DIEGO ADREAZZI DUARTE

PARTICIPAÇÃO DA SIRTUÍNA NA RETINOPATIA DIABÉTICA - MECANISMOS DE REGULAÇÃO DA NEURODEGENERAÇÃO

PARTICIPATION OF SIRTUIN ON DIABETIC RETINOPATHY - MECHANISMS OF REGULATION OF THE NEURODEGENERATION

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Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Clínica Médica na área de concentração Clínica Médica.

Thesis presented to the Faculty of Medical Sciences, State University of Campinas as part of the requirements for obtaining the title of Doctor of Clinical Medicine in the area of Concentration Clinical Medical.

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ESTE EXEMPLAR CORRESPONDE À VERSÃO
FINAL DA TESE DEFENDIDA PELO
ALUNO DIEGO ANDREAZZI DUARTE, E ORIENTADO
PELA PROFª. DRª. JACQUELINE MENDONÇA LOPES DE FARIA.

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DEDICATÓRIA

À minha mãe:

À minha mãe, Lucrécia Pereira da Silva, que embora as dificuldades impostas pela vida sempre esteve ao meu lado como meu porto seguro. A Deus por abençoar o meu caminho.

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"Tudo na vida tem um propósito, propósito esse que nos leva a crer que nascemos cada um com uma missão, seja ela aprender ou ensinar coisas, construir relacionamentos através de amizade, amor e companheirismo, refletir sobre atitudes, aprimorar e crescer com o tempo; e ao fim de nossas vidas em um futuro longínquo poderemos nos orgulhar de tudo que fizemos."

(autoria própria)

SUMÁRIO

LISTA DE ABREVIATURAS

8-OHdG	8-hidrox-2`-deoxiguanosina
AIF	proteína indutora de apoptose
AMPK	proteína quinase ativada
BRB	barreira hematoretiniana
CT	células-tronco
Cu^{+}	Cobre
DM	diabetes *mellitus*
DNA	ácidos desoxiribonocleicos
EC	Epicatequina
ECG	epicatequina gallate
ECM	matriz extracelular
EGC	Epigalocatequina
EGCG	epigalocatequina gallate
EOC-CM	meio condicionado de células *early outgrowth*
EOCs	células *early outgrowth*
ET-1	endotelina 1
$Fe2^{+}$	íon ferroso
FN	fibronectina
GAPDH	gliceraldeído-3-fosfato nitrosilado
GFAP	proteína acídica fibrilar glial
GSH	glutationa reduzida
H_2O_2	peróxido de hidrogênio
HG	alta glicose
ICAM-1	molécula de adesão intercelular 1
IL-1 β	interleucina-1β
iNOS	óxido nítrico-sintase induzível
IκB	proteína inibitória do NF-κB

NAC	N-acetilcisteína
NAD^+	nicotinamida adenina dinucleotídeo
NF-κB	fator nuclear kappa B
NG	glicose normal
NMDA	N-Methil-D-aspartato
•NO	radical óxido nítrico
O_2^-	radical ânion superóxido
$O2•^-$	radical superóxido
$ONOO^-$	radical ânion peroxinitrito
PAR	ADP-ribose
PARP-1	poli(ADP-ribose) polimerase-1
RD	retinopatia diabética
rMC-1	células de linhagem Müller da retina
ROS	espécies reativas de oxigênio
SIRT1	sirtuína 1
SOD	superóxido dismutase
SUMO	*small ubiquitin-related modifier proteins*
TGFβ-1	fator de transformação do crescimento beta-1
TNF-α	fator de necrose tumoral α
VCAM-1	molécula de adesão vascular

RESUMO

A retinopatia diabética (RD) é uma doença devastadora que está entre as maiores causas de cegueira entre pessoas na idade adulta em todo o mundo. Considerada multifatorial e progressiva, a RD afeta células neurais e gliais, e também elementos vasculares da retina. Sabe-se que diversas vias estão envolvidas na patogênese da RD, no entanto, os mecanismos que levam a exacerbação da inflamação e morte de células gliais/neurais o que caracteriza a neurodegeneração da retina, ainda permanecem desconhecidos. Diante disso, a redução desses fatores tem sido extensivamente estudada como alvo no combate a RD. As Sirtuínas, histonas desacetilases dependentes de nicotinamida adenina dinucleotídeo (NAD^+), atuam em resposta a vários estresses e atualmente tem sido relacionadas às importantes funções moleculares na regulação de várias doenças. Considerado um redox sensível, a SIRT1 pode estar reduzida em condição de doença, o que agravar ainda mais a situação patológica. No entanto, não se sabe ao certo o mecanismo de modulação e/ou atuação da SIRT1 frete às doenças neurodegenerativas, tais como a RD.

