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ESTADUAL DE LONDRINA

PRISCILA SAITO

**AVALIAÇÃO DO EFEITO DA RESOLVINA D1 NA
INFLAMAÇÃO E ESTRESSE OXIDATIVO INDUZIDOS PELA
RADIÇÃO UVB EM CAMUNDONGOS SEM PELO**

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

Orientadora: Profa. Dra. Rúbia Casagrande

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BANCA EXAMINADORA

Orientadora: Profa. Dra. Rúbia Casagrande
Universidade Estadual de Londrina - UEL

Profa. Dra. Renata Micheli Martinez
Universidade Estadual de Londrina - UEL

Profa. Dra. Sandra Regina Georgetti
Universidade Estadual de Londrina - UEL

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Dedico este trabalho:

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RESUMO

A pele é o maior órgão do corpo humano e a principal barreira de proteção do organismo contra agressores externos. Entre os fatores externos destaca-se a exposição à radiação UVB que é uma das principais causas de danos na pele. A exposição aguda à radiação UVB acarreta uma série de efeitos adversos na pele 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. Neste contexto, a utilização de mediadores lipídicos anti-inflamatórios/pró-resolução como as resolvinas D1 (RvD1) para enriquecer o sistema de proteção endógeno e controlar os processos lesivos induzidos pela radiação UVB tornam-se uma alternativa promissora. As RvD1 de maneira geral inibem a produção de citocinas, o recrutamento de células pró-inflamatórias e induzem a expressão de moléculas antioxidantes. No presente trabalho foram avaliados os efeitos terapêuticos e mecanismos de ação da RvD1, administrada via intraperitoneal (ip), nos danos cutâneos inflamatórios e oxidativos induzidos pela radiação UVB em camundongos sem pelo. Os resultados *in vivo* demonstraram que o tratamento sistêmico ip com RvD1 reduziu a inflamação cutânea e protegeu a pele do estresse oxidativo induzidos pela radiação UVB. A melhora no processo inflamatório foi constatada pela diminuição do edema de pele, do recrutamento de neutrófilos, da atividade/secreção da metaloproteinase-9 e da produção de diferentes citocinas induzidas pela radiação UVB. Houve também a diminuição do número de queratinócitos apoptóticos, de mastócitos, do espessamento da epiderme e da degradação das fibras de colágeno. O tratamento com RvD1 também protegeu a pele do estresse oxidativo, por manter os níveis da glutathiona reduzida (GSH) e a atividade da catalase a níveis basais, e ainda diminuiu a produção de hidroperóxidos lipídicos, de ânions superóxidos e a expressão de RNAm para gp91^{phox} (subunidade da nicotinamida adenina dinucleotídeo fosfato oxidase). A RvD1 também foi capaz de aumentar a expressão de RNAm para NADPH: quinona oxidoreductase 1 (Nqo-1), fator nuclear [derivado eritróide-2] tipo 2 (Nrf2) e heme-oxigenase 1 (OH-1). Esses resultados sugerem o uso do mediador lipídico RvD1 como estratégia promissora para controlar doenças cutâneas causadas pela exposição à radiação UVB.

Palavras chaves: Resolvina. Inflamação. Estresse oxidativo. Radiação UVB.

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ABSTRACT

The skin is the largest organ of the human body and the main barrier of protection of the organism against external aggressors. Among the external factors, exposure to UVB irradiation is one of the main causes of skin damage. Acute exposure to UVB irradiation lead a number of adverse effects as such as edema, sunburn, erythema, inflammation and immunosuppression, and chronic exposure can lead to premature aging and the development of skin cancer. In this context, the use of anti-inflammatory/pro-resolution lipid mediators such as resolvins D1 (RvD1) to enrich the endogenous protection system and thus control the harmful processes induced by UVB irradiation become a promising alternative. RvD1 in general inhibits cytokine production, inhibits recruitment of pro-inflammatory cells and induced the expression of antioxidant molecules. In the present study, the therapeutic effects and mechanisms of action of RvD1, administered intraperitoneally (ip), on inflammatory and oxidative skin damage induced by UVB irradiation in hairless mice were evaluated. In vivo results demonstrated that systemic ip treatment with RvD1 reduced skin inflammation and protected the skin from oxidative stress induced by UVB irradiation. The improvement in the inflammatory process was evidenced by the decrease of skin edema, neutrophil recruitment, metalloproteinase-9 activity/secretion and the production of different cytokines induced by UVB irradiation. There was also a decrease in the number of sunburn cells, mast cell, epidermal thickness and degradation of collagen fibers. Treatment of mice with RvD1 also protected the skin from oxidative stress by maintaining the levels of reduced glutathione (GSH) and catalase activity to basal levels, and even decreased production of lipid hydroperoxides, production of superoxide anions and mRNA expression for gp91^{phox} (subunit of NADPH oxidase). RvD1 was also able to increase mRNA expression for NAD(P)H: quinone oxidoreductase 1 (Nqo-1), nuclear factor [2 erythroid-derived] type 2 (Nrf2) and heme oxygenase 1 (OH-1). These results suggest the use of the lipid mediator RvD1 as a promising strategy to control UVB-induced cutaneous diseases.

Keywords: Resolvin. Inflammation. Oxidative stress. UVB irradiation.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de variância
AP-1	Ativador de proteína-1
ARE	Elemento de resposta antioxidante
CaCl ₂	Cloreto de cálcio
DNA	Ácido desoxirribonucleico
DHA	Ácido docosaheptaenoico
DTNB	Ácido 5,5'-ditio-bis-(2-nitrobenzóico)
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Ensaio imunoenzimático
ERK	Quinase regulada por sinal extracelular
EROS	Espécies reativas de oxigênio
GSH	Glutathiona reduzida
GPx	Glutathiona peroxidase
GST	Glutathiona-S-transferase
H&E	Hematoxilina e eosina
HCl	Ácido clorídrico
HO [•]	Radical hidroxil
HO-1	Hemeoxigenase-1
HTAB	Brometo de hexadecil trietil amônio
H ₂ O ₂	Peróxido de hidrogênio
IκB	Fator inibidor de κB
IL	Interleucina
Ip	Intraperitoneal
JNK	Quinase c-Jun n-terminal
KCl	Cloreto de potássio
K ₂ HPO ₄	Fosfato de potássio dibásico
KOH	Hidróxido de potássio
KH ₂ PO ₄	Fosfato de potássio monobásico
LOOH	Hidroperóxidos lipídicos
LPO	Peroxidação lipídica

MAPK	Proteína ativada por mitógeno
MMPs	Metaloproteinases da matriz
MPO	Mieloperoxidase
RNA _m	Ácido ribonucleico mensageiro
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NF-κB	Fator nuclear-κB
Nqo-1 NADPH	quinona oxidoreductase 1
Nrf2	Fator nuclear [derivado eritróide-2] tipo 2
O ₂ •-	Radical superóxido
O ₂	Oxigênio molecular
¹ O ₂	Oxigênio singlete
OH	Radical hidroxil
OH	1 Heme-oxigenase 1
ONOO ⁻	Peróxido nitrito
PCR	Reação em cadeia da polimersase
RUV	Radiação ultravioleta
RvD1	Resolvina D1
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

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1. INTRODUÇÃO

1.1. PELE

A pele é o maior órgão do corpo humano e a principal barreira de proteção do organismo contra agressores externos, tais como microrganismos patógenos, substâncias químicas e a radiação ultravioleta (RUV). Além disso, a pele impede a perda excessiva de água e eletrólitos do corpo e é importante na regulação da temperatura corporal (AFAQ; ADHAMI; MUKHTAR, 2005; FONSECA et al., 2011a; KHAVKIN; ELLIS, 2011).

A pele é composta basicamente por três camadas: epiderme, derme e hipoderme, sendo a epiderme a camada mais externa e a hipoderme a mais interna. A epiderme pode ser definida como um epitélio escamoso estratificado que sofre constante renovação. As camadas que compõem a epiderme são: estrato basal ou estrato germinativo, estrato espinhoso, estrato granuloso, estrato lúcido e estrato córneo. As principais células presentes na epiderme são os queratinócitos que representam aproximadamente 95% da sua composição (FONSECA et al., 2011a; KHAVKIN; 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 pele como 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 (ITO; WAKAMATSU; OZEKI, 2000; VINCENSI et al., 1998).

A derme está localizada sob a epiderme, é composta sobretudo por colágeno e apresenta vasos sanguíneos e terminações nervosas (KHAVKIN; ELLIS,

2011), sendo responsável por fornecer suporte mecânico para a epiderme (FISHER et al., 1997). Além disso, na derme estão presentes folículos pilosos, glândulas sebáceas e sudoríparas. Uma grande quantidade de células imunes e fibroblastos, que participam ativamente de muitas respostas fisiológicas na pele, também estão presentes na derme (D'ORAZIO et al., 2013).

A hipoderme, camada mais profunda da pele, está localizada logo abaixo da derme sendo responsável por proteger o corpo contra lesões mecânicas, por isolá-lo e pelo estoque de energia. As células encontradas em maior quantidade na hipoderme são os adipócitos (KHAVKIN; ELLIS, 2011).

A RUV é o fator físico presente em maior quantidade e a principal causa de danos na pele. A exposição aguda à RUV acarreta uma série de efeitos adversos tais 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; MUKHTAR, 2005; FONSECA et al., 2011a; MARTINEZ et al., 2015; TEWARI et al., 2013a).

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 acarretaram o aumento significativo da quantidade de RUV a qual as pessoas estão expostas, o que conseqüentemente aumentou a incidência de câncer de pele em humanos (AFAQ; ADHAMI; 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 30% de todos os tumores malignos registrados (INCa 2017), 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 (AFAQ; ADHAMI; MUKHTAR, 2005).

1.2. RADIAÇÃO ULTRAVIOLETA (UV)

A luz solar é um espectro eletromagnético contínuo, classificada didaticamente em três diferentes tipos de acordo com a faixa de comprimento de onda: luz visível, infravermelho e radiação ultravioleta. A RUV corresponde a 5% da radiação solar que atinge a terra e é subdividida 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 (BALOGH; PEDRIALI; KANEKO, 2011; GONZÁLEZ; FERNÁNDEZ-LORENTE; GILABERTE-CALZADA, 2008; PALM; O'DONOGHUE, 2007; SVOBODOVA; WALTEROVA; VOSTALOVA, 2006; TEWARI et al., 2013a).

A RUV é predominantemente composta de UVA (90% -95%) e UVB (5% - 10%) em menor proporção. A RUV penetra na pele de uma maneira dependente do comprimento de onda, quanto maior o comprimento de onda mais profundamente ela irá penetrar na pele. A UVA alcança as camadas mais profundas e a UVB é absorvida predominantemente nas camadas mais superficiais da pele, principalmente pelos queratinócitos, sendo a UVB a mais lesiva ao DNA (FERNÁNDEZ-GARCÍA, 2014; SAMPLE; HE, 2017; TEWARI et al., 2013a).

A RUV ao atingir a pele provoca um processo complexo associado a reações químicas e morfológicas cumulativas, como a formação de espécies reativas de oxigênio (EROs), alterações histoquímicas, espessamento da camada espinhosa e retificação da junção dermoepidérmica (BALOGH; PEDRIALI; KANEKO, 2011).

O espectro da radiação UVB é considerado o mais prejudicial e lesivo da RUV para pele humana (AFAQ; ADHAMI; MUKHTAR, 2005; FIGUEIREDO et al., 2014; HUPEL; POUPART; AR GALL, 2011). Diferentemente da radiação UVA que não é absorvida pelo DNA, a radiação UVB tem como principal consequência os danos diretos no DNA, além dos danos indiretos ocasionados pela produção de EROs, e desta forma é mais relevante para a fotocarcinogênese (EMRI; HORKAY; REMENYIK, 2006). Os danos diretos são causados, pois ao absorver a radiação UVB o DNA, principalmente dos queratinócitos, pode sofrer mutações que, posteriormente, podem acarretar em transformações malignas nas células que culminam no tumor de pele. Além disso, a exposição à RUV ativa componentes do sistema imunológico cutâneo, gerando resposta inflamatória por diferentes mecanismos, tais como: ativação direta de queratinócitos na epiderme e outras células que liberam mediadores inflamatórios (BALOGH; PEDRIALI; KANEKO, 2011; BOWDEN, 2004; HILDESHEIM; AWWAD; FORNACE, 2004; MAVERAKIS et al.,

2010; OLIVEROS et al., 2009).

1.3. RADICAIS LIVRES E ESTRESSE OXIDATIVO

Os radicais livres são definidos como toda espécie que possui um ou mais elétrons desemparelhados (SUN, 1990). Na natureza o principal componente gerador de radicais livres é o oxigênio no estado fundamental (O_2). As espécies radicalares geradas a partir do oxigênio são denominadas espécies reativas do oxigênio (EROs) que inclui também espécies que não possuem elétrons desemparelhados, mas que são muito reativos em decorrência de sua instabilidade, como o peróxido de hidrogênio (MACHADO ROCHA RIBEIRO et al., 2005).

A geração de EROs é uma condição fisiológica associada à vida aeróbica. Em condições de homeostase, 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 (MACHADO ROCHA RIBEIRO et al., 2005). A exposição da pele à radiação UVB leva a uma produção excessiva de EROs, como o ânion superóxido, o radical hidroxil e o radical peroxil (IVAN et al., 2014). A pele responde espontaneamente ao aumento dos níveis de EROs, no entanto esta resposta pode não ser suficiente para evitar danos às células (AFAQ; ADHAMI; MUKHTAR, 2005).

No organismo, os principais meios de defesa antioxidantes podem ser divididos em dois grupos: enzimáticos e não enzimáticos. No sistema enzimático incluem-se as enzimas glutathiona peroxidase (GPx), superóxido dismutase (SOD) e a catalase (CAT). Já no sistema não enzimático podemos citar a glutathiona reduzida (GSH), o alfa-tocoferol (vitamina E), o ácido ascórbico, o β -caroteno e o ácido úrico (POLETTA; SIMONIELLO; MUDRY, 2016).

A diminuição dos antioxidantes endógenos ou o aumento da geração de espécies oxidantes leva ao estresse oxidativo. Esse desequilíbrio entre a geração e a remoção dos radicais livres no organismo leva a um estado pró-oxidante que pode acarretar em lesões celulares, inclusive em morte celular (JÚNIOR et al., 2001).

Quantidades excessivas de EROs podem ter efeitos deletérios em muitas moléculas, incluindo proteínas, lipídios, RNA e DNA, uma vez que são altamente reativas (LÜ et al., 2010), além disso o estresse oxidativo é responsável por uma

variedade de doenças relacionadas a inflamação e ao estímulo oxidativo, como envelhecimento precoce e câncer de pele (IVAN et al., 2014).

As enzimas antioxidantes atuam cooperativamente e uma mudança em qualquer uma delas pode afetar o estado de equilíbrio e conduzir ao estresse oxidativo (KATIYAR; MEERAN, 2007). A exposição à radiação UVB induz estresse oxidativo pelo excesso de formação de radicais livres que, conseqüentemente, levam a depleção de antioxidantes, como glutathiona reduzida (GSH) e SOD (HALLIWELL, 2009; ZAID et al., 2007).

Uma das moléculas antioxidantes endógenas mais importantes é a GSH que é um oligopéptido composto por três aminoácidos: a cisteína, a glicina e a glutamina. A GSH age doando elétrons aos radicais livres, neutralizando-os. Ao doar elétrons a glutathiona deixa a forma reduzida e passa a se apresentar na forma oxidada (GSSG), sem ação antioxidante. Na presença de NADPH (doador de elétrons) a enzima glutathiona redutase (GR) é capaz de restaurar o GSSG ao seu estado basal, tendo como objetivo impedir a paralisação do ciclo metabólico da glutathiona. O excesso de glutathiona oxidada em relação a reduzida pode indicar a presença de estresse oxidativo (D'ORAZIO et al., 2013; SCHALLREUTER et al., 1999).

A SOD é uma das mais importantes enzimas antioxidantes responsável pela dismutação do radical superóxido a peróxido de hidrogênio e oxigênio molecular. O peróxido de hidrogênio pode ser degradado por outras enzimas, como a CAT e a GPx. A CAT converte o peróxido de hidrogênio em água e oxigênio molecular (D'ORAZIO et al., 2013; SCHALLREUTER et al., 1999). A GPx é uma seleno-enzima que também pode reduzir o peróxido de hidrogênio ou hidroperóxidos orgânicos (incluindo hidroperóxidos derivados de lipídeos) a água e álcoois na presença de GSH (GIROTTI, 1990). Conforme ilustrado na figura 1.

