulations were analyzed for to its consistency, color and odor and phase separation [32]. We also measure the pH of the formulations using a 1:10 (w/w) aqueous dispersion. In this way, 1g of each formulation was weighed and 9g of deionized water was added. After completing homogenization on a plate with magnetic stirring, the pH of the samples was measured using a digital pH meter. The readings were carried out in duplicates [33,34].

Finally, the centrifugation test that allows checking the stability by evaluating whether there was phase separation of the formulation, i.e. allows analyzing whether there was physical-chemical instability [35]. Each formulation was weighed in the amount of 2g in centrifuge test tubes, with a conical and graduated shape. The samples were subjected to 3600rpm for 30 minutes, at room temperature, observing the possible occurrence of phase separation [34].

2.5 Treatment of animals with topical formulation

The animals were treated with a topical formulation containing MaR2 or treated with the control formulation (without MaR2). The formulations were applied to the entire back of the animals with the aid of a brush, approximately 0.5g of formulation, 10 minutes before irradiation. The concentration of MaR2 used in the formulation was 10ng. The animals were treated with a topical formulation containing MaR2 or treated with a control formulation (without MaR2). The formulations were applied to the entire back of the animals with the aid of a brush, approximately 0.5g of formulation, 10 minutes before irradiation. The concentration of MaR2 used in the formulation was 10ng. The dose was chosen according to the results of MaR1 (IP) [36]

The animals were randomly allocated into three groups containing six animals each group, as follows:

- Group 1: Non-irradiated and untreated control
- Group 2: Control irradiated and treated with the control formulation (TFC)
- Group 3: Irradiated and treated with the topical formulation of MaR2 (TFcMaR2)

3. ASSESSMENT OF THE THERAPEUTIC EFFICACY OF MAR2

3.1 FRAP assay.

Animal skin samples were collected and homogenized in 500µL of KCl (1.15%) using a turrax and centrifuged at 1,000g for 10min at 4°C. For the FRAP reaction, the supernatant and 1 mL of FRAP reagent were used (2.5 mL of a 10 mM solution of 2,4,6 tripyridyl-S-triazine (TPTZ) in 40 mM HCl, 2.5 mL of FeCl3 .6H2O 20mM and 25 mL of 0.3 mM acetate buffer (pH 3.6) incubated at 37°C for 30 min). The reaction was incubated for 30 min at 37°C and read at 595 nm. Standard solutions with different concentrations of trolox (0.5 to 20 µM) were used for calibration. The results were expressed as µM Trolox equivalent/mg of skin [37].

3.2 ABTS assay.

For the ABTS reaction, 30 µL of the supernatant was added to 1 mL of diluted ABTS solution. ABTS solution was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate. The mixture was stored in an amber bottle at room temperature for at least 16 hours before use. The ABTS solution for use in the reaction was diluted with phosphate buffer (pH 7.4) to an absorbance of 0.700 at 730nm. The reaction was incubated for 6 min and read at 730 nm. Standard solutions with different concentrations of trolox (1 to 25 µM) were used for calibration. The results were expressed as µM Trolox equivalent/mg of skin [5].

3.3 GSH assay.

The collected skin samples were homogenized in 0.02 M EDTA using a turrax. 50% trichloroacetic acid (TCA) was added to the homogenate in a ratio of 1:0.2 of EDTA and TCA, respectively. The mixture was centrifuged and to carry out the experiment, the supernatant of each sample was added with 0.4 M Tris buffer (pH 8.9) and a solution of 1.9 mg/mL of 5,5'-dithio-bis-acid. (2-nitrobenzoic acid) (DTNB) in methanol. Absorbance was determined after 5 minutes of incubation at 405 nm. The analytical curve was prepared with 0 to 150 µM GSH. The results were expressed in µM of GSH/mg of skin [38-40].

3.4 Catalase assay.

The collected skin samples (100mg) will be diluted in 0.02 M EDTA and homogenized with the aid of turrax. The homogenate will be centrifuged and the final supernatant will be removed for analysis. The determination of CAT activity in the skin will be carried out in a microplate by adding a 1M Tris-HCl buffer sample with 5 mM EDTA pH 8.0, deionized water and 200 mM H2O2. A blank will be included in the test for each sample. The speed at which H2O2 is reduced by the action of CAT will be evaluated through the decrease in the absorbance value by the difference between the initial reading and the reading 30 seconds

after the addition of 200 mM H2O2. The reading will be carried out on a spectrophotometer at 240 nm with a temperature maintained at 25ºC. Catalase values will be expressed as catalase unit/mg of skin/minute [41].

3.5 Assessment of superoxide anion production (NBT).

The skin samples will be homogenized in 0.02 M EDTA with the aid of Turrax, centrifuged and the supernatant removed for analysis. For the reaction, the homogenate will be incubated in microplates for 1 hour. Then the supernatant removed and NBT (1 mg/mL) will be added to the fixed cells. After 15 minutes, the supernatant will be carefully removed and 20 µL of methanol will be added to the precipitate to fix it, 120 µL of 2 M KOH and 140 µL of dimethyl sulfoxide to solubilize the compound formed from the reduction of NBT, formazan. NBT reduction will be measured spectrophotometrically at 620 nm. (CAMPANINI et al., 2013).[42].