No Artigo I, foram avaliados os possíveis efeitos protetores do cacau rico em polifenóis na retina diabética. As células Müller da retina (rMC-1) foram expostos por 24h à glicose normal (NG), alta glicose (HG) ou peróxido de hidrogênio (H_2O_2) e submetidas ao tratamento com cacau na presença ou não de um inibidor da SIRT1 ou siRNA. O estudo animal foi desenvolvido em ratos experimentalmente diabéticos induzidos por estreptozotocina e randomizado para receber tratamentos com cacau com baixa, intermediária, ou elevada dose de polifenol (0,12 mg; 2,9 mg; 22,9 mg/kg/dia) por gavagem durante 16 semanas. As células expostas a H_2O_2 ou HG apresentaram aumento de proteína acídica fibrilar glial (GFAP) e acetil-RelA/p65 e diminuição da atividade/expressão da SIRT1. Estes efeitos foram anulados pelo cacau, que diminuiu a produção de espécies reativas de oxigênio e reduziu a ativação da poli(ADP-ribose) polimerase-1 (PARP-1); melhorou os níveis intracelulares de NAD^+ e consequentemente aumentou da atividade da SIRT1. As retinas dos ratos diabéticos exibiram os primeiros marcadores de retinopatia acompanhada pela eletrorretinografia prejudicada. A presença de diabetes levou a ativação da PARP-1 e diminuição dos níveis de NAD^+, resultando em comprometimento da

SIRT1. O aumento na acetilação do RelA/p65 levou na hiperexpressão do GFAP. A administração oral de cacau polifenol restaurou as alterações acima referidas. Este estudo revelou que o cacau enriquecido com polifenóis teve efeito protetor da retina diabética restabelecendo a via da SIRT-1.

No Artigo II, foi investigado o possível efeito terapêutico de células derivadas de animais saudáveis (Dock7 m +/+ Leprdb db/m) e diabéticos (BKS.Cg-Dock7 m +/+ Leprdb/J, db/db) na retinopatia diabética (RD). Os camundongos db/db (espontaneamente diabéticos) com 8 semanas de idade foram randomizados para receber uma única injeção intravenosa de PBS ou células *early outgrowth* (EOCs) de doadores db/m ou db/db. Quatro semanas mais tarde, os animais foram sacrificados e os olhos enucleados. Para estudo *in vitro*, o meio condicionado das EOCs (EOC-CM) foi gerado a partir do cultivo de EOCs de animais db/m e db/db. As células rMC-1 foram expostas por 24h a NG ou HG e submetidas ao tratamento com db/m ou db/db EOC-CM, em presença ou não de um inibidor farmacológico (EX527) ou gênico (siRNA) da SIRT1. Nos ratos diabéticos, houve um aumento de marcadores de RD e do dano oxidativo, acompanhado por uma diminuição da proteína SIRT1 e seguido pelo aumento da acetilação da lisina-310 do complexo p65-NFkB. A terapia celular com EOCs reduziu significativamente todas as alterações mencionadas acima. As rMC-1 expostas a HG apresentaram aumento da expressão de GFAP, fator de crescimento do endotélio vascular e Nox4, acompanhado pelo aumento dos níveis de espécies reativas de oxigênio e acetil-lisina-310-p65-NFkB. Além disso, a expressão/atividade da SIRT1 foram reduzidas em ambiente diabético. O tratamento com EOC-CM impediu todas estas alterações. Este estudo demonstra que a capacidade parácrina das EOCs, na secreção de fatores, é eficaz no restabelecimento da via de SIRT1 retina, e assim, proteger a retina dos insultos diabéticos.