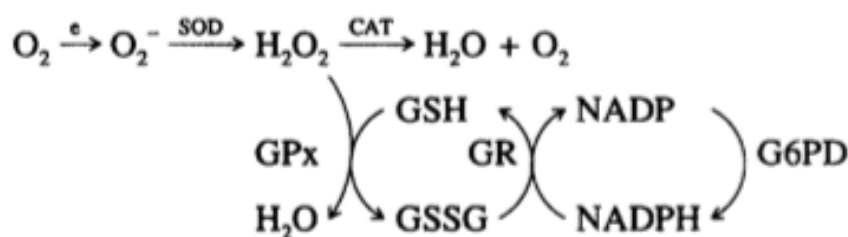


Figura 1: Imagem ilustrativa da ação das enzimas antioxidantes no organismo (SUN, 1990)

Entre as principais fontes geradoras de EROs podemos citar as mitocôndrias. Além disso, a atividade de algumas enzimas como a xantina oxidase, o citocromo P450- oxidase, monoaminooxidases, 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).

Em alguns casos, as ações destrutivas das EROs são benéficas para o organismo. No processo inflamatório, a grande quantidade de EROs liberada tem como objetivo eliminar microrganismos invasores ou degradar estruturas do tecido danificadas. No entanto, a falta de especificidade das EROs podem induzir estresse oxidativo em células normais adjacentes, levando ao desencadeamento de processos patológicos, visto que todos os componentes celulares são suscetíveis ao ataque de EROs (BICKERS; ATHAR, 2006; POLETTA; SIMONIELLO; MUDRY, 2016).

As EROs produzidas na pele após a exposição à radiação UVB, induzem ativação de fatores de transcrição como o NF- κ B, um fator transcricional sensível ao oxidante, que desempenha um papel crucial na ativação de múltiplos genes alvos envolvidos na expressão de várias moléculas pró-inflamatórias, incluindo citocinas inflamatórias (IVAN et al., 2014)

Durante o estresse oxidativo, o sistema de defesa antioxidante é regulado por diferentes moléculas sinalizadoras, dentre as quais se destacam o fator de transcrição Nrf2 (fator nuclear [derivado eritróide-2] tipo 2) e a via do elemento de resposta antioxidante (ARE), que modulam a expressão de enzimas antioxidantes e desintoxicantes, conhecidas como enzimas de fase II. Nesse grupo de enzimas, incluem-se a heme oxigenase-1 (HO-1), NADPH: quinona oxidoreductase 1 (Nqo-1) e glutathione-S-transferase (GST), que protegem nosso corpo de oxidantes prejudiciais. HO-1 é uma importante molécula endógena anti-inflamatória e antioxidante, que catalisa o heme em biliverdina, ferro livre e monóxido de carbono (LEE; CHAU, 2002; OTTERBEIN et al., 2003; PONKA, 1999). O fator de transcrição Nrf2 é regulado

negativamente por uma proteína Kelch 1 associada a ECH (Keap-1). Em condições normais, o Nrf2 está no citoplasma formando um complexo inativo com o Keap-1, o que evita a sua migração para o núcleo. No estresse oxidativo, Nrf2 é dissociado do Keap1, migrando para o núcleo e se ligando com ARE, que está localizado na região promotora das enzimas desintoxicantes de fase II (CHOI et al., 2013). Além disso, enzimas antioxidantes como a catalase, a glutatona peroxidase e superóxido dismutase também são reguladas pelo Nrf2 (KOBAYASHI; YAMAMOTO, 2005).

Nrf2 é um fator chave para a citoproteção como anticancerígeno, efeito neuroprotetivo e estímulo a resposta anti-inflamatória (KOBAYASHI; YAMAMOTO, 2005). A interrupção Nrf2 aumenta a regulação dos principais fatores de transcrição inflamatória, como o fator nuclear kappa B (NF-κB), metaloproteinase de matriz-9 (MMP-9) e aumento da expressão de macrófagos inflamatórios. Nrf2 interfere com vias de sinalização inflamatória inibindo a ativação de NF-κB através da manutenção do estado redox celular (WANG et al., 2010).

A ativação da via de sinalização de NF-κB mostrou ser sensível ao excesso de EROs e é importante na geração do processo inflamatório (WANG et al., 2010). A ativação e translocação do NF-κB para o núcleo têm um importante papel regulador na inflamação, proliferação celular e carcinogênese (KATIYAR; MEERAN, 2007). A ativação de NF-κB pelos estímulos extracelulares depende da fosforilação e posterior degradação da proteína IκB. A interação do NF-κB com a proteína IκB as mantém no citoplasma, evitando a migração de NF-κB para o núcleo. Ao ser fosforilada, NF-κB é liberado da sua associação inibitória e translocado até o núcleo onde ativa a transcrição de genes importantes para as respostas inflamatórias e imunes (BENDER et al., 1998). O NF-κB é comumente ativado por oxidantes, incluindo H₂O₂ e a RUV (KATIYAR; MEERAN, 2007).

Os danos cutâneos causados pela exposição à RUV estão relacionados a absorção dessa radiação por diferentes moléculas presentes na pele (BALOGH; PEDRIALI; KANEKO, 2011; MAVERAKIS et al., 2010). As moléculas presentes na pele capazes de absorver a RUV são denominadas de cromóforos. Essas moléculas apresentam em sua estrutura dupla ou tripla ligações de carbono com carbono (TEWARI et al., 2013a). Podemos citar como exemplos: o ácido urocânico, aminoácidos aromáticos, RNA, melanina e seus precursores e metabolitos, além do

DNA, cromóforo de maior importância na exposição à RUV (BALOGH; PEDRIALI; KANEKO, 2011; YOUNG, 1997). Ao absorver a RUV, o cromóforo passa para um estado transitório e excitado. Ao retornar ao seu estado fundamental, ele libera energia na forma de luz ou calor. Durante esse processo, o cromóforo pode sofrer mudanças químicas ou transferir energia para outras moléculas. Somente quando a luz é absorvida pode ocorrer reação fotoquímica, causando alterações celulares, que eventualmente acarretam em uma resposta clínica (BARON; SUGGS, 2014).

As bases de pirimidina do DNA, como a timina, citosina e 5-metilcitosina, são os principais cromóforos da radiação UVB. Ao serem estimuladas pela radiação UVB essas bases nitrogenadas estimulam a produção de fotoprodutos do DNA que estão relacionados ao câncer de pele. Na pele, o DNA dos queratinócitos, células de Langerhans e fibroblastos ao absorverem a radiação UVB formam dímeros de ciclobutano de pirimidina (CPDs) e de fotoprodutos de (6-4) pirimidina pirimidona (6-4PP) (BARON; SUGGS, 2014; TEWARI et al., 2013b) que correspondem a 65% e 35% das lesões no DNA induzida por UVB (LIPPKE et al., 1981; MITCHELL; JEN; CLEAVER, 1992).

O CPD foi identificado como possível cromóforo para a imunossupressão induzida pela RUV (TEWARI et al., 2013b), pois o CPD desencadeia a liberação de citocinas imunossupressoras, como a interleucina 10 (IL-10). Corroborando com isso, em seu estudo VINK; YAROSH (1996) concluiu que a imunossupressão ocasionada pela exposição à RUV está relacionada a formação de fotoprodutos não reparados do DNA de queratinócitos, que desencadeiam a produção de citocinas imunomoduladoras. Ao atingir a circulação, essas citocinas induzem respostas imunes mediadas por células e podem contribuir para a expansão de células supressoras (VINK; YAROSH, 1996).

Os CPDs são formados quando as ligações duplas presentes nas bases de pirimidina do DNA helicoidal absorvem os fótons da RUV, formando o anel de ciclobutano entre as bases adjacentes. Os CPDs destroem a capacidade de codificação do DNA, o que impede que as bases se juntem com os seus opostos. Remover ou reparar os CPDs é essencial para a não depleção do DNA e para isso as células dispõem de um sistema enzimático composto por mais de 20 enzimas

reparadoras, porém o sistema não é capaz de recuperar a produção exacerbada de fotoprodutos após a exposição à RUV (YAROSH, 2004).

Além disso, a absorção da RUV por cromóforos endógenos não-DNA (fotosensibilizadores) podem levar a danos indiretos no DNA pela formação de EROs. O dano pode ocorrer através da quebra de cadeias de DNA pela geração de ânions superóxido, peróxido de hidrogênio e radicais hidroxila, sendo este último muito reativo com todas as bases de DNA (TEWARI et al., 2013a).

A radiação UVB é capaz de desencadear respostas oxidativas que podem persistir após a interrupção da irradiação. Assim, EROs podem ser geradas após a fotoativação de cromóforos que induzem respostas celulares, inclusive em células não irradiadas, e processo inflamatório na pele (CADET; DOUKI; RAVANAT, 2015).

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. O termo peroxidação lipídica se refere à degradação oxidativa de fosfolipídeos, colesterol e outros ácidos graxos insaturados na presença de oxigênio. Níveis elevados de peroxidação estão associados a efeitos prejudiciais nos sistemas biológicos, como perda de fluidez, inativação de enzimas de membrana e receptores, além do aumento da permeabilidade de íons, que pode levar à ruptura da membrana celular (Figura 2). Ademais os produtos de peroxidação também podem danificar o DNA (GIROTTI, 1990; KATIYAR; MEERAN, 2007). As EROs podem oxidar os lipídeos diretamente, oxidando as ligações duplas do lipídeo ou indiretamente, através de uma reação em cadeia de lipídios oxidados. As membranas lipídicas danificadas são então processadas pelo organismo por reações enzimáticas e não-enzimáticas que podem resultar na expressão de genes de resposta ao estresse ou na produção de prostaglandinas que medeiam reações inflamatórias (BARON; SUGGS, 2014).

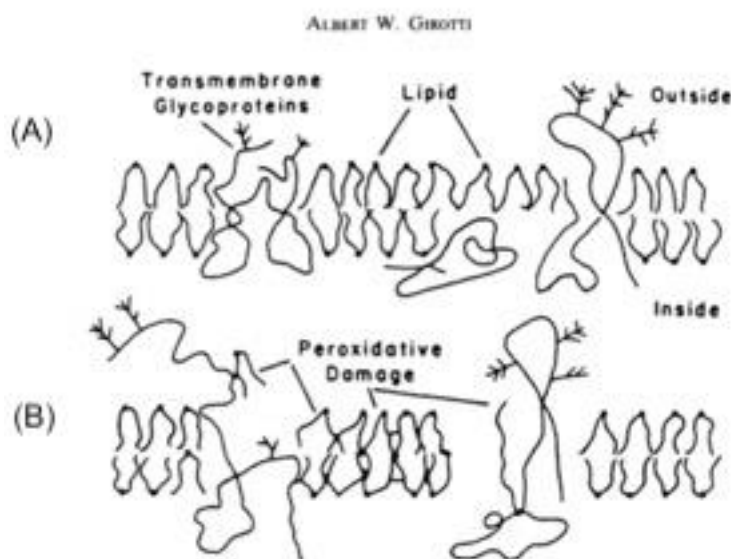


Figura 2: Representação esquemática do dano estrutural causado pela peroxidação lipídica nas membranas biológicas. (A) Arranjo natural dos lipídeos e proteínas na membrana que não sofreu peroxidação. (B) Alteração no arranjo na membrana que sofreu peroxidação: alterações conformacionais em lipídios e proteínas causadas por modificações monofuncionais e ligação cruzada (lipídio-lípido, proteína-lípido ou proteína-proteína) (GIROTTI, 1990).

1.4. RESPOSTA INFLAMATÓRIA INDUZIDA PELA RADIAÇÃO UVB

O processo inflamatório desencadeado pela exposição à radiação UVB ocorre através da estimulação dos queratinócitos que participam ativamente da imunidade inata. Os queratinócitos estimulados produzem e liberam citocinas que conduzem ao processo inflamatório como a IL-1, fator de necrose tumoral alfa (TNF- α), IL-6 e membros da família das quimiocinas (BANGERT; BRUNNER; STINGL, 2011). Além dos queratinócitos outros tipos de células também produzem citocinas quando estimulados pela RUV, como por exemplo as células de langerhans e os mastócitos (DURÁN-ANIOTZ et al., 2013).

Além disso, a exposição à RUV induz secreção de citocinas anti-inflamatórias. Essas citocinas tem como função regular negativamente a resposta imune, como por exemplo 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 sistemicamente por UVB. A diminuição na imunidade celular observada após a exposição da pele à radiação UVB pode levar a alterações na capacidade do organismo de responder a uma variedade de micro-organismos virais e intracelulares (CLYDESDALE; DANDIE; MULLER, 2001). Além da IL-10, podemos citar também a produção da citocina anti-inflamatória TGF- β que apresenta propriedade regulatória e está envolvida em vários tipos de cânceres, inclusive no melanoma (DURÁN-ANIOTZ et al., 2013).

Os queratinócitos protegem a epiderme contra a propagação de células potencialmente cancerosas que podem surgir após a exposição crônica à RUV. As respostas protetivas dos queratinócitos ao estresse genotóxico incluem: ativação da morte celular programada, ativação da resposta inflamatória para eliminação de células danificadas e substituição das células malignas por hiperproliferação. Além disso, o estímulo da RUV leva a ativação da via de sinalização MAPK (proteínas quinases ativadas por mitógenos) nos queratinócitos, processo central no seu sistema de vigilância (HILDESHEIM; AWWAD; FORNACE, 2004). 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. Esses queratinócitos apoptóticos podem ser identificados microscopicamente por apresentarem núcleos picnóticos (BAYERL et al., 1995). Outra consequência, é o aumento da espessura da epiderme chamada de hiperqueratose que ocorre devido ao acúmulo de queratinócitos. Horas após o estímulo da RUV 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 RUV na pele (COELHO et al., 2009; D'ORAZIO et al., 2013; SCOTT et al., 2012).

Os membros da família MAPK são mediadores da transdução de sinal da superfície celular para o núcleo e desempenham um papel importante no desencadeamento e coordenação de respostas genéticas. Fazem parte dessa família as proteínas ERK (quinase regulada por sinal extracelular), JNK (quinase c-Jun n-terminal) e p38 (VAYALIL; ELEMENTS; KATIYAR, 2003). A fosforilação dessas proteínas controla as atividades de vários fatores de transcrição implicados na

proliferação, diferenciação e promoção de tumores. A ativação do JNK regula a transcrição do ativador de proteína-1 (AP-1) em resposta ao estresse ambiental, como a RUV. O aumento da atividade de AP-1 tem sido implicado na inflamação, metástase, angiogênese e também na promoção e progressão de vários tipos de cânceres (KATIYAR; MEERAN, 2007). Além disso, a AP-1 também participa da degradação da matriz extracelular dérmica na pele através da expressão de uma série de metaloproteinases responsáveis pela degradação do colágeno.

A radiação UVB induz a produção de H_2O_2 nas células alvo, que por sua vez inicia a fosforilação das proteínas MAPK e a ativação dos sinais e expressão de genes que tem ligação direta com o câncer de pele (MANTENA; KATIYAR, 2006).

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 ($O_2^{\cdot-}$) 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; 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 $O_2^{\cdot-}$ induzida pela radiação UVB. O $O_2^{\cdot-}$ é uma espécie reativa intermediária utilizada como substrato para geração de espécies mais reativas como o radical hidroxila ($\cdot OH$), H_2O_2 , oxigênio singlete (1O_2) e peróxido nitrito ($ONOO^-$) (ANRATHER; RACCHUMI; IADECOLA, 2006; GARCIA et al., 1999; ROBINSON; OHIRA; BADWEY, 2004). A produção de EROs pela $gp91^{phox}$ é crucial para o estabelecimento do estresse oxidativo (ANRATHER et al., 2006).

O propósito do *burst* respiratório de fagócitos é gerar compostos microbicidas. Esses compostos fazem parte do sistema de defesa antimicrobiano na resposta imune inata. Em locais de inflamação, o aumento da produção de radicais livres está associado à ativação da NADPH oxidase de neutrófilos. Além disso, a exposição à RUV leva a ativação do fator de transcrição nuclear (NF- κ B). NF- κ B

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 $O_2^{\cdot-}$ 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; WINROW et al., 1993).

A partir da ativação celular dos neutrófilos, ocorre também a liberação de enzimas microbicidas presentes em seus grânulos azurófilos, como a mieloperoxidase (MPO) (WITKO-SARSAT et al., 2000). A MPO utiliza o H_2O_2 formado durante o *burst* respiratório para produzir o ácido hipocloroso, um potente agente citotóxico (ANRATHER; RACCHUMI; IADECOLA, 2006). Além disso, a MPO interage com outros substratos gerando uma grande variedade de subprodutos. Os oxidantes derivados da MPO também estão implicados em processos não relacionados à defesa do hospedeiro, como a carcinogênese (LONDON; LEHMAN; TAYLOR, 1997; WITKO-SARSAT et al., 2000). Portanto, a exposição substancial à RUV pode levar a efeitos prejudiciais, como o dano tecidual devido em parte aos mecanismos de vigilância do organismo tentando eliminar as células danificadas através da apoptose e do processo inflamatório (CLYDESDALE; DANDIE; MULLER, 2001).