3.6 LOOH assay.

The assay was conducted based on the measurement of the quimioluminescence (QL) induced by the tert-butyl hydroperoxide [43]. The skin samples were homogenized in 800L of Phosphate Buffered Saline (PBS) containing NaCl 137mM, KCl 2.7 mM, Na2HPO4 10mM, KH2PO4 1.8mM, pH 7.4, and centrifuged at 2.000xg for a duration of 2 minutes. The supernatant, which was 70 L, was mixed with 420L of reaction buffer (20 mM KH2PO4 with 0.9% NaCl pH 7.4), and subsequently, 10L of tert-butyl and 10L of luminol were added. For 3600 seconds, each sample was analyzed, resulting in the same number of points of chemioluminescence. The analysis of the reaction buffer without the sample was performed to check if there was interference in the chemiluminescence reading using the Glomax 20/20 (Madison, Wisconsin, USA) The outcomes were expressed in terms of relative light units per mg of skin.

3.7 Assessment of skin edema

With the aid of a mold, a constant area of skin from the back of each animal (5 mm in diameter) was collected and subsequently weighed [44]. The analysis was based on the difference in weight between the different groups. The result was expressed in mg of skin.

3.8 Histology

The histologic processing was performed with fixation in formaldehyde and embedding in paraffin. Histological sections were prepared and stained with hematoxylin and eosin to quantify the presence of sunburn cells in the epidermis at a magnification of x100 [5] and to determine the thickness of the epidermis at a magnification of 40x (18) Masson's trichrome

was used to demonstrate collagen fibers (x10 magnification) and toluidine was used to quantify mast cells (x40 magnification) For each experiment, the number of mice was 6 in each group. This gives a total of 12 mice per group. Using light microscopy and the software Infinity Analyze, all histological sections were analyzed. The digital images were processed with the Image J program.

3.9 Analyses of skin proteinase substrate-embedded enzymography

The dorsal skin of hairless mice was homogenized using Tissue-Tearor (Biospec 985370) in 0.05 M Tris-HCl buffer. The homogenates were centrifuged twice at 1200xg for 10 minutes at 4°C. A portion of 50L of the samples was mixed with 10L of 0.1M Tris-HCl (pH 7.4) containing 20% glycerol, 4% SDS, and 0.005% xylene cyanol. The mixture was then electrophoresed in a gel containing 10% acrylamide and 0.025% gelatin. After electrophoresis, the gels were incubated for 1 h in 2.5% Triton X-100, followed by overnight incubation in Tris-HCl/CaCl2 buffer (pH 7.4) and 0.02% sodium azide at 37°C. The gel was stained with brilliant blue R the following day, and the zone of enzyme activity was analyzed by comparing the groups using the Image J software package (NIH, Bethesda, MD, USA) [45].

3.10 Statistical analysis of results

The results were statistically analyzed using GraphPad Prism® software, version 8 by analysis of variance (ANOVA) with one factor followed by Tukey's multiple comparison test and presented as the mean ± SEM of measurements made with 6 animals in each group per experiment. Results were representative of two separate experiments and were considered significantly different at $p<0.05$.

4. RESULTS

4.1 Physicochemical stability of TFcMaR2

After 24 hours at temperatures of 4°C, RT and 40 ± 2°C at 75% ± 5% RH, the formulations containing or not MaR2 maintained their color, homogeneity and there was no phase separation. The pH value of the control formulation (TFC) was 5.3 and the TFcMaR2 was 5.6, similar to the pH of the skin, which favored the use of this formulation.

4.2 TFcMaR2 inhibits oxidative stress caused by UVB radiation.

UVB irradiation reduces antioxidant defenses and increases skin indicators of oxidative stress [46]. The antioxidant effects of MaR2 minimized skin damage caused by UVB irradiation, as TFcMaR2 reduces UVB-induced oxidative stress. UVB radiation depleted the skin's antioxidant capacity, as noted by the increase in activity/level in the FRAP (Figure 2A), ABTS (Figure 2B), GSH quantification (Figure 2C) and catalase (Figure 2D) assays. Furthermore,

UVB radiation also increased lipid peroxidation and superoxide anion production, which are indicators of oxidative stress. TFcMaR2 restored the skin's antioxidant capacity, as shown in Figures 2A, 2B, 2C and 2D. Furthermore, TFcMaR2 inhibited the production of superoxide anion (O_2^-) (Figure 3A) and lipid peroxidation (LOOH) (Figure 3B). Thus, the antioxidant effects of MaR2 minimized skin damage caused by UVB irradiation.