Em resumo, a presente tese fornece evidências que tanto a administração oral do cacau enriquecido com polifenóis quanto à terapia celular com EOCs, conferem neuroproteção da retina aos insultos do diabetes. Portanto, intervenções que modulem a atividade das sirtuínas são promissoras no tratamento farmacológico da retinopatia diabética.

ABSTRACT

The diabetic retinopathy (RD) is a devastating disease and the principal cause of blindness among people in adulthood worldwide. The RD is considered a multifactorial and progressive disease, affecting neuronal and glial cells, and also vascular elements of the retina. It is known that several pathways are involved in the pathogenesis of RD, however, the mechanisms that lead to exacerbation of inflammation and death of glial/neuronal cell, characterizing retinal neurodegeneration, remain unknown. Therefore, the reduction of these factors have been extensively studied as a therapeutic target against RD. Sirtuin 1 (SIRT1), a family of histone deacetylase enzyme, acts in response to various stresses and, currently, has been related to important molecular functions in the regulation of various diseases. Considered a redox-sensitive, SIRT1 may be reduced under disease condition, whereby aggravate the pathological situation. However, is not known the mechanism of modulation/activity of SIRT1 in neurodegenerative diseases, such as RD.

In the article I, were studies the possible protective effects of cocoa in the diabetic retina were assessed. rMCs exposed to NG, HG or H_2O_2 were submitted to cocoa treatment in the presence or absence of SIRT-1 inhibitor and siRNA. The experimental animal study was conducted in streptozotocin-induced diabetic rats and randomized to receive low, intermediate, or high polyphenol cocoa treatments via daily gavage for 16 weeks (i.e., 0.12 mg/kg/day, 2.9 mg/kg/day, or 22.9 mg/kg/day of polyphenols). The rMCs exposed to HG or H_2O_2 exhibited increased GFAP and acetyl-RelA/p65 and decreased SIRT1 activity/expression. These effects were cancelled out by cocoa, which decreased ROS production and PARP-1 activity, augmented the intracellular pool of NAD^+, and improved SIRT1 activity. The rat diabetic retinas displayed the early markers of retinopathy accompanied by markedly impaired electroretinogram. The presence of diabetes activated PARP-1 and lowered NAD^+ levels, resulting in SIRT1 impairment. This augmented acetyl RelA/p65 had the effect of upregulated GFAP. Oral administration of polyphenol cocoa restored the above alterations in a dose-dependent manner. This study reveals that cocoa enriched with polyphenol improves the retinal SIRT-1 pathway, thereby protecting the retina from diabetic milieu insult.

In the article II, were investigated the possible therapeutic effect of cells derived from control (db/m) and spontaneously diabetic (db/db) mice on diabetic retinopathy. The db/db mice with 8 weeks of age were randomized to receive a unique intravenous injection of PBS or $0{,}5x10^5$ db/m EOCs or $0{,}5x10^5$ db/db EOCs. Four weeks later, the animals were euthanized and the eyes enucleated. For *in vitro* study, EOC-CM was generated from db/m and db/db EOCs cultures. rMCs were exposed for 24h to NG or HG combined or not with db/m or db/db EOC-CMs. In diabetic rats, there was an increase of DR and oxidative damage markers, accompanied by decrease in SIRT1 protein followed by lysine-310-p65-NFκB acetylation. The treatment with cells from db/m significantly reduced all the above-mentioned, but interestingly the treatment with cells from db/db mice fully restored the above alterations to normal levels. rMCs exposed to HG displayed GFAP and VEGF expression up regulated, accompanied by increase in Nox4 expression and ROS levels, and acetyl-lysine-310-p65-NFκB. SIRT1 protein expression and activity were markedly reduced in diabetic milieu conditions. The treatment with both EOC-CMs prevented all these abnormalities, but db/db EOC-CM fully restored to NG conditions. This study demonstrates that endocrine capacity of EOCs is effective in improving retinal SIRT1 pathway thus protecting the retina from diabetic milieu insult.

In summary, compelling novel evidence is provided herein that either through oral administration of polyphenol enriched cocoa or cell therapy with EOCs, conferred retinal neuroprotection against diabetic insults in animal models. The identification of SIRT-1 as a potential therapeutic target in the treatment of diabetic retinopathy may provide new perspective in the pharmacological treatment of this diabetic complication.