Os neutrófilos podem produzir também metaloproteinases (MMPs) (JOHN; TUSZYNSKI, 2001). As metaloproteinases de matriz (MMPs) constituem uma família composta por mais de 25 enzimas proteolíticas que atuam sobre os componentes da matriz extracelular (MEC) e são secretadas em uma variedade de processos fisiológicos e patológicos envolvendo a pele (HENRIET; BLAVIER; DECLERCK, 1999; NAKOPOULOU et al., 2002; RIBEIRO et al., 2008; STERNLICHT; WERB, 2001; TOI; ISHIGAKI; TOMINAGA, 1998). 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 entre MMPs 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 (TALWAR et al., 1995). O remodelamento da MEC é feito pelas MMPs e seus inibidores (TIMP). A MMP-1, MMP-3 e MMP-9 são altamente induzidas por RUV em queratinócitos residentes e fibroblastos. Uma das causas do fotoenvelhecimento seria a hidrólise da 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 et al., 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 e degradação de fibras de colágeno, perdendo sua força e elasticidade, o que acarreta em mudanças na aparência da pele como o aparecimento de rugas e pigmentação irregular (BARON; 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, fatores ativados pela RUV como os fatores como AP-1 e NF- κ B 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 do endotélio e epitélio, infiltração de leucócitos polimorfonucleares, macrófagos inflamatórios e linfócitos no local da lesão que conseqüentemente levam ao edema de tecido (COTRAN; COLLINS, 2009). A exposição à RUV também leva a um aumento 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 o imunossupressão sistêmica induzida pela radiação UVB (HART et al., 1998).

1.5. MEDIADOR LIPÍDICO PRÓ-RESOLUÇÃO: RESOLVINA D1

O processo inflamatório é a primeira resposta do sistema imunológico à infecção ou lesão tecidual, tem função protetora para o hospedeiro e deve ser "auto-limitada", porém respostas inflamatórias excessivas ou inadequadas contribuem para uma série de doenças humanas agudas e crônicas (SEKI et al., 2010; SERHAN, 2010). A resposta imunológica inicia-se pelo processo inflamatório agudo que pode progredir para o crônico. Para que não haja transição da inflamação aguda para a crônica, são produzidos mediadores locais para resolução da inflamação, denominados de mediadores lipídicos pró-resolução. Esses mediadores atuam em conjunto para o reestabelecimento da homeostase do tecido após lesões, controlando a magnitude e a extensão dos eventos inflamatórios. Pertencem a esse grupo as resolvinas, as maresinas, as lipoxinas e as protectinas (CHIURCHIU et al., 2016).

Durante anos, a pesquisa se concentrou em identificar fatores que iniciam e perpetuam a inflamação, porém com a identificação de compostos que ativam mecanismos pró-resolução durante a inflamação houve uma mudança na ênfase da pesquisa para outra extremidade do processo inflamatório, ou seja, sua resolução. O objetivo agora é entender como as respostas inflamatórias mediadas pelo sistema imune são encerradas, uma vez que a inflamação descontrolada acarreta o desenvolvimento de muitas doenças (BUCKLEY; GILROY; SERHAN, 2014).

Com o isolamento e a caracterização desses mediadores endógenos, evidenciou-se que a resolução da inflamação é um processo ativo e que envolve a biossíntese de mediadores como as resolvinas (SERHAN et al., 2002; SERHAN; CHIANG, 2009), foco do nosso trabalho.

Os mediadores lipídicos são produzidos principalmente por neutrófilos e macrófagos a partir de ácidos graxos poli-insaturados. Esses mediadores inflamatórios receberam destaque nos últimos anos devido a sua capacidade de reduzir o processo inflamatório em diversos modelos de doenças. Desde a sua descoberta, os mediadores lipídicos endógenos pró-resolução provaram atuar como iniciadores do processo de resolução da inflamação aguda, seja reduzindo o tráfico de granulócitos, a produção de citocinas e de EROs extracelular, bem como diminuindo a magnitude da resposta inflamatória de maneira geral e também aumentando a depuração de detritos celulares e micro-organismos invasores mediado por macrófagos (SERHAN, 2014).

O fracasso na resolução da inflamação aguda leva a inflamação crônica, ao excesso de dano tecidual, a desregulação da cicatrização de tecidos e tem sido implicada em múltiplos tipos de doença, inclusive na patogênese do câncer (SERHAN, 2014; SOMMER; BIRKLEIN, 2011) quando associada ao estresse oxidativo (NISHISGORI, 2015; WANG et al., 1991).

É importante ressaltar que existe um ciclo de estimulação recíproca entre citocinas e EROs. Citocinas induzem a expressão e ativação de enzimas como a NADPH oxidase, resultando em uma maior produção de $O_2^{\cdot-}$. Por outro lado, a produção de $O_2^{\cdot-}$ é essencial para manter o direcionamento de neutrófilos e induzir a produção de citocinas (BOWIE; O'NEILL, 2000; VILELA et al., 2016). Portanto, a inflamação e o estresse oxidativo induzidos pela exposição à RUV estão estreitamente relacionados.

Considerando o efeito sinérgico da produção de EROs e de mediadores inflamatórios, a melhora do sistema antioxidante endógeno e a resolução da inflamação tornam-se abordagens promissoras para prevenir e/ou tratar os danos cutâneos induzidos pela radiação UVB (FONSECA et al., 2010). Neste contexto, a avaliação de mediadores lipídicos anti-inflamatórios/pró-resolução para enriquecer o sistema de proteção endógeno e assim controlar os processos lesivos induzidos

pela radiação UVB tornam-se uma alternativa interessante, visto que o uso de protetores solares não é totalmente seguro. Estudos demonstraram que os protetores solares podem penetrar na pele e induzir a produção de EROs, além disso o seu uso pode estimular a exposição prolongada ao sol, pois retardam a ocorrência de queimaduras solares (HANSON; GRATTON; BARDEEN, 2006).

Entre os mediadores destacamos a resolvina D1 (RvD1) que é um metabólito bioativo gerado em resposta à inflamação por conversão enzimática do ácido docosahexaenoico (DHA). A denominação resolvina foi utilizada, pois esses compostos foram originalmente identificados na fase de resolução da inflamação aguda e o termo é uma abreviação do inglês “*resolution phase interaction products*” (produtos de interação da fase de resolução) (SEKI et al., 2010).

O DHA pertence à família dos ácidos graxos poli-insaturados ômega-3, são biossintetizados em pequena quantidade nos seres humanos e, portanto devem ser obtidos a partir da dieta, (CALDER, 2013) com alimentos e/ou suplementos (MOLFINO et al., 2014). A RvD1 é o principal mediador lipídico derivado do DHA (CHEN et al., 2016).

As resolvinas desempenham papéis importantes na fase de resolução da inflamação através do estímulo à fagocitose, a remoção de detritos e invasores microbianos e também a esferocitose. Na esferocitose, os fagócitos removem do local da inflamação células mortas e neutrófilos apoptóticos. Quando a esferocitose não funciona bem, os neutrófilos não são removidos e continuam liberando citocinas inflamatórias prolongando assim a inflamação, logo sugere-se que as resolvinas tenham potencial clínico para retardar a progressão das doenças crônicas (CHAN; MOORE, 2010; MORO et al., 2016; RECCHIUTI; SERHAN, 2012).

As resolvinas exercem suas ações biológicas através da ativação de receptores específicos acoplados a proteína G de forma estereoseletiva. A RvD1 atua sobre os receptores ALX/FPR2 e GPR32 e a sinalização através desses receptores medeia seus efeitos, melhorando a fagocitose dos macrófagos e neutrófilos apoptóticos (SANSBURY; SPITE, 2016).

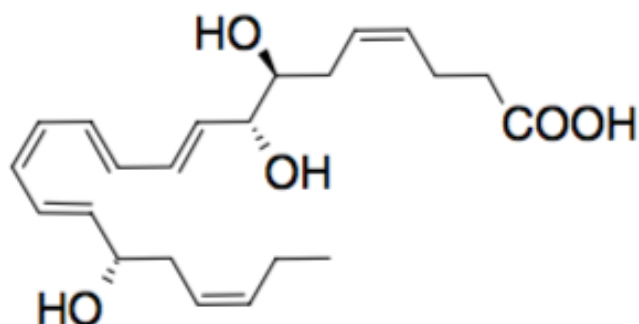


Figura 3: Estrutura química da RvD1 (Adaptado (SERHAN; CHIANG, 2009))

Estudos demonstraram que a RvD1 é capaz de inibir a resposta inflamatória e promover a resolução da inflamação através da diminuição da expressão das citocinas pró-inflamatórias TNF α e IL-1 β e aumento da expressão da citocina anti-inflamatória IL-10 em modelos de camundongos com lesão pulmonar aguda induzida por lipopolissacarídeos (LPS). Além disso, foi constatada a capacidade da RvD1 em reduzir o estresse oxidativo na lesão pulmonar através do aumento da transcrição do gene que codifica a enzima OH-1. Por último, foi também observado um aumento do nível de SOD e diminuição da formação de malondialdeído (MDA) (WANG et al., 2014). Outros estudos, evidenciaram a melhora das respostas inflamatórias em modelos de peritonite, de lesão isquêmica renal e resposta alérgica das vias aéreas com o uso da RvD1 (HAWORTH; LEVY, 2007; ROGERIO et al., 2012), além de suprimir a produção de citocinas pró-inflamatórias induzidas pelo extrato de fumo de cigarro em modelos de lesão pulmonar em camundongos (HSIAO et al., 2013).

Esses resultados reforçam o potencial benéfico desse mediador no controle da inflamação cutânea e melhora do sistema antioxidante. É importante destacar que apesar do estabelecimento do papel endógeno e efeito anti-inflamatório e pró-resolução em vários modelos de doença, ainda não existia evidências do papel ou efeito terapêutico da RvD1 na inflamação e estresse oxidativo induzido pela radiação UVB.

2. OBJETIVOS

2.1. OBJETIVO GERAL

O objetivo geral deste trabalho foi avaliar o efeito da resolvina D1 na inflamação e estresse oxidativo induzidos pela radiação UVB.

2.2. OBJETIVOS ESPECÍFICOS

Avaliar os efeitos da RvD1 quando administrada via ip em camundongos sem pelo, no modelo de lesão cutânea induzida pela radiação UVB com enfoque na (o):

- Edema de pele;
- Migração do infiltrado leucocitário (ensaio da mieloperoxidase);
- Atividade/secreção de metaloproteinase-9;
- Níveis do antioxidante endógeno GSH e atividade da catalase
- Produção de hidroperóxidos e de ânion superóxido
- Produção de citocinas pró-inflamatórias (IL-1 β , IL-6, IL-33 e TNF- α) e anti-inflamatórias (TGF- β e IL-10);
- Expressão de RNAm para gp91^{phox}, Nrf2, Nqo-1 e OH-1 por reação em cadeia da polimerase (PCR) quantitativa;
- Avaliação histopatológica pelas colorações de hematoxilina eosina (H & E), azul toluidina e tricrômico de masson.

3. MATERIAIS E MÉTODOS

3.1. MATERIAIS

3.1.1. Reagentes

Resolvina D1 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 hidroperóxido, azul brilhante R, glutathione 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), enzima transcriptase reversa (Superscript[®] III), Oligo (dT)12-18 primers, Platinum SYBRGreen[®] e primers da Invitrogen (Carlsbad, CA, USA). Kits de Ensaio imunoenzimático (ELISA) para dosagem de citocinas da eBioscience (San Diego, CA, USA). Todos os outros reagentes utilizados foram de grau analítico.

3.1.2. Equipamentos

Agitador mecânico, Fisatom[®]; Balança analítica, HR-120, A&d[®], precisão de 4 casas; Banho-maria, 314/2 DN, Nova Ética[®]; Banho-maria 100, Fanem; Centrífuga refrigerada, Rotina 46R, Hettich Zentrifugen[®]; Câmara de madeira projetada para irradiação; Deionizador de água, Purebal Option-Q, Elga[®]; Estufa 0-120°C, De Leo & Cia[®]; Fonte elétrica para eletroforese MS 300V, Major Science[®]; Homogeneizador de tecidos Tissue-Tearor (Biospec 985370); Lâmpada ultravioleta fluorescente PHILIPS TL/12 40W RS-UVB, MedicalHoland[®]; Leitor de microplaca, Asys Expert Plus, Biochrom[®]; Leitor de microplaca, Enspire, Perkin Elmer[®]; Leitor de microplaca, Multiskan GO, Thermo Scientific; pHmetro, Tec-3MP, TECNAL[®]; Radiômetro IL 1700 Research Radiometer. Detectores: SED240 – filtro UVB (290nm), SED005 – filtro UV (350nm); Sistema de eletroforese Mini Vertical, Mini-Protean[®] Tetra System, Bio-RAD[®]; Spray drier, model SD-05, Lab Plant[®]; Termociclador, TX96, Amplitherm[®]; Ultra-som, TSO, Thornton[®].

3.2. MÉTODOS

3.2.1. Animais experimentais

Foram utilizados camundongos sem pelo da linhagem HRS/J, de ambos os sexos, adultos, pesando em média 25g. Os camundongos foram mantidos no Biotério do Centro de Ciências da Saúde/UEL onde foi utilizado ciclo claro/escuro (12/12 h) com temperatura controlada ($22 \pm 2^{\circ}\text{C}$) e livre acesso a água e a ração. O projeto foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Estadual de Londrina e os experimentos foram realizados segundo suas normas (registro no CEUA sob o nº 017/2015, processo nº 1447.2015.10).

3.2.2. 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 λ 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 $4,14 \text{ J/cm}^2$ (CAMPANINI et al., 2013). A lâmpada foi instalada em uma caixa retangular de madeira com capacidade para 6 caixas menores, desenvolvida especificamente para esse fim. Os camundongos foram distribuídos nessas caixas menores e sobre eles foi colocada uma tela que restringe a altura da caixa e garante a exposição total do dorso dos animais a luz UV. Durante toda a irradiação foi realizado o rodízio das caixas para assegurar igual dose de irradiação nos animais, pois ocorre variações de emissão de luz ao longo da lâmpada

3.2.3. Protocolo experimental de avaliação da eficácia da resolvina D1 em camundongos via intraperitoneal

3.2.3.1. Tratamento dos animais

Os animais foram alocados aleatoriamente em seis grupos contendo cinco animais 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 RvD1 3,0 ng/animal
- Grupo 4: Irradiado e tratado com a dose RvD1 10,0 ng/animal
- Grupo 5: Irradiado e tratado com a dose RvD1 30,0 ng/animal
- Grupo 6: Irradiado e tratado com a dose RvD1 100,0 ng/animal

Os animais foram tratados com 200 μ L de diferentes concentrações de RvD1 (3, 10, 30 e 100 ng/animal) via intraperitoneal, em dois diferentes tempos: 1h antes do início da irradiação e 7h após o início da irradiação (MARTINEZ et al., 2015). Os animais dos grupos controles receberam tratamento apenas com o veículo (salina) utilizado na diluição da droga. As doses de RvD1 usadas nos tratamentos foram selecionadas com base nos efeitos terapêuticos de estudos previamente publicados em outros modelos de doença (HSIAO et al., 2013; SPITE et al., 2009; WANG et al., 2014; XIE et al., 2016) e nos experimentos de dose resposta realizados nesse trabalho.

3.2.3.2. Coleta das amostras de pele

Os animais foram terminalmente anestesiados com 3% de isofurano e as peles coletadas 12h após o final da irradiação. Para avaliação de outros parâmetros, os animais foram anestesiados (3% de isofurano), decapitados e as peles coletadas 2h e 4h 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, MPO, atividade/secreção da MMP-9 e histologia as peles dos animais foram coletadas 12 horas após a o fim da irradiação. Já para as análises de atividade da catalase e produção de ânion superóxido (NBT) as peles foram coletadas 2h após a irradiação e para as análises de citocinas,

peroxidação lipídica e PCR, 4 horas após a irradiação (Figura 4) (CAMPANINI et al., 2013; MARTINEZ et al., 2015).