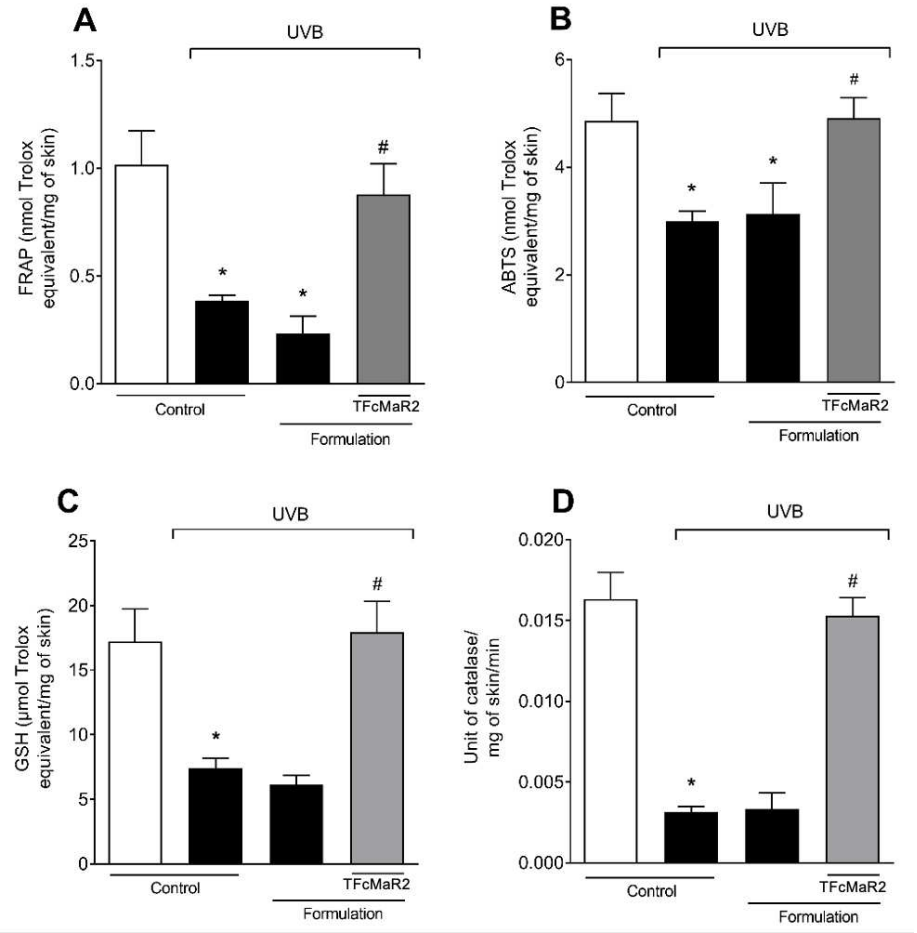

Figure 2 – TFcMaR2 on antioxidant capacity of skin after UVB radiation. The antioxidant capacity was determined by ABTS (A), FRAP (B) and GSH (C) tests on samples collected after 12h and for CAT (D) on samples collected after 2h of the end of irradiation. The bars represent mean ± SEM of 6 mice per group per experiment and are representative of two separate experiments. [*$p<0.05$ compared to the non-radiated control group (white bar); #$p<0.05$ compared to the radiated control groups (bars in black)].

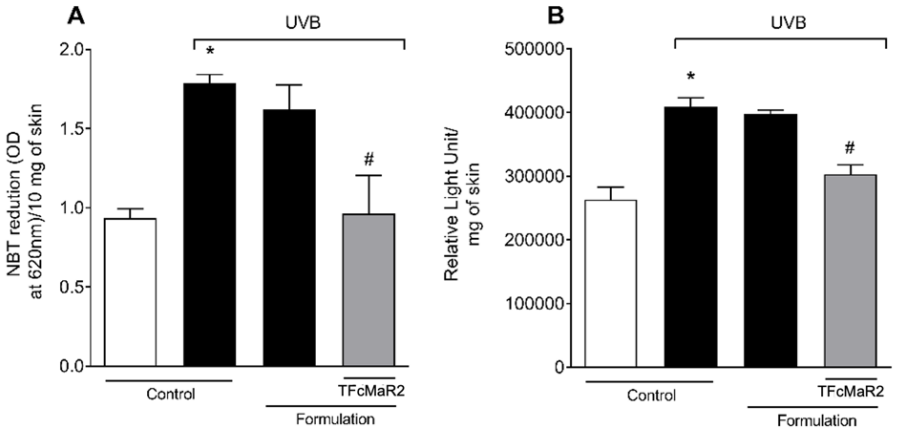

Figure 3 - TFcMaR2 inhibited UVB-radiation induced superoxide anion production and LOOH. The t-butyl LOOH-initiated chemiluminescence (QL) (B) and *nitroblue tetrazolium* (NBT) reduction (A) were determined in samples collect 2 h after UVB exposure. Data are expressed as the mean ± SEM of 6 mice per group and are representative of two separate assays. [*$p<0.05$ compared to the non-radiated control group (white bar); #$p<0.05$ compared to the radiated control groups (bars in black)].

4.3 TFcMaR2 reduced edema, MMP-9 activity and collagen fiber degradation triggered upon UVB stimulation

In order to evaluate the effects of MaR2 on UVB irradiation, we analyzed the production of skin edema. The control group had increased skin weight compared to the sham (Figure 4A). However, the FTcMaR2 technique showed a significant decrease in edema induced by UVB irradiation. Furthermore, MMP-9 activity was demonstrated by the degradation of the polyacrylamide gel in the bands corresponding to the irradiated groups and those treated with the topical formulation without MaR2 (Figure 4B). MMP-9 scavenging activity was lower in the TFcMaR2-treated group. Collagen degradation was assessed using the intensity of blue color in dermal areas of the skin as an indicator of collagen fiber density [5]. Figure 5E demonstrates the proportion of degradation of collagen fibers, caused by UVB irradiation, corroborating the MMP-9 activity data. TFcMaR2 intervened in reducing the degradation of collagen fibers (Figure 5D). As shown in Figure 5, UVB exposure induced collagen degradation and TFcMaR2 inhibited this degradation. This result is in agreement with the inhibited activity of MMP-9 with TFcMaR2 shown in Figure 4.