1 - INTRODUÇÃO

1.1 - Retinopatia Diabética

1.1.1 - Definição

Atualmente a retinopatia diabética (RD) é considerada uma doença neurovascular decorrente da hiperglicemia crônica, o que leva à degeneração de neurônios retinianos acompanhada por extensas alterações vasculares. Caracterizada como uma complicação do Diabetes Mellitus (DM), a RD está presente tanto no DM tipo 1 como no DM tipo 2 (Barber, 2004; Zheng e cols., 2007). Portanto, a RD está diretamente associada ao tempo de duração do DM (Klein e cols., 1984) e ao controle glicêmico (DCCT, 1993; UKPDS, 1998).

1.1.2 – Epidemiologia

Estima-se que 7,5% da população mundial sejam acometidas pela DM. No último Congresso da Associação Europeia para o Estudo da Diabetes (EASD, 2014) foi divulgado pela Federação Internacional de Diabetes que atualmente no mundo 382 milhões de pessoas são acometidas pelo DM, alcançando uma proporção epidêmica, o que tem sido um dos desafios de saúde pública do século XXI, principalmente nos países desenvolvidos e emergentes (Figura 1) (INTERNATIONAL DIABETES FEDERATION, 2011).

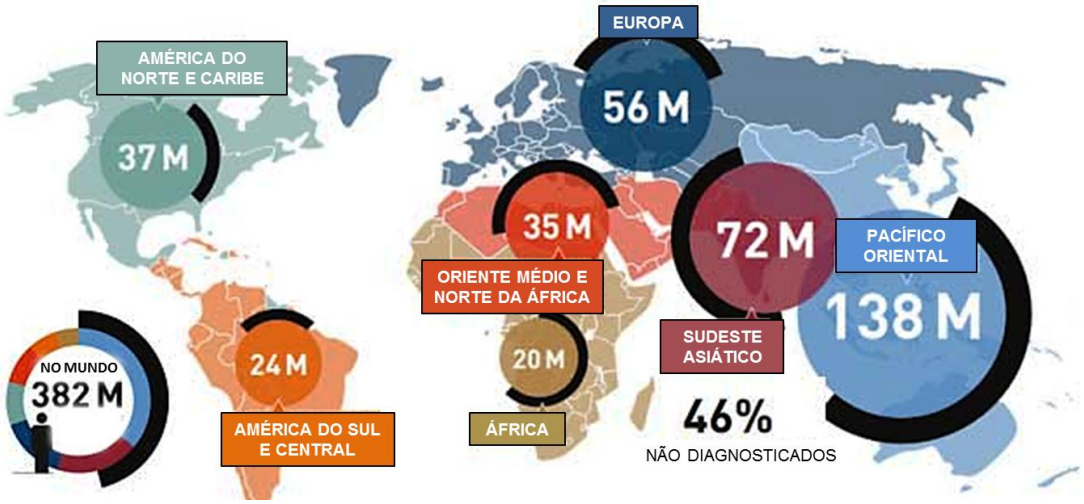

Figura 1. Prevalência de pessoas acometidas pelo DM nos diversos continentes no ano de 2013 (Federação Internacional do Diabetes, 2013).

Aproximadamente 25 a 44% das pessoas com DM desenvolvem alguma forma de RD em algum momento da doença (WESDR, 2009). A RD em algum grau ocorre em cerca de 95% dos pacientes com DM tipo 1 e em mais de 60% dos pacientes com DM tipo 2, prevalência que varia de acordo com o tempo de DM (Figura 2) (Fong e cols., 2004).