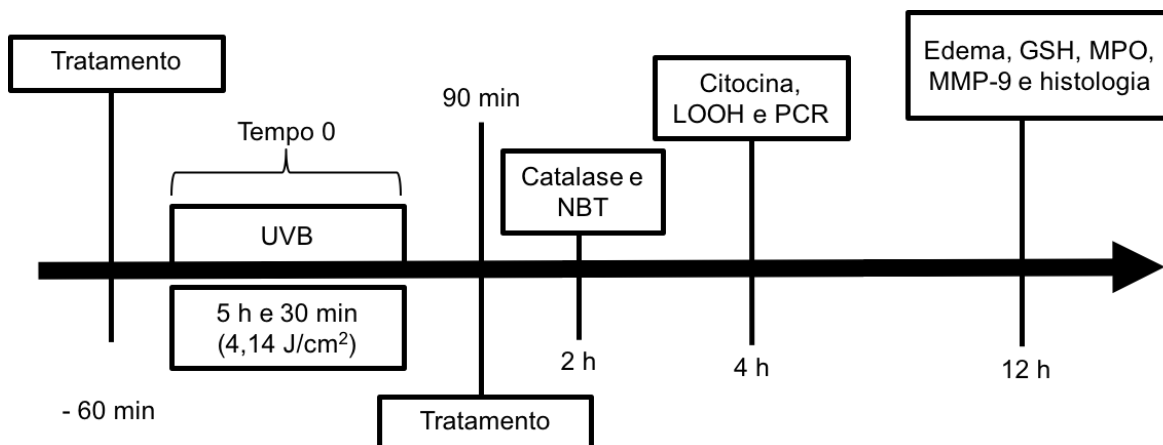


Figura 4. Fluxograma dos tempos de tratamentos e coleta das peles dos animais de acordo com os protocolos experimentais pré-estabelecidos para avaliação da eficácia da RvD1 administrada via intraperitoneal. Os animais foram submetidos à radiação UVB por um período de 5 horas e 30 min ($4,14 \text{ J/cm}^2$). Nos animais foi realizado um pré-tratamento com RvD1 1h antes e um 7h após início da irradiação (3, 10, 30 e 100 ng/animal). Os animais foram eutanasiados e as amostras de pele foram coletadas em diferentes tempos: 2 horas, 4 horas e 12 horas após o fim da irradiação UVB de acordo com o teste a ser realizado.

3.2.4. Avaliação do edema de pele

No processo inflamatório agudo, desencadeado pela exposição à RUV, ocorre a liberação de mediadores químicos que aumentam a permeabilidade do endotélio vascular, levando a formação de edema como resultado do extravasamento de fluidos e proteínas para pele (DUSTING; 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 (5 mm diâmetro). O edema foi expresso pela variação do peso de pele entre os diferentes grupos controles e tratados (IVAN et al., 2014).

3.2.5. Determinação da atividade da mieloperoxidase (MPO)

Foi realizada a quantificação da atividade da enzima mieloperoxidase (MPO) como marcador do infiltrado de leucócitos (monócitos/macrófagos e neutrófilos) na pele após a irradiação UVB (KATIYAR; MEERAN, 2007).

As amostras de pele foram coletadas em tampão fosfato 50mM (pH 6,0) contendo 0,5% de brometo de hexadeciltrimetil amônio (HTAB), homogeneizadas com auxílio do Tissue-Tearor (Biospec 985370) e centrifugadas (16,100 g por 2 min a 4°C). Para realização do ensaio, 30 µL do sobrenadante de cada amostra foram misturados com tampão fosfato 50mM (pH 6,0) contendo 0,167mg/mL de o-dianisidina e 0,015% de peróxido de hidrogênio. A atividade da MPO foi determinada espectrofotometricamente em 450nm (EnSpire, Perkin Elmer). A atividade da MPO das amostras foi obtida através da comparação com a atividade da MPO de uma curva padrão de neutrófilos. Os resultados obtidos foram expressos em números de neutrófilos/mg de pele (CASAGRANDE et al., 2006).

3.2.6. Determinação da atividade/secreção de metaloproteinase-9 (MMP-9)

Para avaliação da MMP-9, foi utilizado a técnica de zimografia em gel de poliacrilamida com dodecil sulfato de sódio (SDS), método utilizado para detecção de proteases. A análise detecta enzimas que degradam a gelatina presente no gel (FONSECA et al., 2011a; KIM et al., 2007).

Foi feito um pool das amostras de pele de cada grupo e armazenadas em eppendorfs. As amostras foram homogeneizadas com auxílio do homogeneizador de tecidos Tissue-Tearor (Biospec 985370), na proporção 1:4 em tampão fosfato Tris/HCl 50mM (pH 7,4) com cloreto de cálcio (CaCl₂) e 1% de inibidores de proteinases (fenantrolina, fluoreto de fenilmetilsulfonila e N-etilmaleimida).

Os homogenatos foram centrifugados duas vezes a 12.000 g por 10 min a 4°C e os sobrenadantes foram submetidos ao ensaio de zimografia. Alíquota de 50 µL do sobrenadante 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°C durante 8 minutos imediatamente antes de ser aplicada no gel de eletroforese.

A espessura do gel utilizado foi de 1 mm, composto por um gel de separação e um gel de concentração, preparado conforme Tabela 1.

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

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

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

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

Ao término da eletroforese o gel de poliacrilamida foi lavado por 1 hora com solução de Triton X-100 2% sob constante agitação, posteriormente o gel foi incubado por 16 horas a 37 °C em tampão Tris/HCl 50 mM (pH 7,4) contendo CaCl_2 10 mM. 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 miliQ. Para visualização das bandas o gel foi descorado com ácido acético 20% (FONSECA et al., 2011b). A atividade proteolítica foi analisada por meio da comparação das diferenças de densidades de cor entre as bandas de cada grupo pelo programa ImageJ[®] (NIH, Bethesda, MD, USA).

3.2.7. Avaliação dos níveis do antioxidante endógeno glutathiona 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 aumento da 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 que pode predispor a célula a uma defesa menor contra o estresse oxidativo (HALLIWELL, 2009; ZAID et al., 2007).

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 uma solução 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 5 minutos de incubação, em 405 nm. A curva padrão foi preparada com 0 a 150 µM de GSH. Os resultados foram expressos em µM de GSH/mg de pele (SRINIVASAN; SABITHA; SHYAMALADEVI, 2007).

3.2.8. Avaliação da atividade da catalase (CAT)

O método é baseado no decaimento da concentração de H_2O_2 que é diretamente proporcional a diminuição da absorbância a 240 nm. A diferença de absorbância por unidade de tempo é a medida da atividade da catalase (AEBI, 1984).

As amostras de pele dos animais foram homogeneizadas em 500 μ L de EDTA 0,02M utilizando o homogeneizador Tissue-Tearor (Biospec 985370). O homogenato foi centrifugado a 2.700 g por 10 minutos a 4°C. O sobrenadante final foi retirado para análise. A determinação da atividade da CAT na pele foi realizada em microplaca por meio da adição de 10 μ l amostra, 160 μ L tampão Tris-HCl 1M com EDTA 5 mM pH 8,0, 20 μ L água deionizada e 20 μ L H_2O_2 200 mM. Foi incluído um branco para cada amostra preparado com 10 μ L do sobrenadante da amostra, 180 μ L de tampão Tris-HCl 1M com EDTA 5 mM pH 8,0 e 20 μ L de água deionizada. A velocidade com que o H_2O_2 é reduzido pela ação da CAT foi avaliada através da diminuição no valor da absorbância pela diferença entre a leitura inicial e a leitura 30 segundos após a adição do H_2O_2 200 mM. A leitura foi realizada em espectrofotômetro de microplacas (EnSpire, Perkin Elmer) a 240 nm com temperatura mantida em 25°C. Os valores de catalase foram expressos como unidade de catalase/mg de pele/minuto (AEBI, 1984).

3.2.9. Avaliação da produção de hidroperóxido (LOOH)

A peroxidação lipídica é uma das expressões orgânicas mais importantes do estresse oxidativo (YAGI, 1987). A oxidação dos lipídeos foi mensurada a partir da formação de hidroperóxidos que são os produtos primários na peroxidação lipídica (LINGNERT; VALLENTIN; ERIKSSON, 1979).

A produção de hidroperóxido foi avaliada por método de quimiluminescência descrito anteriormente (MARTINEZ et al., 2015). O método é baseado na determinação de quimioluminiscência iniciada pelo tert-butil hidroperóxido (GONZALEZ FLECHA; LLESUY; BOVERIS, 1991).

As amostras de pele dos animais foram homogeneizadas em 800 μ L de tampão fosfato (pH 7,4) com auxílio do Tissue-Tearor (Biospec 985370). Em seguida, foram centrifugadas a 700 g por 2 minutos a 4 °C. Para o ensaio, 250 μ L do sobrenadante foram adicionados a 1730 μ L de meio de reação (KCl 120mM, tampão fosfato pH 7,4 30 mM) e 20 μ L de tert-butil hidroperóxido 3 mM. Este ensaio foi

realizado em contador β marca Beckman[®] LS 6000 (FULLERTON, CA, EUA) em uma faixa de contagem não coincidente com a resposta entre 300 e 620 nm. Todo o experimento foi realizado ao abrigo da luz para evitar a fosforescência dos frascos, a 30 °C, durante 120 minutos. Os resultados foram medidos em contagem por minuto (cpm) por mg de pele.

3.2.10. Avaliação da produção de ânion superóxido ($O_2^{\cdot-}$)

A produção de ânion superóxido é aumentada após a exposição a radiação UVB (YASUI et al., 2006). A dosagem de ânion superóxido foi realizada através do ensaio de redução do *nitroblue tetrazolium* (NBT) (CAMPANINI et al., 2013). As amostras de pele foram homogeneizadas com o Tissue-Tearor (Biospec 985370) em EDTA 0,02 M e centrifugadas (2000 g por 20 segundos a 4°C). Para a reação, o sobrenadante (50 μ L) foi incubado em microplacas por 1 hora. Em seguida, o sobrenadante foi removido e adicionado NBT (1mg/mL) as células fixadas. Após 15 minutos, o NBT foi cuidadosamente removido e ao precipitado foram adicionados 20 μ L de metanol 100% para fixar. O composto formado pela redução do NBT (formazan) foi solubilizado com 120 μ L de KOH 2M e 140 μ L 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).

3.2.11. Dosagens das citocinas pró-inflamatórias: IL-1 β , IL-6, IL-33 e TNF- α e citocinas anti-inflamatórias: TGF- β e IL-10

As amostras de pele foram homogeneizadas em salina (500 μ L) com o Tissue-Tearor (Biospec 985370) e centrifugadas a 2000 g por 15 min a 4 °C. Os sobrenadantes foram utilizados para quantificação das citocinas IL-1 β , IL-6, IL-33, TNF- α , TGF- β e IL-10 através da técnica de enzimaensaio (ELISA) do tipo sanduíche com diferentes kits comerciais disponíveis de acordo com as instruções de uso do fabricante. Os resultados foram obtidos comparando as densidades ópticas em 490 nm das amostras com as densidades das respectivas curvas

padrões das citocinas (VERRI et al., 2008).

3.2.12. Expressão de RNAm para gp91^{phox}, Nrf2, Nqo-1 e OH-1 por reação em cadeia da polimerase (PCR) quantitativa

As amostras de pele foram coletadas em reagente trizol e homogeneizadas para a extração do RNA total. O RNA total foi transcrito por ação da enzima transcriptase reversa, e o DNAc obtido foi usado para amplificação específica do RNAm de interesse. A expressão quantitativa de RNAm para gp91^{phox}, Nrf2, Nqo-1 e OH-1 foi analisada por meio de reações de PCR em tempo real, em que foram utilizados o reagente SYBRGreen Master Mix (o qual contém fluoróforo SYBRGreen; enzima DNA polimerase; DNTPs e fluoróforo ROX), a solução de DNAc, água MiliQ tratada com dietilpirocarbonato e uma solução contendo cada primer. O sistema utilizado realiza as reações de amplificação e detecção, e quantifica as amostras no termociclador ABI5700 (APPLIED BIOSYSTEMS, WARRINGTON, REINO UNIDO) por meio da análise do nível de fluorescência gerado pela incorporação de nucleases fluorogênicas (SYBRGreen) aos produtos de amplificação durante o curso da reação. Os resultados foram analisados com base no valor do limiar do ciclo, onde a amplificação das amostras atinge um limiar que permite a análise quantitativa da expressão do fator avaliado (VERRI et al., 2008).

3.2.13. 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., 2016).

3.2.14. Análise estatística dos resultados

Os resultados foram analisados estatisticamente por análise de variância com um fator seguido do teste de comparações múltiplas de Tukey e apresentados pela média \pm erro padrão da média (EPM) de mensurações feitas com 5 animais em cada grupo e representativos de 2 experimentos separados. As análises estatísticas foram realizadas usando o software GraphPad Prism 7 (GraphPad Software Inc., San Diego, EUA). Os resultados foram considerados significativamente diferentes para $p < 0,05$.

4. RESULTADOS E DISCUSSÃO – ARTIGO CIENTÍFICO

4.1. Resolvin D1 inhibits UV irradiation-induced inflammation and oxidative stress in the skin of hairless mice

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Priscila Saito^a, Cristina P. B. Melo^a, Renata M. Martinez^a, Victor Fatorri^a, Talita L. C. Cezar^a, Ingrid C. Pinto^a, Alan J. C. Bussmann^a, Sandra R. Georgetti^a, Marcela M. Baracat^a, Waldiceu A. Verri Jr.^b, Rúbia Casagrande^{*a}

^aDepartamento de Ciências Farmacêuticas, Universidade Estadual de Londrina-UEL, Avenida Robert Koch, 60, Hospital Universitário, 86039-440 Londrina, Paraná, Brazil

^bDepartamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina-UEL, Rodovia Celso Garcia Cid, Km 80, PR445, Cx. Postal 10.011, 86057-970, Londrina, Brazil

*Corresponding author. Address: Avenida Robert Koch, 60, Vila Operária, CEP 86039-440 Londrina, Paraná, Brazil. Tel.: +55 43 33712475. E-mail address: rubiacasa@yahoo.com.br (R. Casagrande).

Highlights

- RvD1 protected the skin from the deleterious effects of UVB irradiation.
- RvD1 inhibited UVB-induced inflammatory edema and neutrophil recruitment.
- RvD1 suppressed the secretion of MMP-9 induced by UVB-irradiation.
- RvD1 inhibited UVB-induced skin oxidative stress and gp91^{phox} mRNA expression.
- RvD1 reduced UVB-induced production of a wide range of cytokines.

Abbreviations

CAT	catalase
DHA	docosahexaenoic acid
DTNB	5,5' - dithiobis(2 - nitrobenzoic acid)
GSH	reduced glutathione
H ₂ O ₂	hydrogen peroxide
HTAB	hexadecyltrimethylammonium bromide
IL	interleukin
KOH	potassium hydroxide
LPO	lipid peroxidation
LOOH	lipid hydroperoxides
MAPKs	mitogen activated protein kinases
MMP	matrix metalloproteinase
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium

NOX 2	NADPH oxidase 2
Nqo-1	NADPH: quinone oxidoreductase 1
Nrf2	nuclear factor [2 erythroid-derived] type 2
$O_2^{\cdot-}$	superoxide anion
OH-1	heme-oxygenase 1
ROS	reactive oxygen species
RvD1	resolvin D1
SDS	sodium dodecyl sulfate
SEM	standard error mean
TGF - β	transforming growth factor <i>beta</i>
TNF - α	tumor necrosis factor - α
UV	ultraviolet

Abstract

UV irradiation-induced oxidative stress and inflammatory responses have been implicated in skin diseases and skin disorders, including premature aging of the skin and cancers. In this study, we evaluated the anti-inflammatory and pro-resolution effect of the lipid mediator resolvin D1 (RvD1) in inhibiting UVB irradiation-induced inflammation and stress oxidative in the skin of hairless mice. RvD1 is a bioactive metabolite generated in response to inflammation by enzymatic conversion of docosahexaenoic acid (DHA). *In vivo* results demonstrated that systemic intraperitoneally treatment with RvD1 reduced skin inflammation and protected the skin from oxidative stress induced by UVB irradiation. The improvement in the inflammatory process was evidenced by the decrease of skin edema, neutrophil recruitment, metalloproteinase-9 activity/secretion, degradation of collagen fibers, and the production of different cytokines induced by UVB irradiation. There was also a decrease in epidermal thickness, number of sunburn cells and mast cell. Treatment of mice with RvD1 also protected the skin from oxidative stress by maintaining the levels of reduced glutathione (GSH) and catalase activity in basal levels, and even decreased production of lipid hydroperoxides, superoxide anions and gp91^{phox} mRNA expression (subunit of NADPH oxidase). RvD1 was also able to increase mRNA expression for nicotinamide adenine dinucleotide (phosphate) quinone oxidoreductase 1 (Nqo-1), nuclear factor [2 erythroid-derived] type 2 (Nrf2) and heme-oxygenase 1 (OH-1). These results suggest the use of the lipid mediator RvD1 as a promising strategy to control UVB-induced cutaneous diseases.