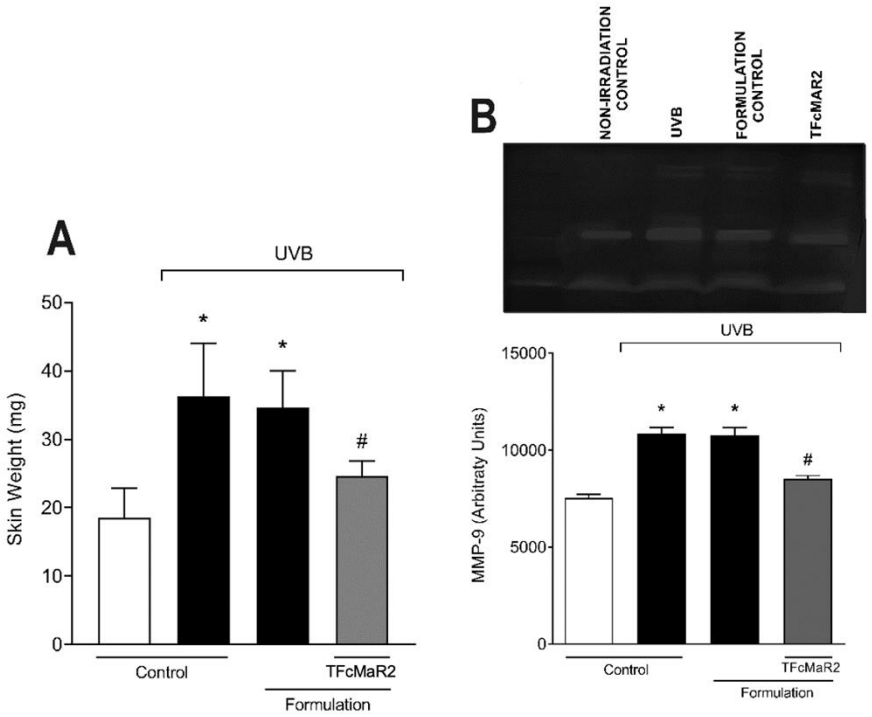

Figure 4 - TFcMaR2 reduces UVB radiation-induced skin edema (A) and skin matrix metalloproteinase-9 (MMP-9) (B) activity. The skin edema and MMP-9 were determined in samples collected 12 h after the end of radiation. Data are expressed as the mean ± SEM of 6 mice per group and are representative of two separate assays. [*$p<0.05$ compared to the non-radiated control group (white bar); #$p<0.05$ compared to the radiated control groups (bars in black)].

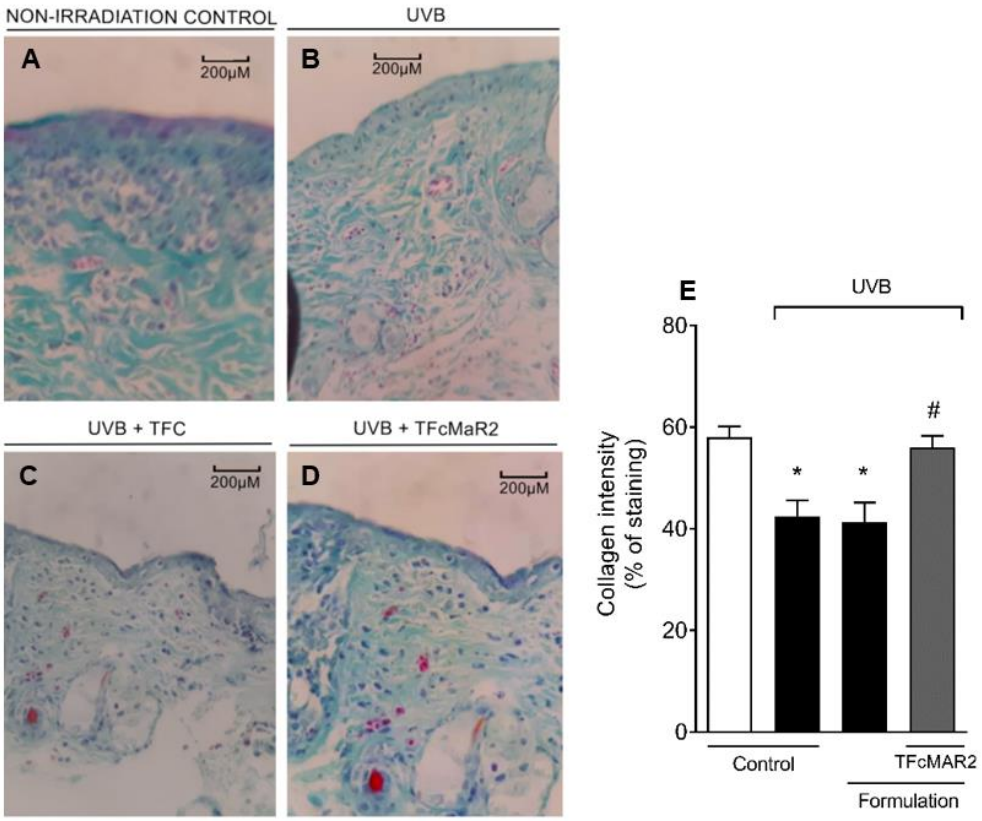

Figure 5 – TFcMAR2 inhibits UVB radiation-induced collagen fiber damage. The intensity of collagen fibers was measured using Masson's Trichrome stain on skin samples collected 12 hours after the end of irradiation. Representative images of the groups: non-irradiated control (A), UVB (B), UVB + TTFC (C) and UVB + TFcMaR2 (D) (10x magnification). Quantitative analysis of collagen degradation of experimental groups is presented as percentage of staining in panel (E). Data are expressed as the mean ± SEM of 6 mice per group and are representative of two separate assays. [*p<0.05 compared to the non-radiated control group (white bar); #p<0.05 compared to the radiated control groups (bars in black)].

4.4 TFcMaR2 prevents the keratinocyte apoptosis process, inhibits epidermal thickening and decreases the number of skin mast cells after UVB stimulation

UVB radiation has been shown to be capable of inducing keratinocytes to undergo apoptosis due to its action. Therefore, we investigated apoptotic keratinocytes after TFcMaR2 treatment. The results showed that TFcMaR2 treatment inhibited sunburn cells compared to the irradiated group without treatment and the formulation without MaR2 (Figure 6). One of the consequences of prolonged exposure to UVB radiation is the occurrence of inflammation that results in enlargement of the epidermis due to increased edema, cellular infiltration and cellular proliferation [47]. Analysis of the tissue sections revealed that the epidermal thickness of the dorsal skin increased significantly after radiation in the untreated group and in the one treated with the formulation without MaR2, compared to the control group. However, epidermal

hypertrophy was significantly reduced with the TFcMaR2 technique (Figure 7). The formulation without MaR2 had a significant effect on reducing cell and sunburn thickness. The data presented demonstrate the protective effect of TFcMaR2 after keratinocyte apoptosis induced by UVB irradiation and epidermal thickness.