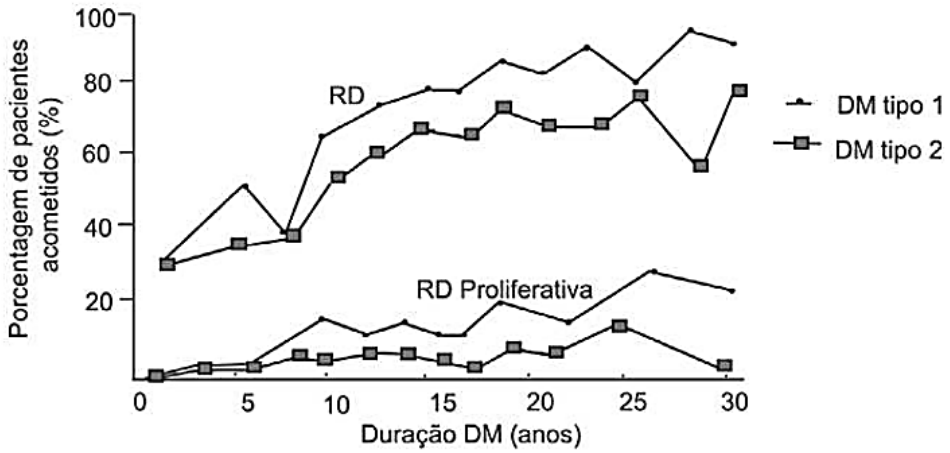

Figura 2. Prevalência de RD e RD proliferativa em pacientes com DM tipos 1 e 2 (ETDRS, 1985).

A RD é a principal causa de cegueira em adultos em idade produtiva em países desenvolvidos. Sua prevalência apresenta uma variação muito grande segundo a literatura, dependendo basicamente da população em estudo, variando de 18% a 40%. Calcula-se que 1 a 3% da população mundial esteja acometida pela doença. Aproximadamente 85% dos casos se manifestam após os 40 anos de idade, sendo apenas 5% abaixo dos 20 anos (Imesch, 1997).

Segundo Hirschi (1997) e Polak. (1997), no continente americano, a RD é responsável por 12% de todos os casos de perda de visão anuais em americanos com idade entre 20 a 74 anos. Em especial no Brasil, as estatísticas são insuficientes, mas provavelmente apresenta incidência semelhante aos países ocidentais.

1.1.3 - Patogênese

A patogênese da RD envolve várias alterações anatômicas da retina, tais como: hemodinâmicas, bioquímicas e endócrinas que culminam em alterações de elementos vasculares e neurais (Fong e cols., 2004). Há uma série de fatores relacionados ao desenvolvimento e progressão da RD, alguns mais fortemente associados com a gravidade da retinopatia, outros menos (Hirschi e D'Amore, 1997).

A RD é caracterizada por alterações neurogliais seguidas por extensivas alterações vasculares. As manifestações vasculares são principalmente caracterizadas por capilares acelulares, perda de pericitos, formação de microaneurismas, espessamento da membrana basal e quebra da barreira hematoretiniana (Cogan e cols., 1961) Por outro lado, a concomitante relação entre os elementos neuronais e gliais permite deduzir que estas alterações possam interferir na homeostase vascular. Há evidências crescentes de que a incapacidade funcional precede as manifestações clínicas mais precoces de doença vascular da retina do diabético (Hyvrinen,1983; Dosso, 1996; Sokol, 1985).

Levantamentos clínicos e experimentais ajudam a elucidar a fisiopatologia da RD. Estudos eletrofisiológicos mostraram em pacientes diabéticos alterações funcionais da retina, no entanto, sem qualquer alteração de fundo de olho através da eletrorretinografia (Puvanendran e

cols., 1983; Lopes de Faria e cols., 2001, Ghirlanda e cols., 1991). Estas evidências caracterizam a RD como uma doença neurodegenerativa, em que a disfunção visual começa no início do diabetes. Portanto, as células neurais e gliais estão envolvidas na cascata de eventos fisiopatogênicos da RD.

A influência do diabetes em células neurais da retina também foi analisada ao nível molecular. Sob condições diabéticas, o aumento de superóxido na retina neural leva a uma diminuição da biodisponibilidade do óxido nítrico, que é originalmente induzida na retina para a proteção do tecido; a diminuição da ação do óxido nítrico aumenta a formação de peroxinitrito, que inibe a sinalização de fatores neuroprotetores e leva à senescência neurocelular (Lambooij e cols., 2000).