Keywords: Resolvin, inflammation, oxidative stress, UVB irradiation

1. Introduction

The skin is the largest organ of the human body and the main protection barrier of the organism against chemical, physical and biological aggressors [1–3]. External aggressors include exposure to UV irradiation, the main cause of skin damage. Acute exposure to UV irradiation leads to a number of adverse effects such as edema, sunburn, erythema, inflammation and immunosuppression, and chronic exposure can lead to premature aging and skin cancer [2–5].

The skin damage induced by UV irradiation occurs due to overproduction of reactive oxygen species (ROS), such as the superoxide anion [6], consequently leading to depletion of endogenous antioxidant, such as reduced glutathione (GSH) [7,8]. The imbalance between generation and removal of free radicals in the body leads to a pro-oxidant state that can lead to cell damage, including cell death [9]. The UVB irradiation spectrum is considered the most damaging and harmful UV irradiation for the human skin [3,10,11], because its main consequence is direct DNA damage, mainly keratinocytes. In addition, exposure to UVB irradiation activates the skin immune system components, triggering inflammatory response through the release of inflammatory mediators [12–16]. Considering the synergistic effect of the production of ROS and inflammatory mediators, the improvement of the endogenous antioxidant system and the resolution of inflammation become promising approaches to prevent and treat UVB irradiation-induced skin damage [17].

Resolvin D1 (RvD1) is a bioactive metabolite generated in response to inflammation by enzymatic conversion of docosahexaenoic acid (DHA) [18]. The RvD1 belongs to the group of anti-inflammatory/pro-resolution lipid mediators that play important roles in the resolution phase of inflammation [19–21]. These inflammatory mediators have been prominent in recent years due to their ability of reducing the inflammatory process in various disease models. Since their discovery, endogenous pro-resolution lipid mediators have proven to act as initiators of the acute inflammation resolution process, either by reducing the traffic of granulocytes, the production of cytokines and ROS, or by decreasing the magnitude of the inflammatory response in general and also increasing macrophage-mediated clearance of cell debris and invading microorganisms [22].

Studies have shown that RvD1 is able to inhibit the inflammatory response and promote the resolution of inflammation by reducing the production of the pro-inflammatory cytokines TNF- α and IL-1 β in models of mice with acute lung injury induced by lipopolysaccharides (LPS). In addition, the ability of RvD1 to reduce oxidative stress in lung injury was demonstrated through increased transcription of the gene encoding the enzyme heme-oxygenase 1 (HO-1) [23]. Other studies have demonstrated the improvement of inflammatory responses in asthma and acute lung injury with the use of RvD1, besides suppressing the production of chemokines and oxidative stress induced by cigarette smoke extract [24–26].

These results reinforce the beneficial potential of this mediator in the treatment of cutaneous inflammations and improvement of the cutaneous antioxidant system. It is important to note that, despite the establishment of the endogenous role and anti-inflammatory and pro-resolution effect in several disease models, still there was no evidence of the role or therapeutic effect of RvD1 on UVB-induced skin inflammation and oxidative stress. Thus, in this study we have attempted to investigate the effects of RvD1 on UVB irradiation-induced skin inflammation and oxidative stress in hairless mice.

2. Materials and methods

2.1. Chemicals

Resolvin D1 from Cayman Chemical (Ann Arbor, Michigan, USA). Brilliant blue R, reduced glutathione (GSH), hexadecyltrimethylammonium bromide (HTAB), *o*-dianisidine dihydrochloride, 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). Tris was obtained from Amresco (Solon, OH, USA). ELISA kits for determination of cytokine were obtained from eBioscience (San Diego, CA, USA). Acrylamide, sodium dodecyl sulfate (SDS), platinum SYBRGreen and superscript III kits were obtained from Invitrogen. All other reagents used were from pharmaceutical grade.

2.2. Animals

Sex matched hairless mice (HRS/J) weighing 20 - 30 g were obtained from the University Hospital of Londrina State University under the following conditions: 12 h dark/12 h light cycle and $23 \pm 2^\circ\text{C}$ temperature. The mice were maintained with free access to water and food throughout the experiment. The animal protocol used in this study was approved by Animal Ethics Committee (CEUA process number 1447.2015.10) of the Londrina State University.

2.3. Experimental protocol

The animals were randomly assigned to six groups containing five animals each. The groups were: non-irradiated control treated with vehicle (saline), irradiated control treated with vehicle (saline), irradiated treated with RvD1 3,0 ng/mice, irradiated treated with RvD1 10 ng/mice, irradiated treated with RvD1 30 ng/mice, irradiated treated with RvD1 100 ng/mice.

The animals were treated with different concentrations of RvD1 (3, 10, 30 and 100 ng / animal) intraperitoneally, 1 h before and 7 h after the beginning of UV

irradiation [27]. Animals in the control groups received treatment with vehicle (saline) used in the dilution of the drug. The doses of RvD1 used in the treatments were selected based on the therapeutic effects of studies previously published in other disease models [23,28,26] and on dose-response curves tested in the present study.

Based on the results obtained in the assays (edema, GSH, MPO and MMP-9) the choice of the best dose of RvD1 treatment was made. Thereby, the other parameters were evaluated only with the best dose of RvD1 treatment.

2.4. Irradiation

The light source used in the experiments to induce oxidative stress and acute inflammatory process in hairless mice was a fluorescent UVB lamp model PHILIPS TL / 12 40W RS (MEDICAL-NETHERLANDS). The lamp emits irradiation in the range of 270 to 400nm with a peak emission at 313 nm. The dose of irradiation used to induce inflammation and oxidative stress was 4.14 J/cm² [29].

All animals were irradiated simultaneously. The animals were kept at a distance of 20 cm from the lamp as previously described (CAMPANINI et al., 2013). The hairless mice were terminally anesthetized with 3.0% isoflurane (Abbott [Abbott Park, IL, USA]) 12 h after the end of UVB irradiation and the full thickness of the dorsal skins were removed for edema, MPO activity, MMP-9 activity and GSH assays and histology. Moreover, the hairless mice were anesthetized with 3.0% isoflurane, following by decapitation at 2 h for catalase and NBT assays, and 4 h for evaluation of production hydroperoxides, cytokines measurement and PCR after the end of UVB exposure and the dorsal skins were removed. Each parameter was evaluated at a specific time, which was previously determined [4,29]. The dorsal skin samples were collected and stored at - 80°C until analysis. The samples collected by verification of cutaneous edema were weighed immediately after collecting and by histology were fixed in buffered formaldehyde.

2.5. Skin edema

In the acute inflammatory process, triggered by the UV-irradiation, the release of chemical mediators that increase the permeability of the vascular endothelium, leading to the formation of edema as a result of fluids and proteins extravasation to the skin [30].

In order to evaluate the skin edema associated with the inflammatory process, the dorsal skin samples were collected from the animals with the aid of a mold with a fixed area (5 mm diameter). Edema was expressed by the variation of skin weight between the different control and treated groups [6].

2.6. Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) activity was quantified to be used as a marker of the leukocyte infiltrate (monocytes / macrophages and neutrophils) on the skin after UVB irradiation [31].

The skin samples were collected in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB), homogenized with Tissue-Tearor (Biospec 985370) and centrifuged (16,100 g for 2 min at 4 ° C). Briefly, 30 μ L of the resulting supernatant from each sample were mixed with 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine and 0.015% hydrogen peroxide. MPO activity was determined spectrophotometrically at 450nm (EnSpire, Perkin Elmer). The MPO activity of the samples was obtained by comparison with the MPO activity of a standard neutrophil curve. The results were expressed in neutrophil numbers per mg of skin [32].

2.7. Analyses of skin proteinase substrate-embedded enzymography

For the analyze of MMP-9, the polyacrylamide gel zymography technique with sodium dodecyl sulfate was applied (SDS), a method used to detect proteases. The analysis detects enzymes that degrade the gelatin present in the gel [2,33].

Skin samples were diluted (1: 4) and homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM calcium chloride (CaCl_2) and 1% proteinase inhibitors (phenanthroline, phenylmethylsulfonyl fluoride and N - ethylmaleimide) with the aid of

Tissue-Tearor (Biospec 985370). Thereafter, the homogenates were centrifuged (12,000 g, 10 min, 4°C) twice. Supernatant aliquots (25 µL) were mixed with 5 µL of 0.1 M Tris-HCl (pH 7.4) containing 20% glycerol, 4% SDS and 0.005% xylene cyanol and applied on electrophoresis gel (13.5% acrylamide and 0.025 % gelatin). After electrophoresis, the gels were washed for 1 h with 2.5% Triton X-100 under constant shaking, incubated overnight in 0.05 M Tris-HCl (pH 7.4) and 0.01M CaCl₂ at 37 ° C. The next day, the gels were stained with brilliant blue R and destaining in 20% acetic acid. The zones of enzymatic activity were detected as regions of negative staining against a dark background. The proteolytic activity was analyzed quantitatively by comparing the results of the samples of the treated animals with the controls not treated by the Image J program (NIH, Bethesda, MD, USA) [32,34].

2.8. Quantification of endogenous antioxidant reduced glutathione (GSH)

Skin samples were diluted in 0.02M EDTA and triturated using Tissue-Tearor (Biospec 985370). Whole homogenates were treated with 50% trichloroacetic acid. The mixture was then centrifuged at 2700 g for 10 minutes at 4°C. The supernatant was removed and recentrifuged at 2700 g for a further 15 minutes at 4°C. The final supernatant was removed for analysis. For the assay, the reaction mixture contained 50 µL of the sample supernatant, 100 µL of 0.4 M Tris buffer pH 8.9 and 5 µL of a 1.9 mg/mL solution of 5,5'-dithio-bis- (2-nitrobenzoic acid) (DTNB) in methanol. The absorbance was determined in a spectrophotometer (EnSpire, Perkin Elmer) after 5 minutes of incubation at 405 nm. The standard curve was prepared with 0 to 150 µM GSH. The results were expressed in as µM of GSH per mg of skin [35].

2.9. Levels of the of endogenous antioxidant catalase (CAT)

The method is based on the concentration decay of hydrogen peroxide (H₂O₂) which is directly proportional to the absorbance decrease at 240 nm. The difference in absorbance per unit time is the measure of catalase activity [36].

Skin samples were homogenized in 500 μL of 0.02M EDTA using the Tissue-Tearor homogenizer (Biospec 985370). The homogenate was centrifuged at 2700 g for 10 minutes at 4°C twice. The determination of CAT activity on skin was performed on microplate by addition of 10 μL sample, 160 μL 1M Tris-HCl buffer with 5 mM EDTA pH 8.0, 20 μL deionized water and 20 μL 200 mM H_2O_2 . A white was included for each sample prepared with 10 μL of the sample supernatant, 180 μL of 1M Tris-HCl buffer with 5 mM EDTA pH 8.0 and 20 μL of deionized water. The rate at which H_2O_2 is reduced by the action of CAT was evaluated by decreasing the absorbance value by the difference between the initial reading and reading 30 seconds after the addition of 200 mM H_2O_2 . The reading was performed on a microplate spectrophotometer (Enspire, Perkin Elmer) at 240 nm with a temperature maintained at 25 ° C. The catalase values were expressed as unit of CAT/ mg skin/ minute [36].

2.10. Assay for lipid peroxidation (LPO)

Lipid peroxidation is one of the most important organic expressions of oxidative stress (YAGI, 1987). Oxidation of lipids was measured by the formation of hydroperoxides, which are the primary products in lipid peroxidation [37].

The hydroperoxide production was evaluated by the chemiluminescence method previously described [4]. The method is based on the determination of the chemiluminescence initiated by the tert-butyl hydroperoxide [38].

The dorsal skin samples were homogenized in 800 μL of phosphate buffer (pH 7.4) using a Tissue-Tearor (BIOSPEC 985370) and centrifuged at 700 g for 2 minutes at 4°C. For the assay, 250 μL of the supernatant was diluted to 1730 μL of reaction medium (120 mM KCl, 30 mM phosphate buffer pH 7.4) and 20 μL of 3 mM tert-butyl hydroperoxide. The reading was conducted in a β - counter Beckman ® LS 6000SC (FULLERTON, CA, USA) in a non - coincident counting for 30 s with a response range between 300 and 620 nm. The experiment was performed in the dark in order to avoid vial phosphorescence activated by light at 30°C for 2 hours. The results were measured in counts per min (cpm) per mg of skin.

2.11. Evaluation of the production of superoxide anion ($\text{O}_2^{\cdot-}$)

Superoxide anion assay was performed through the nitroblue tetrazolium assay (NBT). Skin samples were homogenized with Tissue-Tearor (BIOSPEC 985370) in 0.02 M EDTA and centrifuged (2000 g for 20 seconds at 4°C). For the reaction, the supernatant (50 µL) was incubated in microplates for 1 hour. Then the supernatant was removed and NBT (1mg/mL) added to the fixed cells. After 15 minutes, the NBT was carefully removed and 20 µL of 100% methanol was added to the precipitate to settle. The compound formed by the reduction of NBT (formazan) was solubilized with 120 µL of 2M KOH and 140 µL of dimethylsulfoxide (DMSO). The reduction of NBT to formazan was measured in a microplate spectrophotometer reader (ENSPIRE, PERKIN ELMER) at 620 nm and the results are presented as optical density (OD) per 10 mg of skin [29].

2.12. Cytokine measurement

The dorsal skin samples were homogenized in saline (500 µL) with Tissue-Tearor (Biospec 985370) and centrifuged at 2000 g for 15 min at 4°C. The supernatants were used to quantify the cytokines IL-1, IL-6, IL-33, TNF- α , TGF- β and IL-10 by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience). The results were obtained by comparing the optical densities at 490 nm of the samples with the densities of the respective cytokine standard curves [39].

2.13. Quantitative polymerase chain reaction (qPCR)

Followed the method described elsewhere [29,40]. Briefly, samples of skin were homogenized in trizol reagent, and total RNA was extracted. The purity of total RNA was measured with a spectrophotometer, and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. Reverse transcription of total RNA to cDNA was carried out using a Superscript III kit (Invitrogen) and oligo (dT)₁₂₋₁₈ primers. Real-time PCR (qPCR) was performed with Platinum SYBRGreen kits (Invitrogen) in a 50 µL reaction volume following the manufacturer's cycling conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 45 cycles of 95 °C for

15 s and 55 °C for 30 s. Melting curve analysis was performed (65–95 °C) in order to verify that only one product was amplified. Samples with more than one peak were excluded. qPCR was performed in a LightCycler Nano Instrument (Roche). The relative gene expression was measured using the comparative $2^{-\Delta\Delta Cq}$ method. The expression of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA was used as a control for tissue integrity in all samples. The primers used were gp91^{phox}, sense: 5'-AGCTATGAGGTGGTGTAGTGG-3', antisense: 5'-CACAATATTTGTACCAGACAGACTTGAG-3'; HO-1, sense: 5'-CCCAAACTGGCCTGTAAAA-3'; antisense: 5'-CGTGGTCAGTCAACATGGAT-3'; Nrf2, sense: 5'-TCACACGAGATGAGCTTAGGGCAA-3', antisense: 5'-TACAGTTCTGGGCGGCGACTTTAT-3'; Nqo-1, sense: 5'-TGGCCGAACACAAGAAGCTG-3', antisense: 5'-GCTACGAGCACTCTCTCAAACC-3' and Gapdh sense: 5'-ATGACATCAAGAAGGTGGTG-3, antisense: 5'-CATAACCA-GGAAATGAGCTTG-3';

2.14. Skin histologic evaluation

The dorsal skin samples were collected in formol 10%, fixed in paraformaldehyde 4%, dehydrated in ascending concentrations of ethanol, cleared in xylene, embedded in paraffin and sectioned to a thickness of 5 µm. The sections were stained with hematoxylin and eosin, toluidine blue and Masson's trichrome stain

The sections stained with H & E were examined using light microscopy at 40x magnification for determination of epidermal thickness [41] and a 100x magnification for counting the number of sunburn cells [42]. For mast cell count, the sections were stained with toluidine blue and analyzed under light microscopy at 40x magnification. Both analysis were done with the software Infinity Analyze (Lumenera® Software). The sections stained with masson's trichrome were examined using light microscopy at a magnification of 10x to visualize changes in collagen fibers by analyzing the intensity of the blue coloration in the dermal areas of the skin exposed to UVB with the aid of the Image J software (NIH) [43].

2.15. Statistical analysis

The bars in the results indicate the mean values \pm standard error of the mean (SEM) of 5 mice per group per experiment and are representative of two separate experiments. Data were statistically analyzed by ANOVA followed by Tukey's t test. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA). Results were considered significantly different when $p < 0.05$.

3. Results

3.1. Resolvin D1 reduces UVB irradiation-induced skin edema and MPO activity

The anti-inflammatory action of RvD1 was first assessed by the edema assay and MPO activity (neutrophil marker). Skin edema was inhibited by RvD1 treatment only at the dose of 30 ng/mice (Figure 1 A). On the other hand, all four doses of RvD1 showed a similar inhibition in MPO activity (Figure 1 B).