Exposure to UVB radiation induces an increase in epidermal thickness due to the hyperproliferation of keratinocytes [38]. Histological analysis of hematoxylin and eosin-stained tissue sections indicated that epidermal thickness was significantly increased following exposure to UVB. On the other hand, epidermal hypertrophy was reduced compared with the irradiated control group when mice were treated with TFcMaR2 (Figure 8).

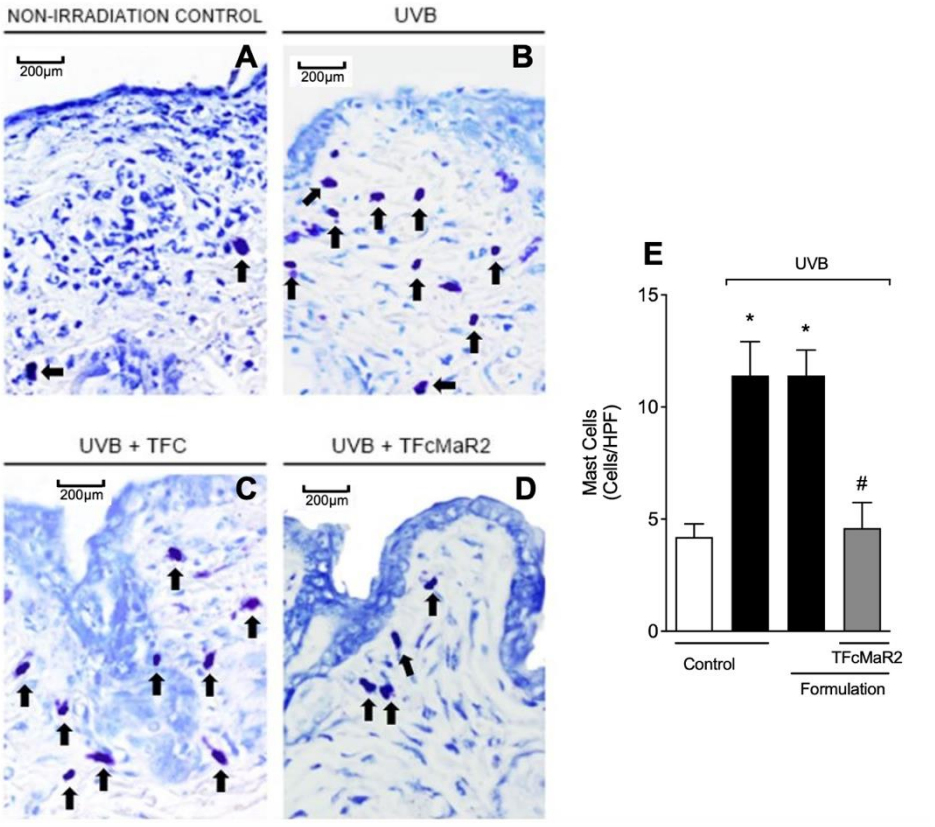

Figure 6 - TFcMAR2 diminishes the mast cells counts in the skin after UVB exposure. Mast cells were evaluated by toluidine blue. Representative images of the groups: non-irradiated control (A), UVB (B), UVB + TFC (C) and UVB + TFcMaR2 (D) (40x magnification). Quantitative analysis of mast cell recruitment from experimental groups is presented as percentage of staining in panel (E). Data are expressed as the mean ± SEM of 6 mice per group and are representative of two separate assays. [*$p<0.05$ compared to the non-radiated control group (white bar); #$p<0.05$ compared to the radiated control groups (bars in black)].

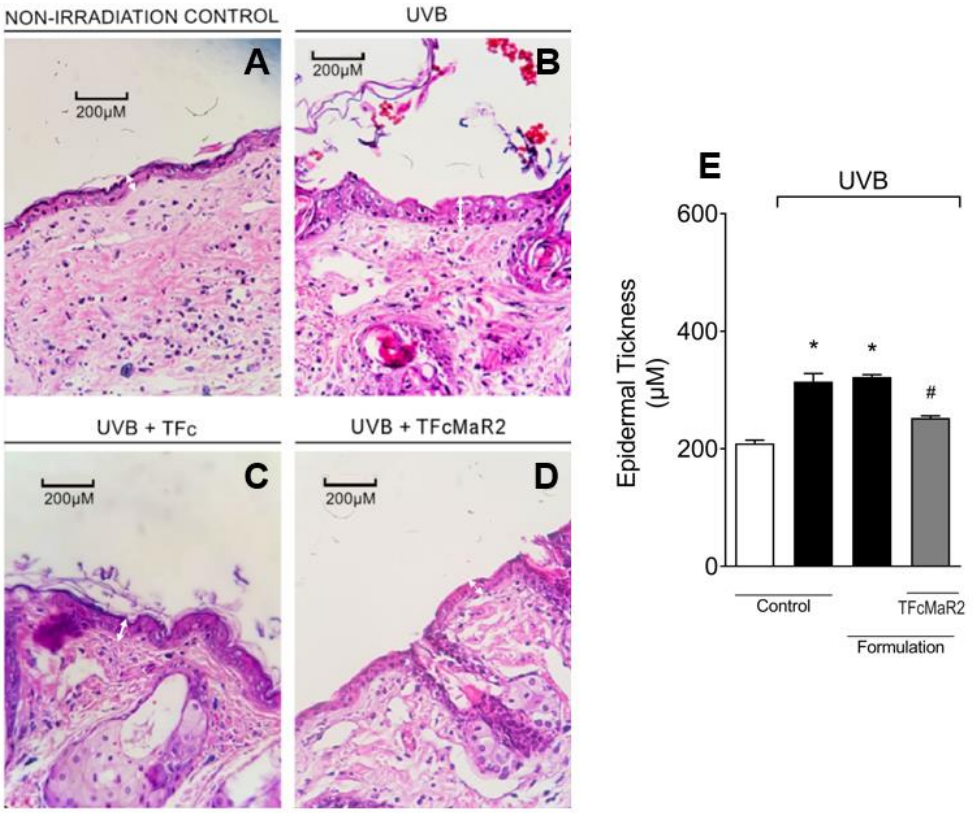

Figure 7 – TFcMaR2 reduced UVB irradiation-induced increase of epidermal thickness. The epidermal thickness was determined in samples collected 12 h after the end of irradiation and stained with hematoxylin and eosin staining (H&E). Representative images of the groups: non-irradiated control (A), UVB (B), UVB + TFC (C) and UVB + TFcMaR2 (D) (40x magnification). Quantitative analysis of the epidermal thickness of the experimental groups is presented as percentage of staining in panel (E). Data are expressed as the mean ± SEM of 6 mice per group and are representative of two separate assays. [*$p<0.05$ compared to the non-radiated control group (white bar); #$p<0.05$ compared to the radiated control groups (bars in black)].

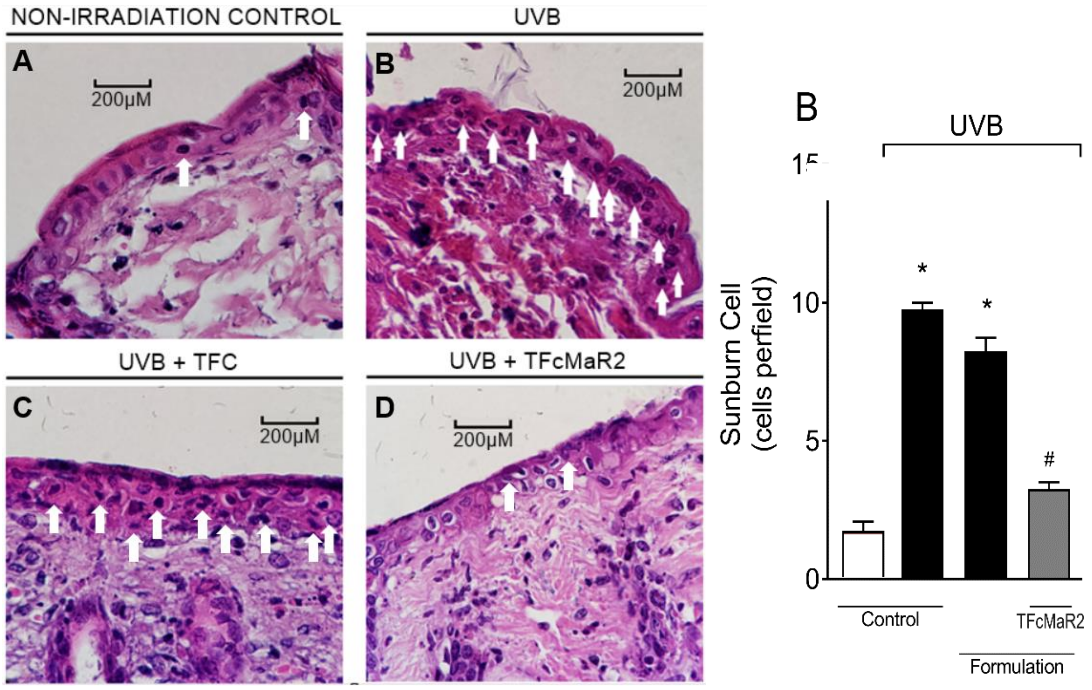

Figure 8 - TFcMaR2 diminishes UVB radiation-induced sunburn cell counts. Sunburn cell (black arrows) counts were evaluated using hematoxylin and eosin-stained slices of skin samples collected 12 h after the end of irradiation. Representative images of the groups: non-irradiated control (A), UVB (B), UVB + TFC (C) and UVB + TFcMaR2 (D). Quantitative analysis of sunburn cells from experimental groups is presented as percentage of staining in panel (E). Data are expressed as the mean ± SEM of 6 mice per group and are representative of two separate assays. [*p<0.05 compared to the non-radiated control group (white bar); #p<0.05 compared to the radiated control groups (bars in black)].

5. DISCUSSION

MaR2 was added to a topical formulation. MaR2 was added to a topical formulation. The dose and skin collection times were previously described in a previous study with MaR1 [22] and, when exposing hairless mice to UVB radiation, it was noted that, when treated with TFcMaR2, the photooxidative and inflammatory damage was alleviated. To date, the use of MaR2 in topical emulsions has not been investigated in any model.

The use of an emulsion allows the drug to be administered topically to directly treat the injured area or deliver the drug to other tissues [48]. Therefore, knowledge of skin physiology is important. It is known that the skin is a multifunctional organ with multiple compartments, presenting a lipophilic stratum corneum composed of proteins and lipids that can reversibly or irreversibly bind to medications [49]. Thus, this stratum limits the diffusion of compounds into the viable epidermis. However, other epidermal structures may be absorption pathways for substances, such as hair follicles and sweat glands [50]. Then, some variables allow adequate skin permeation: molecular mass less than 600 Da, solubility in

water and oil, high partition coefficient and high skin hydration [51,52].