3.2. Resolvin D1 reduces UVB irradiation-induced skin MMP-9 activity

After the exposure to UVB irradiation, there was a significant increase in the secretion/activity of MMP-9 from skin cells in hairless mice. In this study, treatment with RvD1 at dose of 30 and 100 ng/mice suppressed the secretion of MMP-9 (Figure 2).

3.3. Resolvin D1 reduces UVB irradiation-induced decrease of skin antioxidants

The effects of RvD1 were determined by reduced glutathione (GSH) assay and catalase assay. The dose of UVB irradiation used in the experiment (4.14 J/cm^2) was able to significantly reduce the endogenous antioxidant GSH and catalase in the irradiated control group compared to the non-irradiated control group. The treatment with 30 ng/mice of RvD1 was able to inhibit GSH reduction following UVB irradiation (Figure 3 A). Based on the results obtained in the previous tests, 30 ng/mice was

chosen as the best treatment, therefore all other assays were performed only with this dose.

Catalase assay (Figure 3 B), performed only with at dose of 30 ng/mice, confirmed that this dose is actually able to decrease the depletion of antioxidant activity.

3.4. Resolvin D1 reduces UVB irradiation-induced hydroperoxides (LOOH) and superoxide anion ($O_2^{\cdot-}$) production

UVB irradiation increased the hydroperoxides and superoxide anion production in the skin of irradiated control group compared to non-irradiated control group. Treatment with RvD1 at the dose of 30 ng/mice reversed this effect by reducing LOOH and $O_2^{\cdot-}$ production (Figure 4 A and B).

3.5. Resolvin D1 inhibits UVB irradiation-induced gp91^{phox} up-regulation, Nrf2 and Nqo-1 down-regulation and improves HO-1 mRNA expression in the skin

RvD1 (30 ng/mice) treatment was able to inhibit the gp91^{phox} expression induced by UVB exposure (Figure 6 A). UVB irradiation significantly induced HO-1 mRNA expression and decreased Nrf2 and Nqo-1 mRNA expression in the skin. RvD1 (30 ng/mice) treatment enhanced HO-1 mRNA expression and inhibited Nrf2 and Nqo-1 down regulation mRNA expression after UVB irradiation in the skin (Figure 6 B, C and D).

3.6. Resolvin D1 inhibits UVB irradiation-induced skin inflammation by reducing cytokine production

UVB irradiation induced higher levels of secretion of pro-inflammatory (IL-1 β , IL-6, IL-33 and TNF- α) and anti-inflammatory (TGF- β and IL-10) cytokines in hairless mice skin. The behavior of cytokine induction was investigated following the treatment with RvD1 30 ng/mice, and the results showed that RvD1 inhibited the increase of inflammatory cytokines IL-1 β , IL-6, IL-33 and TNF- α (Figure 5 A, B, C

and D) and maintained the secretion of anti-inflammatory cytokines at basal levels compared to non-irradiated control (Figure 5 E and F).

3.7. Resolvin D1 reduces UVB irradiation-induced epidermal thickness and apoptosis of keratinocytes

Epidermal thickness is used as a quantitative parameter to assess inflammation [4]. Measurement of hematoxylin and eosin stained tissue sections indicated that dorsal skin epidermal thickness was significantly increased following UVB irradiation in the irradiated control group compared to the non-irradiated control group. By contrast, epidermal hypertrophy was significantly reduced when mice were treated with RvD1 30 ng/mice before irradiation (Figure 7 A).

One of the consequences of acute exposure to UV radiation is the activation of apoptosis in epidermal keratinocytes. These apoptotic keratinocytes are known as sunburn cells. UV radiation was able to induce numerous sunburn cells, which are defined as shrunken cells within the epidermis that exhibit eosinophilic cytoplasm and condensed nucleus [44]. Thus, we investigated apoptotic keratinocytes after treatment with RvD1 30 ng/mice. The results showed that treatment with dose 30 ng/mice RvD1 inhibited sunburn cells compared with the irradiated control group (Figure 7 B).

3.8. Resolvin D1 reduces UVB irradiation-induced mast cells

After exposure to UVB irradiation, there was a significant increase in the number of mast cells in the skin of hairless mice. In this study, treatment with RvD1 at dose of 30 ng/mice decreased the number of mast cells compared to the irradiated control group (Figure 8).

3.9. Resolvin D1 reduces UVB irradiation-induced damage of collagen fiber

The tissue sections were subjected to Masson's trichrome staining in order to visualize changes in collagen fiber formation in the dermal areas of the UVB-exposed

dorsal skin [43]. Notably, the collagen fibers (blue) in the group pretreated with RvD1 30 ng/mice showed lower levels of damage in collagen fiber compared with irradiated group (Figure 9).

4. Discussion

UV irradiation causes a number of adverse biological effects on the skin, such as premature aging and skin cancer [2,3,27,45]. The deleterious effect of UV irradiation on the skin, in particular UVB (280-315 nm), occurs due to excessive free radical production and direct DNA damage [46]. ROS are directly involved in the induction of inflammatory process and oxidative stress, because they stimulate the secretion of cytokines [6] and the depletion of endogenous antioxidants [7,8]. Although well established in the literature that pro-resolution lipid mediators actively reduce inflammation, there was not yet evidence of the therapeutic effect of lipid mediators on UV irradiation-induced inflammation and oxidative stress in the skin. RvD1 is a bioactive metabolite generated in response to inflammation by enzymatic conversion of docosahexaenoic acid (DHA). Thereby, in this study we demonstrated the anti-inflammatory and pro-resolution effect of the lipid mediator resolvin D1 (RvD1) in model inflammatory induced by UVB irradiation in hairless mice.

The acute inflammatory process triggered by exposure to UV irradiation causes specific cellular events, including increased permeability of the vascular endothelium, infiltration of polymorphonuclear leukocytes, inflammatory macrophages and lymphocytes at the site of the lesion, which consequently lead to skin edema [47]. Systemic treatment with RvD1 was able to inhibit UV-induced skin edema in hairless mice. Moreover, the histopathological analysis found that treatment with RvD1 attenuated the thickening of the epidermis. A previous study reported that RvD1 attenuate pulmonary edema in a model of lipopolysaccharide (LPS)-induced acute lung injury [48].

UV irradiation induces the increase of mast cells in the dermis [49] and the appearance of sunburn cells [44]. The presence of mast cells in the dermis correlates directly with the degree of susceptibility to systemic immunosuppression induced by UVB, and suppression of the immune system allows UV-induced tumors not to be destroyed [50]. Sunburn cells (SBCs) are used as markers of skin damage caused by UVB. When UV irradiation exceeds the protective response threshold of keratinocytes, these cells activate apoptosis and die. The reduction in the number of SBCs indicates an increase in the photoprotection of keratinocytes. Herein, through

the histopathological analysis, it was possible to observe that, in the group treated with RvD1, there was a decrease in the number of mast cells and sunburn cells compared to the irradiated control group.

Interestingly, we found that treatment with RvD1 decreased the recruitment of neutrophils induced by UVB irradiation, determined by MPO activity. Neutrophils have the potential to increase the damage caused by UV irradiation, as they are able to release a variety of substances that are harmful to cells and tissue such as ROS [51,52]. RvD1 treatment with different doses was able to significantly inhibit MPO activity. It has been reported that RvD1 reduced MPO activity in liver injury model induced by carbon tetrachloride (CCl₄) [53]. In addition, in the acute cigarette smoke-induced lung inflammation model, RvD1 was able to inhibit neutrophilic inflammation and increase neutrophil efferocytosis [26]. Another inflammatory parameter evaluated was the activity/secretion of metalloproteinase-9 (MMP-9). MMP-9 are proteolytic enzymes responsible for extracellular matrix collagen degradation during pathological processes such as photoaging; these enzymes are also associated with the invasive and metastatic potential of tumors [7]. RvD1 treatment was able to inhibit the activity/secretion of MMP-9 after exposure to UVB irradiation. Corroborating with this result, the histopathological analysis showed that treatment with RvD1 was able to inhibit UVB irradiation-induced collagen fibers degradation.

ROS that were produced on the skin after exposure to UV irradiation, induced transcription factors such as NF- κ B, an oxidant-sensitive transcriptional factor, which plays a crucial role in the activation of multiple genes involved in the expression of various pro-inflammatory molecules, including cytokines such as IL-1, IL-6, IL-33, TNF- α , IL-10 and TGF- β [6,54–56]. Treatment with RvD1 inhibited the increase of pro-inflammatory cytokines IL-1 β , IL-6, IL-33 and TNF- α , and anti-inflammatory TGF- β and IL-10 induced by UVB exposure. Pro-inflammatory cytokines, such as TNF- α , play a crucial role in the recruitment of cells, such as neutrophils, to injury areas, and are involved in the formation of sunburn cells [42]. Previous studies have demonstrated that RvD1 is able to inhibit the inflammatory process in several disease models. RvD1 inhibited pro-inflammatory cytokines such as IL-1 β in lipopolysaccharide-induced acute lung injury in mice [23] and inhibited IL-6 and TNF- α cytokines in human visceral adipose tissue from obese patients [57]

Moreover, excessive stimulation of TGF- β is an indispensable prerequisite for tumorigenesis and tumor progression. TGF- β affect the recognition and destruction of tumor cells by regulating immune cell function [58]. It has been demonstrated that RvD1 decreased the production of anti-inflammatory cytokines by inhibiting the activation of MAPKs (ERK1/2, JNK and p38) and NF- κ B signaling pathways in model imiquimod-induced mice psoriasiform dermatitis [59]. Taken together, the results show that decreased production of pro-inflammatory and anti-inflammatory cytokines in the skin of hairless mice treated with RvD1 contribute to their protective function against the inflammatory lesions induced by UVB irradiation on the skin.

The accumulated ROS act on the cell biological membrane and the availability of polyunsaturated fatty acids regulates the lipid peroxidation process. High peroxidation levels are associated with harmful effects on biological systems, such as loss of fluidity inactivation of membrane enzymes and receptors, and increasing of the permeability of ions, what can lead to cell membrane rupture. In addition, peroxidation products can damage DNA. LOOH, which are the primary products of lipid oxidation [37], were used as markers of oxidative stress in the present study, and it was found that RvD1 treatment reduced production of LOOH induced by UVB irradiation exposure.

Exposure to UVB irradiation induces the expression of the gp91^{phox} subunit of NADPH oxidase 2 (NOX2) leading to a higher radical O₂⁻ production. The production of ROS by gp91^{phox} is crucial for the establishment of oxidative stress [60]. Treatment with RvD1 decreased the expression of mRNA for gp91^{phox} and production of superoxide anion, improved the anti-inflammatory and antioxidant pathways, maintaining basal levels of mRNA expression for Nrf2 and Nqo-1, and increased mRNA expression of OH-1.

Furthermore, RvD1 also improved the antioxidant capacity of the skin after exposure to UVB irradiation by maintaining GSH and catalase activities at basal levels. GSH plays an important role in protecting skin cells against oxidative damage through the direct elimination of ROS or acting as a coenzyme of glutathione peroxidase [7,8]. In addition, catalase is an antioxidant enzyme that converts hydrogen peroxide in water and molecular oxygen [61,62]. The loss of UVB-mediated

cell viability is associated with a notable decrease in endogenous antioxidants, therefore weakening the cell's defense system against the condition of oxidative stress [7,8].

The data from several studies also showed that RvD1 reduced oxidative stress. RvD1 increased the levels of GSH and HO-1 mRNA expression in carbon tetrachloride (CCl₄)-induced acute liver injury model [53]. Also, RvD1 induced GSH release in human chondrocytes obtained from osteoarthritis patients. Our finding showed that RvD1 increases redox status as indicated by expanded GSH levels. These data are consistent with those of the literature indicating that RvD1 inhibited GSH depletion [63].

Nrf2 is a transcription factor that modulates the expression of antioxidant and detoxifying enzymes, known as phase II enzymes. In this group of enzymes, HO-1, Nqo-1 and catalase [64,65] are included. Exposure to UVB irradiation increased HO-1 mRNA expression and decrease Nrf2 and Nqo-1 mRNA expression. In fact, Nrf2 induces HO-1 mRNA expression in the skin in response to oxidative stress [65]. HO-1 is antioxidant enzyme that is responsive to different stress conditions, including the inflammation process; in this sense HO-1 is essential to main cellular resistance during stress conditions. Enhanced HO-1 mRNA expression was associated with resolution of inflammation and natural killer (NK) cell-mediated cytotoxicity, which may explain why HO-1 production increases while other antioxidant enzymes are inhibited [40,66,67]. Corroborating this, treatment with RvD1 increased Nrf2 mRNA expression, which resulted in an enhancement of HO-1 mRNA expression, and maintenance the GSH levels and Nqo-1 mRNA expression near to basal levels after UVB exposure. In different inflammatory diseases, such as psoriasis and atopic dermatitis, HO-1 expression is increased [66]. Further, RvD1 increasing of HO-1 expression contributed to the protection from the deterioration of tight junction proteins in a model of acute lung injury induced by lipopolysaccharide in mice [48]. Therefore, the increase in the levels of endogenous antioxidants induced by RvD1 contributes to protect the skin from UVB irradiation.

In conclusion, the present study demonstrated for the first time that treatment with RvD1 can prevent oxidative and inflammatory damage induced by exposure to UVB irradiation in hairless mice, maintaining mRNA expression for antioxidant and

anti-inflammatory molecules (Nrf2 and Nqo-1), increasing mRNA expression for OH-1 and suppressing mRNA expression for gp91^{phox}. We also observed that RvD1 treatment was able to reduce neutrophil recruitment, MMP-9 activity/secretion, production of lipid hydroperoxides, superoxide anions production, epidermal thickening, degradation of collagen fibers, mast cell and sunburn cells numbers. RvD1 also decreased the secretion of cytokines and prevented the depletion of endogenous antioxidants. These results imply that RvD1 have strong potential as a novel therapeutic approach to resolve inflammation and oxidative stress caused in skin of hairless mice by UVB exposure.

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Figures

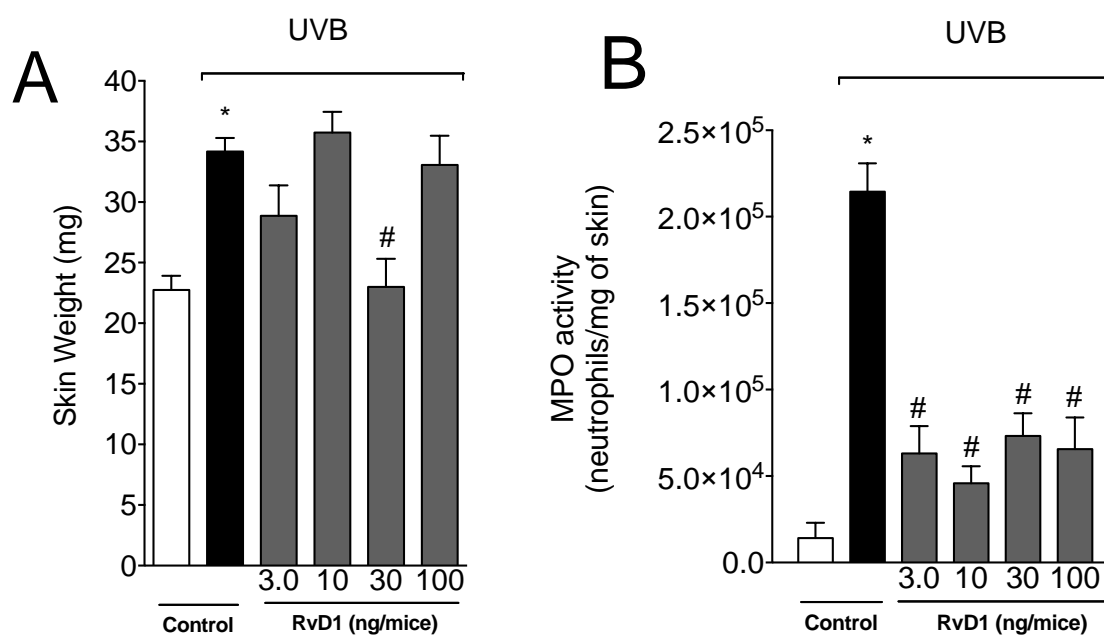


Figure 1 - RvD1 reduces UVB irradiation-induced skin edema and MPO activity. The skin inflammation was determined in samples collected 12 h after the end of irradiation. (A) Skin edema and (B) MPO activity. Bars represent means \pm SEM of 5 mice per group and are representative of two separate experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [* $p < 0.05$ compared to the non-irradiated control group; # $p < 0.05$ compared to the irradiated control group (vehicle)].