In this context, MaR2 (13r 14s-dihydroxy-docosahexaenoic acid) is a lipophilic substance with a molecular mass of 360.5 Da, which was added to the emulsion meeting the requirements mentioned above, combined with other excipients such as the moisturizer propylene glycol. Lipophilic substances can be solubilized during the lipophilic phase of emulsions, which will result in greater solubility [53]. Without a doubt, the vehicle in which the active is administered can have a significant impact on the release of a topical pharmaceutical prescription. Adequate formulation of the topical agent will allow it to exert its maximum activity on the skin. A drug that is in a vehicle needs to reach the skin in adequate form and in adequate quantities [54].

Both formulations, the control and TFcMaR2, maintained their color, odor, and consistency characteristics. The formulations were also stable when the centrifugation test was applied, no phase separation was detected. In addition, the pH value of the TFcMaR2 or control formulation were compatible with skin conditions.

MaR2 was added in topical formulation and the therapeutic potential was characterized by reduced edema, increased thickness of the epidermal layer, increased metalloproteinase-9 activity, reduced formation of sunburn cells, preservation of collagen in the dermis and evaluation of the antioxidant effects, as shown in the results above.

Previous studies have already shown that new families of lipid mediators, called lipoxins, resolvins, protectins and maresins have a potent effect on resolving inflammation [55–63]. We have recently demonstrated that the systemic treatment with lipoxin A4 (LXA4), maresin 1 (MaR1) and resolvin D1 (RvD1) was efficient in reducing inflammation and oxidative stress in model a UVB-induced skin lesion in hairless mice [4], [22], [5]. Other studies also reported that MaR2 was able to attenuate the effects of inflammation and oxidative stress [17,23-25,28].

Solar UVB radiation (290–320 nm) is the most important environmental factor involved in the pathogenesis of skin cancers. The genotoxic properties of UVB radiation include mainly induction of oxidative stress, photoaging and photocarcinogenesis [64,65].

One of the consequences of UVB-irradiation is the recruitment of neutrophils with the release of enzymes from the metalloproteinase family that degrade collagen. In this study, we analyzed MMP-9.

MMPs play a crucial role in photocarcinogenesis, regulating several processes that are related to tumor progression, such as initiation, growth, angiogenesis and metastasis. Furthermore, excessive production of MMP-9 contributed to early skin disease [66].

MMP-9 is an enzyme that requires the activation of AP-1 to be expressed. MMP-9 is predominantly secreted by neutrophils, mast cells and macrophages. Its protective activity has type IV collagen as its main component of the basement membrane [67].

Simultaneous exposure to UVB radiation can cause an increase in the production of ROS in the skin, which results in an increase in the activity of AP-1 which results in a continuous degradation of proteins MMP-1, MMP-3 and MMP-9, reduction the rate of collagen renewal/synthesis. Dysregulation of MMP-9 is associated with inflammatory skin diseases and the invasive and metabolic potential of cancer cells [68]. In this way, MaR2 prevents tissue changes caused by UVB radiation, such as collagen degradation, these results show in also other experiments with pró-resolution lipid [4,5,22,46] and the results showed that the enzyme activity was attenuated in the group treated with TFcMaR2.

Exposure to UVB irradiation stimulates the inflammatory response, causing edema [69]. Recently it was reported that activation of the vascular endothelial growth factor (VEGF) pathway is necessary for initiating the acute pathological effects UV-induced in the epidermis. VEGF is a potent vascular permeability factor mainly produced by epidermal keratinocytes and fibroblasts. It is upregulated following UV exposure in mice, and it induces edema, erythema, epidermal hyperplasia, and increased vascularity. Also, it causes edema and the related immediate impact on the skin while working concomitantly with EROS to induce inflammation [70]. TFcMaR2 reduced skin edema, like the other pro-resolving lipid findings [4,5,22].

Neutrophils are the first cells to migrate to the injury site [71]. These phagocytes produce large amounts of pro-inflammatory cytokines and ROS [71], like superoxide anion ($O_2^{\bullet-}$). The additional production of ROS contributes to the intensification of the inflammatory process and tissue damage [72].

UVB irradiation produces ROS directly and indirectly through the inflammatory response [73]. Excessive ROS production causes depletion of the skin's natural antioxidants, limiting the cells' ability to self-protect and resulting in oxidative stress [74]. Oxidative stress can lead to cellular damage (e.g. lipid peroxidation) [75].

ROS play a critical role in the photoaging of human skin, as well as being responsible for several types of skin cancer and other inflammatory skin diseases [73]

Regarding endogenous antioxidants, reduced glutathione (GSH) is an antioxidant peptide that reduces ROS and inhibits lipid peroxidation, donating a hydrogen atom, in addition to participating in the oxidation-reduction mechanism of glutathione peroxidase (GPX) [76]. The largest reserve of endogenous antioxidants presents in the skin, including GSH, is located in the epidermis [77]. However, the epidermis is the layer of the skin most affected by UVB radiation, which results in a decrease in its antioxidant reserves [78]. Thus, GSH depletion is a previous marker of oxidative stress in the epidermis. MaR1 was analyzed in a previous study for the protection of the antioxidant system and showed an increase in the production of endogenous GSH [27,79].

In our research, the results showed the maintenance of GSH concentration in the

TFcMaR2 group when compared to the untreated irradiated control group and treated with control formulation, other pro-resolution lipids also had this effect [4,5,22,46].

Another endogenous antioxidant that changes when exposed to UVB radiation is catalase, responsible for the decomposition of hydrogen peroxide (H_2O_2), although not highly reactive, it plays an important role in oxidative stress as it is able to easily cross cell membranes and generate the hydroxyl radical (OH^-) [80,81]. The increase in lipid peroxidation and the production of hydroxyl radicals, in addition to the decrease in the skin's antioxidant capacity, observed by the reduction in FRAP activity, and the endogenous antioxidants CAT and GSH have been used as markers of UVB-induced oxidative stress [4,43,45,79,82,83]. TFcMaR2 treatment not only reduced oxidative stress but also improved the skin's antioxidant capacity after exposure to UVB irradiation, maintaining catalase and GSH at basal levels.

What is more, our results suggest that MaR2 can significantly improve UVB-induced oxidative stress by reducing LOOH and $O_2^{\bullet-}$ production, as well as improving skin antioxidant capacity by increasing ferric reduction (FRAP assay). Similarly, the antioxidant properties of MaR2 and other lipid mediators have been reported in many other studies related to oxidative stress [4,5,22,27,84].

Finally, after UVB exposure, keratinocytes undergo apoptosis (sunburn cells) if the damage is sufficiently large and exceeds the cell recovery limit [80]. Apoptosis is the major mechanism preserving cell homeostasis and thus preventing skin carcinogenesis [85]. UVB causes DNA fragmentation in keratinocytes [86], and DNA-damaged cells are prone to undergo subsequently malignant transformation [85].

Histologically, sunburn cells present altered morphology as observed by chromatin condensation and eosinophilic cytoplasm [87]. Schwarz et al. have concluded that TNF- α contributes to the formation of sunburn cells, but it does not seem to be the only mechanism involved. Keratinocytes also release a variety of cytokines which may contribute as well. Therefore, the study supports the concept that UVB-induced formation of sunburn cells and apoptosis is a multifactorial event [86]. Also, the reduction in the number of sunburn cells indicates an increase in the photoprotection of keratinocytes by TFcMaR2.

Hours after the exposure to UV, the signs of damage response decrease, and epidermal keratinocytes begin to proliferate intensely, mediated by various epidermal growth factors. Increased cell division of keratinocytes after exposure to UVB causes epidermal thickening [88]. UVB irradiation induced significant epidermal thickening compared to the non-radiated control group and this effect was inhibited by TFcMaR2.

UVB radiation also stimulates mast cells to secrete hormones that inhibit inflammation and attract other leukocytes, such as neutrophils [89]. Histamine is the main biogenic amine of mast cells and is related to systemic immunosuppression caused by UVB radiation [90]. Exposure to UVB radiation caused a significant increase in mast cells compared to baseline

conditions in the non-irradiated control group. TFcMaR2 also significantly decreased mast cell counts. Hart et al. reported that the downregulation of systemic immune responses in mice following exposure to UVB radiation is directly related to the prevalence of histamine-containing dermal mast cells. Furthermore, it has been reported that mast cells degranulate in response to direct contact with activated T cells and occurrences of TNF-α during the inflammatory process. TNF-α is a cytokine whose main function is to inhibit the manifestation of inflammatory responses in the skin, as well as the positive regulation of MMP-9 expression [91], as demonstrated by our results.

Finally, UVB radiation induces keratinocyte apoptosis and increased number of mast cells [90,92]. Our results showed that TFcMaR2 reduced apoptosis of keratinocytes (sunburned cells) and the number of mast cells. Consistent with our findings, experimental studies have already showed that treatment with pro-resolving lipids suppresses epidermal enlargement, mast cell recruitment and decreases burned cells [5,93]. The topical administration of drugs, in addition to being safer when compared to other routes, has already been shown to be efficient in reducing damage caused by UVB [30,42,43]. Thus, the TFcMaR2 proved to be a promising agent for the treatment of damage caused by UVB.

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6. CONSIDERAÇÕES FINAIS

A exposição a radiação ultravioleta induz uma série de efeitos deletérios na pele, inclusive o envelhecimento precoce e o câncer de pele. A produção excessiva de radicais livres, induzida pela UVB, leva a depleção dos antioxidantes endógenos e também ao processo inflamatório. Assim, o uso de mediadores lipídicos anti-inflamatórios/pró-resolução, como a MaR2, para enriquecer o sistema antioxidante endógeno e controlar os processos lesivos induzidos pela radiação UVB torna-se uma alternativa promissora. No entanto, ao nosso conhecimento, ainda não existiam estudos sobre o uso da MaR2 na melhora ou prevenção de danos inflamatórios e oxidativos causados pela radiação UVB em camundongos.

A MaR2 foi capaz de inibir o estresse oxidativo e a inflamação nas duas vias de administração por manter os níveis de GSH e atividade da catalase. Somando-se a isso, também foi capaz de inibir o edema cutâneo, a atividade da MMP-9, a produção de hidroperóxidos lipídicos e o radical superóxido, também reduziu a espessura epidérmica, o número de células apoptóticas, de mastócitos e a degradação de fibras de colágeno induzidos pela radiação UVB, porém na avaliação do recrutamento de neutrófilos pelo método indireto da atividade da enzima mieloperoxidase (MPO) não observamos diferença estatística significativa nas duas vias de tratamento, para todos os outros parâmetros avaliados neste estudo, como citados acima, ambos os tratamentos obtiveram diferença estatística significativa.

Os tratamentos quando comparados entre si, demonstraram um efeito satisfatórios nas duas vias de administração, porém levando em consideração o uso da radiação como forma de indução do estresse oxidativo e inflamação, entendemos que a via tópica seria uma melhor opção, por agir direto no local da lesão de forma segura e efetiva, não ter efeitos de primeira passagem, irritação gastrointestinal, metabolismo hepático e toxicidade, quando comparado a uma administração sistêmica.

Em conclusão, os resultados do nosso trabalho sugerem que o mediador lipídico MaR2 administrado topicamente ou pela via intraperitoneal (efeito sistêmico), são ótimas estratégias para controlar e/ou prevenir os danos cutâneos causados pela radiação UVB na pele.

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