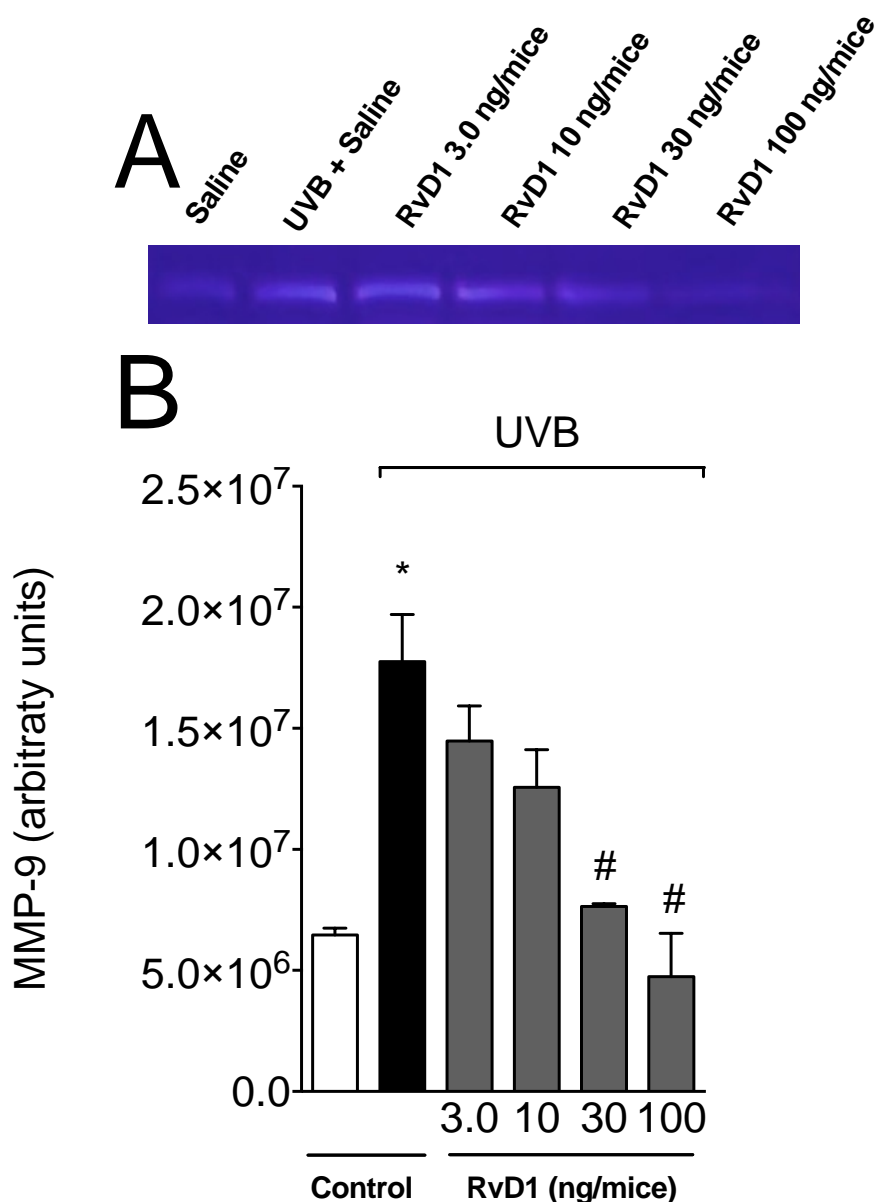


Figure 2 – RvD1 inhibits UVB irradiation-induced increase of MMP-9 activity. The MMP-9 activity was determined in samples collected 12 h after the end of irradiation. (A) Representative image of gelatin zymography and (B) bars represent means \pm SEM of 5 mice per group per experiment and are representative of two separate experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [* p <0.05 compared to the non-irradiated control group; # p <0.05 compared to the irradiated control group (vehicle)].

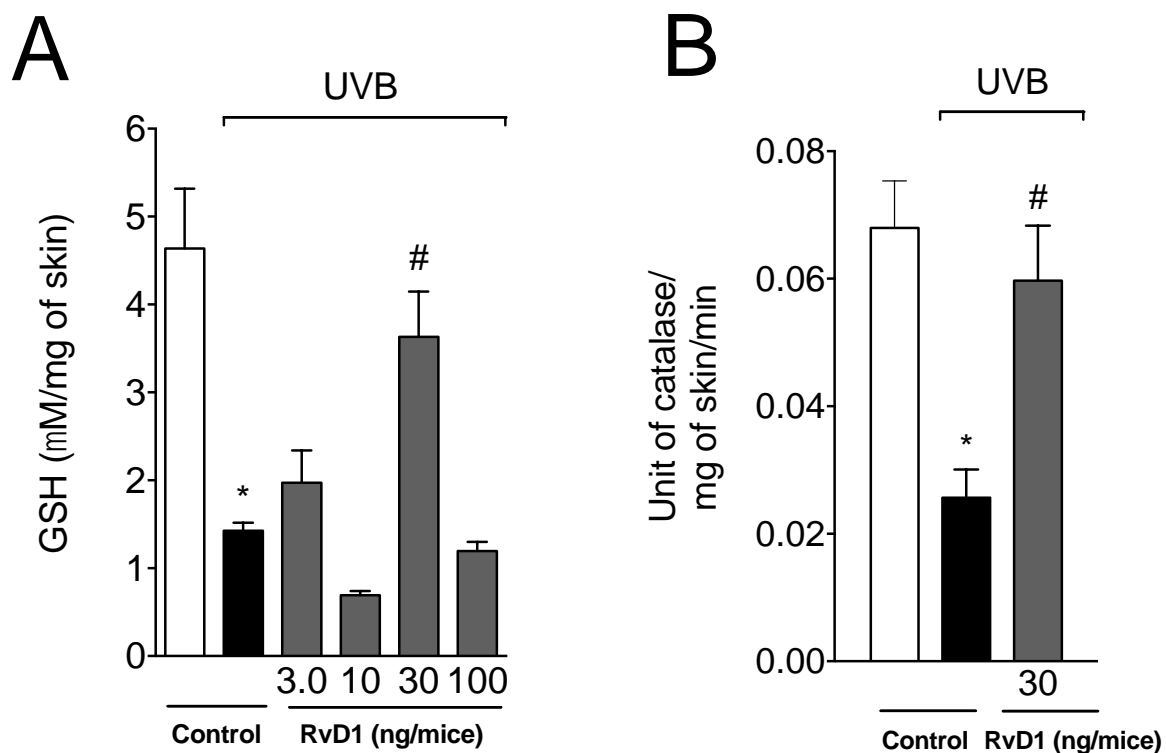


Figure 3 – RvD1 inhibits UVB irradiation-induced GSH and CAT depletion. The GSH levels (A) and CAT activity (B) were determined in samples collected 12 h and 2 h after the end of irradiation, respectively. Bars represent means \pm SEM of 5 mice per group per experiment and are representative of two separated experiments. [$*p < 0.05$ compared to the non-irradiated control group and # $p < 0.05$ compared to the irradiated control group (vehicle)].

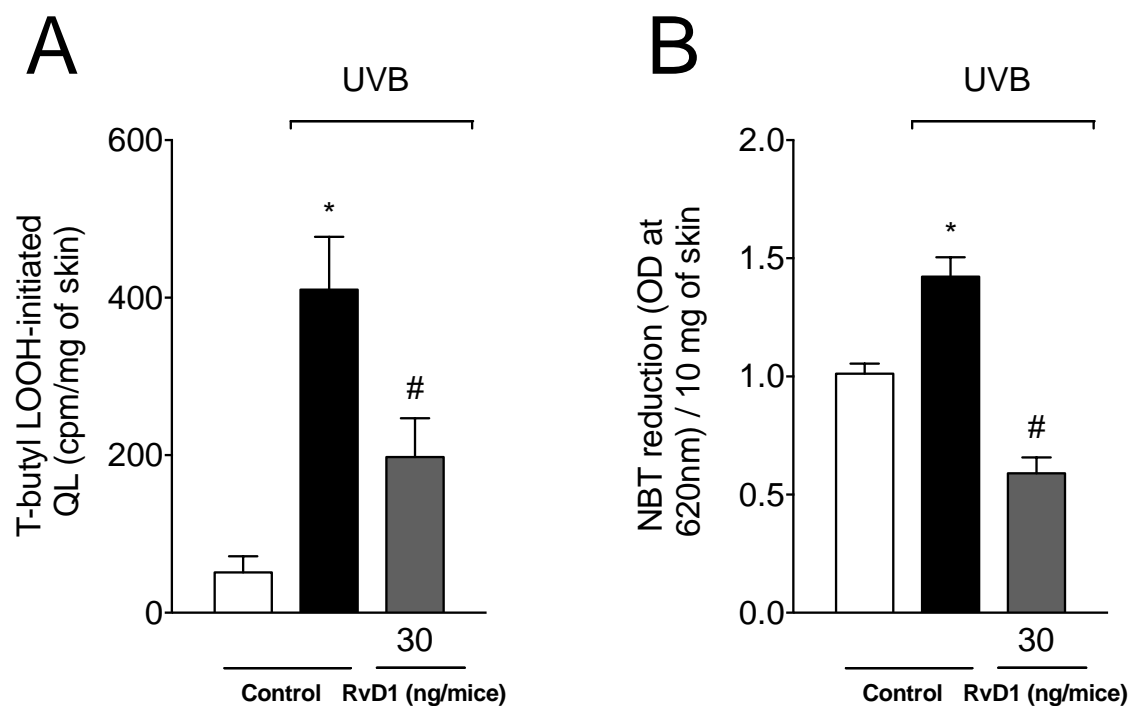


Figure 4 – RvD1 inhibited UVB irradiation-induced lipid peroxidation (LOOH) and superoxide anion production. Lipid peroxidation (A) and superoxide anion production (B) were measured by t-butyl lipid hydroperoxides (LOOH)-initiated chemiluminescence (QL) and nitroblue tetrazolium (NBT) reduction assay in samples collected 4 and 2 h after the end of irradiation, respectively. Bars represent means \pm SEM of 5 mice per group and are representative of two separate experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$*p < 0.05$ compared to the non-irradiated control group; $\#p < 0.05$ compared to the irradiated control group (vehicle)].

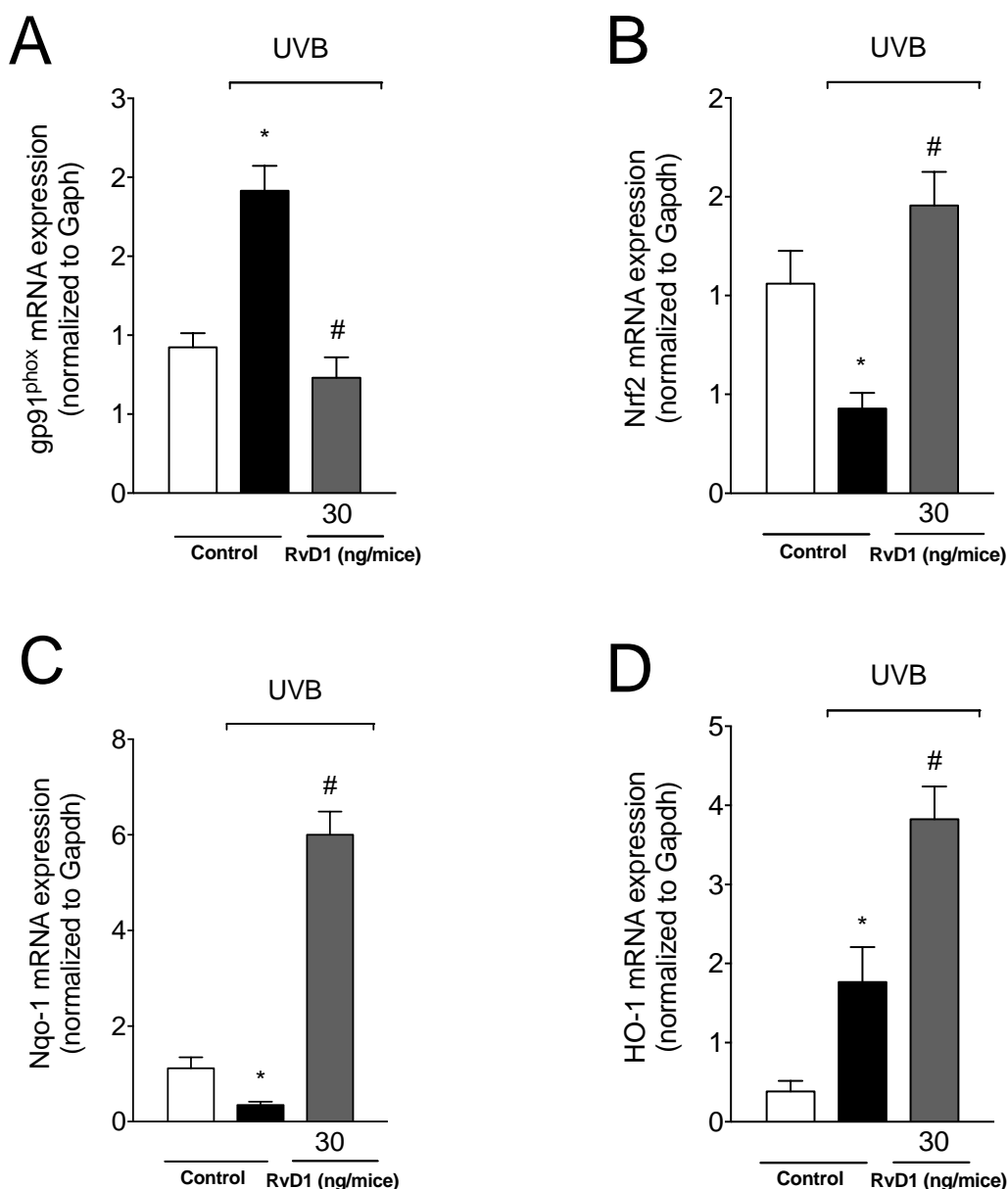


Figure 5 - RvD1 inhibits UVB irradiation-induced gp91^{phox} up-regulation, Nrf2 and Nqo-1 down-regulation and improves HO-1 mRNA expression in the skin. NADPH oxidase sub-unit gp91^{phox} (A), HO-1 (B), Nrf2 (C) and Nqo-1 (D). The gp91^{phox}, HO-1, Nrf2 and Nqo-1 mRNA expression was determined in samples collected 4 h after the end of irradiation by quantitative polymerase chain reaction (qPCR). Bars represent means \pm SEM of 5 mice per group per experiment and are representative of two separate experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [* p <0.05 compared to the non-irradiated control group; # p <0.05 compared to the irradiated control group (vehicle)].

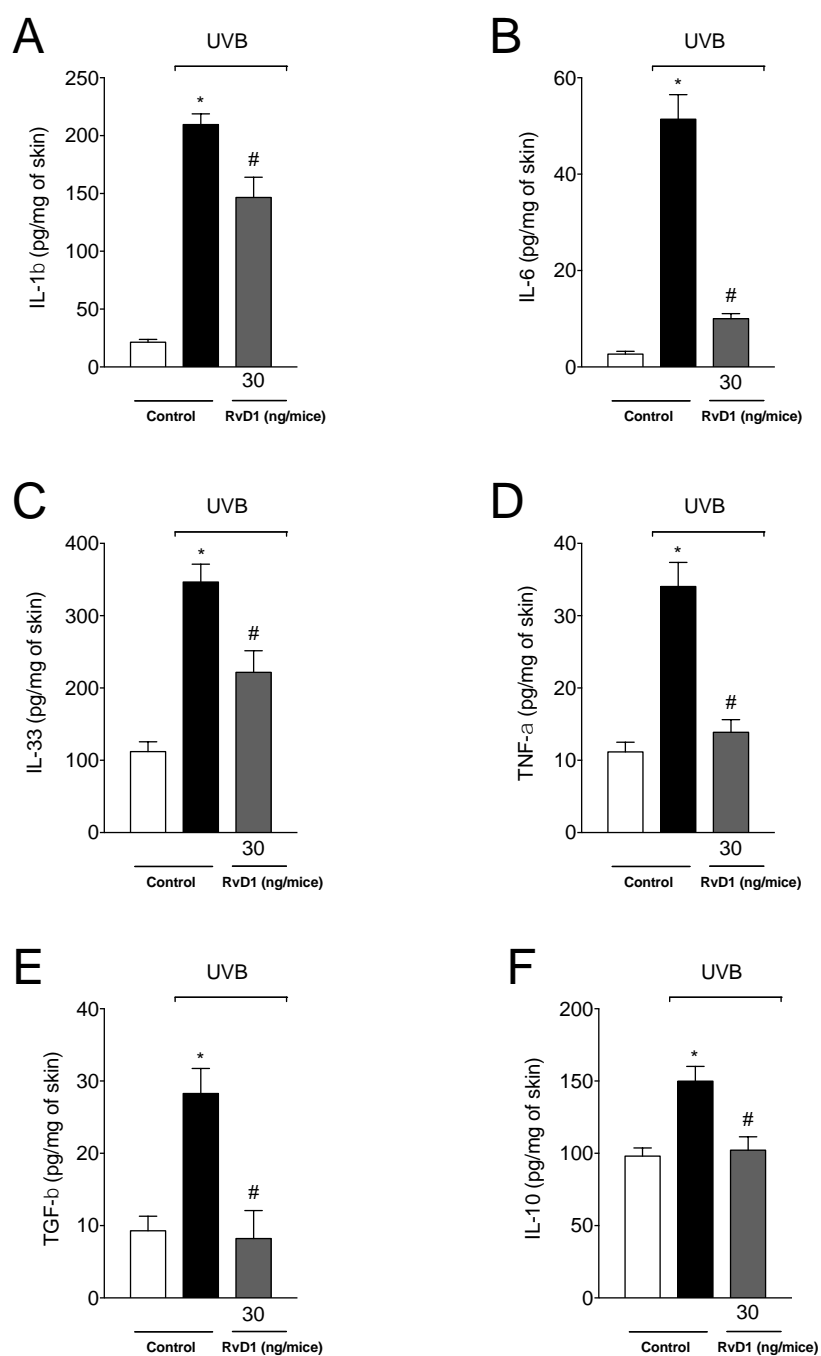


Figure 6 - RvD1 inhibits UVB irradiation-induced pro-inflammatory cytokines production (A) IL-1 β (B) IL-6 (C) IL-33 and (D) TNF- α and inhibits anti-inflammatory cytokines production (E) TGF- β and (F) IL-10. Cytokines were determined in skin samples collected 4 h after the end of irradiation. Bars represent means \pm SEM of 5 mice per group and are representative of two separate experiments. Statistical analysis was performed by one-way

ANOVA followed by Tukey's test. [$*p < 0.05$ compared to the non-radiated control group; $\#p < 0.05$ compared to the radiated control group (vehicle)].

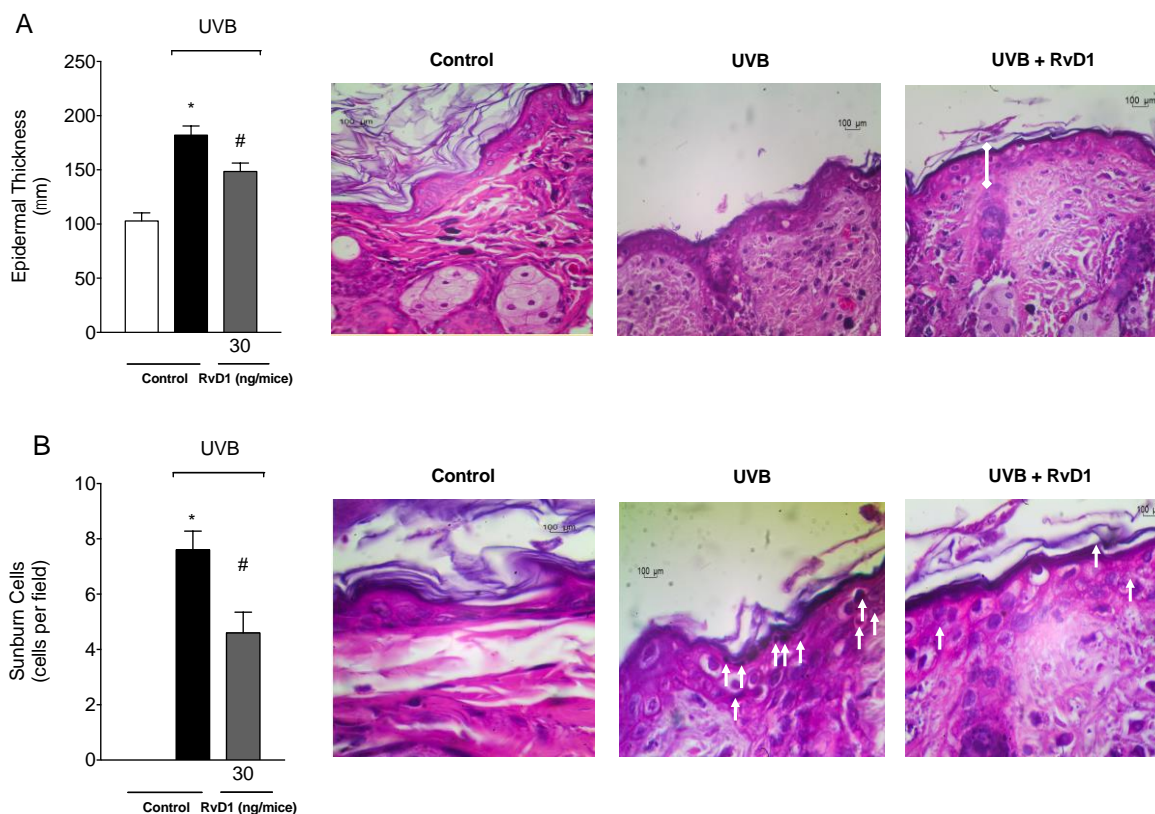


Figure 7 - RvD1 reduces UVB radiation-induced epidermal thickness and sunburn cells. Epidermal thickness and sunburn cells were evaluated using hematoxylin and eosin staining (H & E) in skin samples collected 12 h after the end of irradiation. Epidermal thickness (μm) (A) and the number of sunburn cells (B). The sections stained with H & E were examined using light microscopy at 40x (A) magnification and 100x (B). Bars are representative of two separate experiments and represent means \pm SEM of 5 mice per group per experiment. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$*p < 0.05$ compared to the non-radiated control group; $\#p < 0.05$ compared to the radiated control group (vehicle)].

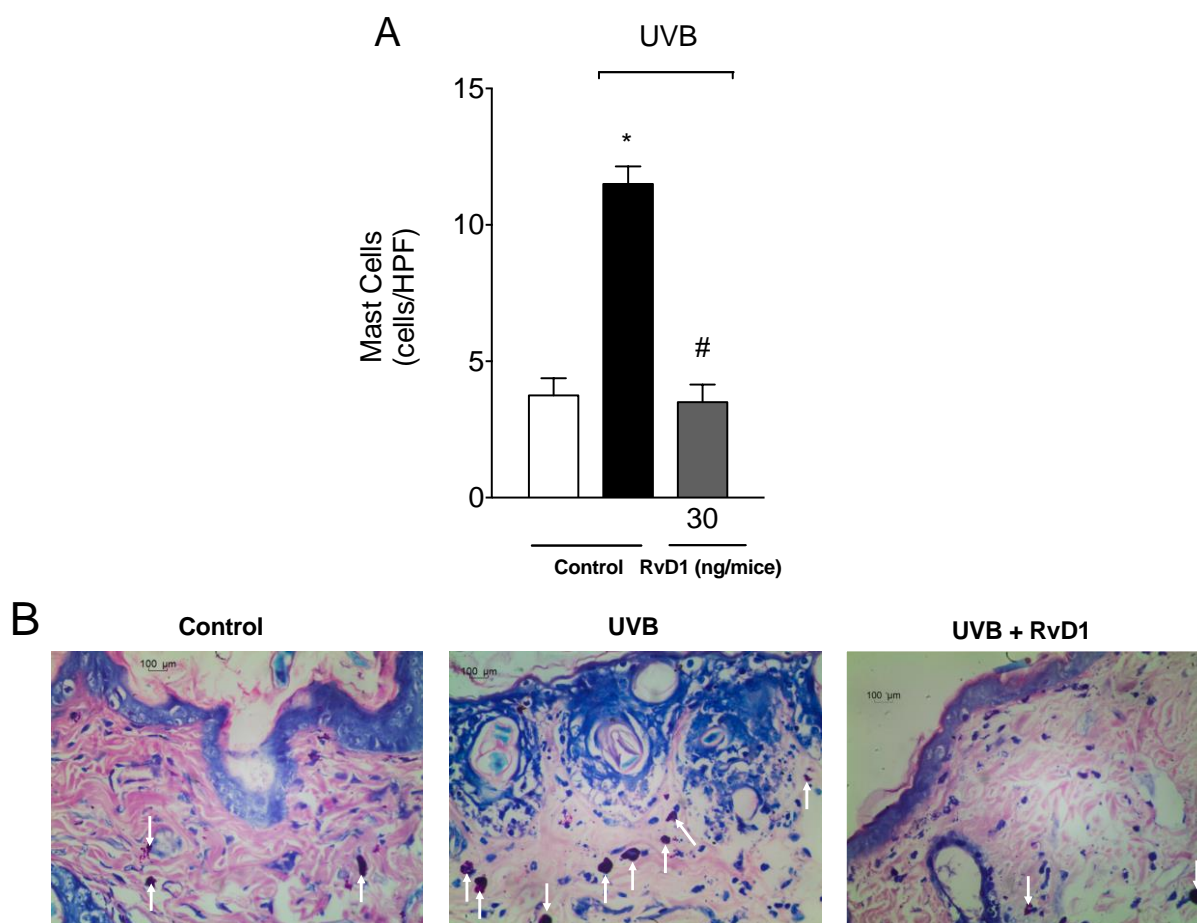


Figure 8 - RvD1 reduces UV irradiation-induced mast cells. Mast cells were evaluated using blue toluidine in skin samples collected 12 h after the end of irradiation. Number of mast cells (A) and the sections stained with blue toluidine were examined using light microscopy at 40x magnification (B). Bars are representative of two separate experiments and represent means \pm SEM of 5 mice per group per experiment. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [* $p < 0.05$ compared to the non-irradiated control group; # $p < 0.05$ compared to the irradiated control group (vehicle)].

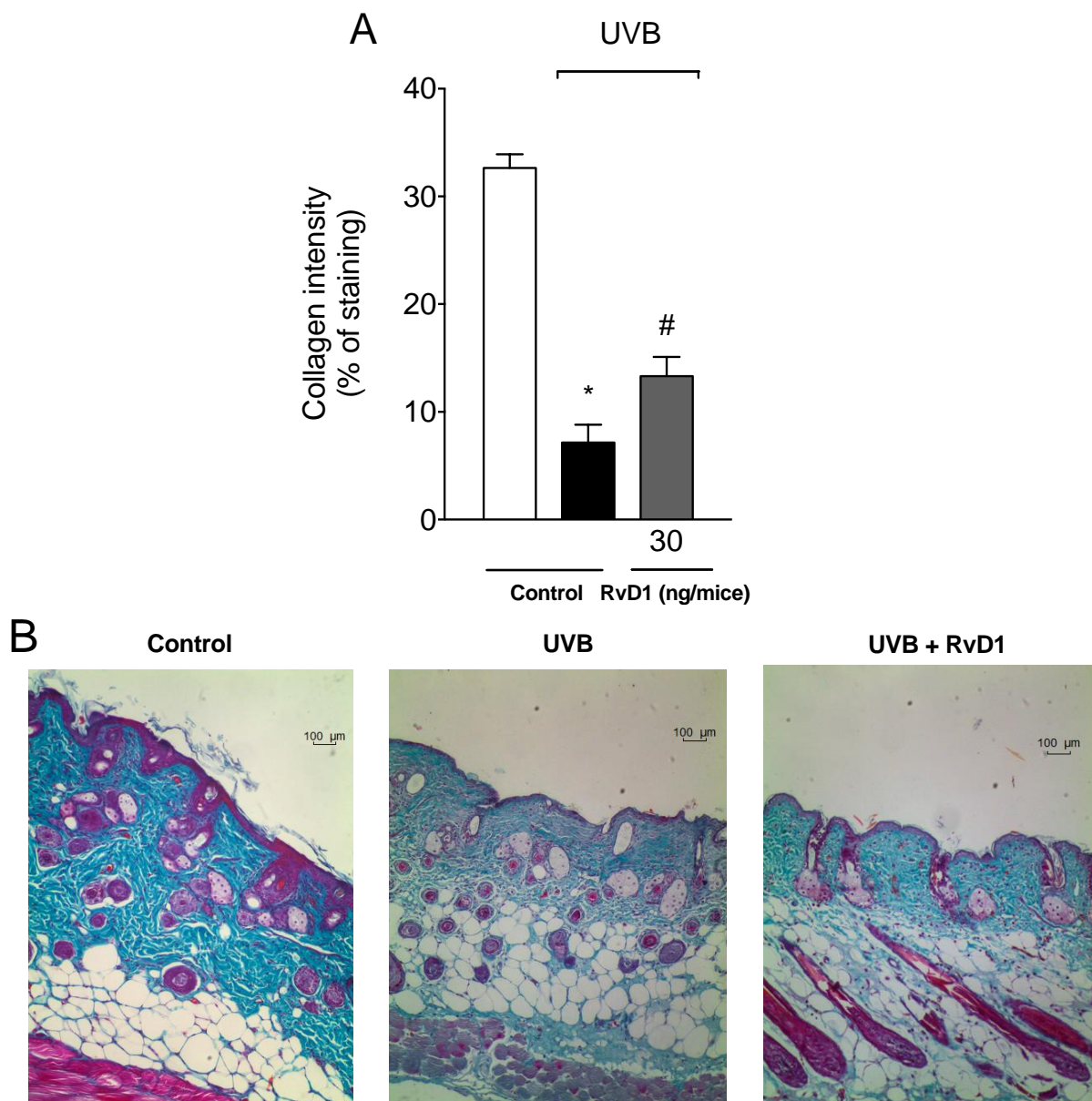


Figure 9 – RvD1 inhibits UVB irradiation-induced collagen fibers damage to the skin of hairless mice. Collagen fiber formation was evaluated using Masson's trichrome staining in skin samples collected 12 h after the end of irradiation. Collagen fiber intensity and bundles shown in blue was analyzed by Image J Program (10x magnification). Bars are representative of two separate experiments and represent means \pm SEM of 5 mice per group per experiment. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$*p < 0.05$ compared to the non-irradiated control group; $\#p < 0.05$ compared to the irradiated control group (vehicle)].

5. CONSIDERAÇÕES FINAIS

A exposição à radiação ultravioleta induz uma série de efeitos adversos na pele, inclusive o envelhecimento precoce e o câncer de pele. A produção excessiva de radicais livres, induzida pela RUV, leva a depleção dos antioxidantes endógenos e consequente processo inflamatório. Assim, o uso de mediadores lipídicos anti-inflamatórios/pró-resolução, como a RvD1, para enriquecer o sistema de proteção endógeno e controlar os processos lesivos induzidos pela radiação UVB tornam-se uma alternativa promissora. Estudos anteriores, elencados neste trabalho, evidenciaram efeitos benéficos do uso da RvD1 na diminuição dos danos oxidativos e inflamatórios em diferentes modelos, como asma e lesão pulmonar aguda. No entanto, ainda não havia estudos sobre o uso da RvD1 na melhora ou prevenção de danos inflamatórios e oxidativos causados pela radiação UVB em camundongos.

Os resultados *in vivo* obtidos nesse estudo demonstraram que o tratamento com RvD1 protegeu a pele contra os danos inflamatórios e oxidativos induzidos pela radiação UVB. A RvD1 foi capaz de inibir o estresse oxidativo por manter os níveis de GSH e atividade de catalase. Além disso, o tratamento com RvD1 também aumentou a expressão de RNAm para moléculas antioxidantes e anti-inflamatórias (Nrf2, OH-1 e Noq-1) e diminuiu a expressão de gp91^{phox}. Somando-se a isso, a RvD1 também foi capaz de inibir o edema cutâneo, o recrutamento de neutrófilos, a atividade da MMP-9, a produção de hidroperóxidos lipídicos, de ânion superóxido e a produção de citocinas inflamatórias (IL-1 β , IL-6, IL-33 e TNF- α) e anti-inflamatórias (TGF- β e IL-10). A RvD1 também reduziu a espessura epidérmica, o número de células apoptóticas, de mastócitos e a degradação de fibras de colágeno induzidos pela RUV.

Dessa forma, os resultados sugerem que o mediador lipídico RvD1 pode ser uma nova estratégia para controlar e/ou prevenir os danos causados pela radiação UVB na pele.

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Anexo I –Informações sobre submissão do artigo a revista Journal of Photochemistry and Photobiology b: Biology

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[1] B.K. Armstrong, A. Kricker, The epidemiology of UV induced skin cancer, J. Photochem. Photobiol. B 63 (2001) 8-18.

Reference to a book:

[2] W. Strunk Jr., E.B. White, The Elements of Style, third ed., Macmillan, New York, 1979. Reference to a chapter in an edited book:

[3] G.R. Mettam, L.B. Adams, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing, Inc., New York, 1994, pp. 281-304.

[4] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. <http://dx.doi.org/10.17632/xwj98nb39r.1>

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