Os mecanismos patológicos que levam a déficits neuronais são desconhecidos. No entanto, parece que as células gliais da retina desempenham um papel essencial na manutenção da função normal e integridade da retina, incluindo células de Müller, as principais células gliais da retina (Tretiach e cols. 2005) (Figura 3). As células gliais são importantes na manutenção da barreira retiniana, no revestimento endotelial da microvasculatura retiniana e também no ambiente extracelular iônico auxiliando na função eletrofisiológica de neurônios. Além disso, as células da glia mantêm os níveis sinápticos de neurotransmissores (Gardner e cols, 1997). O principal neurotransmissor excitatório da retina é glutamato, um aminoácido tóxico para os neurónios da retina, quando presente em quantidades elevadas (Romano e cols., 1995). Assim, a regulação do glutamato na retina pelas células gliais é essencial para a função visual (Riepe e Norenburg, 2000). Um estudo anterior, mostrou que na RD experimental a função da glia e o metabolismo do glutamato são alterados na retina de ratos (Lieth e cols., 1998).

A barreira hematoretiniana (BRB) limita a permeabilidade entre os elementos do sangue e do tecido da retina. Esta barreira tem características de permeabilidade particulares e parece desempenhar um papel de grande importância na fisiopatologia da doença da retina (Runkle e Antonetti, 2011). Um marcador precoce bem estabelecido de RD é o colapso da BRB interna, a qual é formada por junções intercelulares endoteliais dos capilares da retina (Qaum e cols., 2001)

e na BRB externa, constituída também por junções em células do epitélio pigmentado da retina (RPE) (Xu e cols., 2011). As junções intercelulares em ambas as células são essenciais para o controle rigoroso do fluxo eletrolítico da retina, bem como para impedir a entrada de moléculas tóxicas e componentes do plasma para o tecido retiniano (Runkle e Antonetti, 2011). Os componentes vasculares da BRB mantém o ambiente apropriado da retina neural, regulando o equilíbrio osmótico, concentração iónica, e o transporte de nutrientes (açúcares, lípideos, e aminoácidos), ajudando assim a controlar o ambiente especializado da retina (Frank, 2004).

As alterações vasculares iniciais na retina diabética incluem leucoestase (Haimovich e cols., 1993), a agregação de plaquetas (Jian e cols., 2001), o fluxo de sangue alterado (Alder e cols., 1998) e a degeneração de pericitos (Cai J e Boulton, 2002). A permeabilidade vascular pode ser afetada pelo transporte paracelular e transcelular; o paracelular envolve as junções endoteliais, e a via intracelular é mediada por endocitose (Simó e cols., 2010; Brankin e cols. 2005; Barber e cols., 2003; Hofman e cols. 2000; Antonetti e cols., 1998; Cunha-Vaz, 1980; Benolken e cols.,1973).

O epitélio pigmentado da retina (RPE) é um epitélio especializado que se encontra entre a retina neural e a camada coriocapilar e desempenha um papel essencial para o bom funcionamento e manutenção da retina neural, permitindo a difusão de oxigênio a partir da circulação coroideia para cones e bastonetes, que controlam o fluxo de solutos e de fluidos, evitando assim a acumulação de fluido extracelular no espaço sub-retinal (Simó e cols., 2010). Assim, a BRB externa desempenha um papel fundamental no transporte e reciclagem de componentes importantes para a função retiniana (Benolken e cols. 1973). A disfunção da BRB induzida pelo diabetes foi claramente identificada em um estudo realizado por Vinores e cols (1989). Neste estudo, os autores localizaram albumina em sítios extravasculares de pacientes diabéticos em várias fases de retinopatia. A presença de albumina extravascular foi detectada nas camadas internas da retina, no entanto, foram observados somente nos estágios mais avançados da RD (Vinores e cols., 1989). Por eletrofisiologia, as alterações morfológicas na RPE podem ser facilmente detectadas em animais diabéticos, que são refletidas por alteração na onda C da

eletrorretinografia (Pautler e Ennis, 1980; MacGregor e Matschinsky, 1986). Apesar dessas observações, a contribuição da disfunção de BRB externa necessita de mais estudos. O desenvolvimento de terapias inovadoras que permitam o transporte de compostos específicos através das barreira da retina depende da compreensão dos mecanismos de funcionamento da BRB no meio diabético.

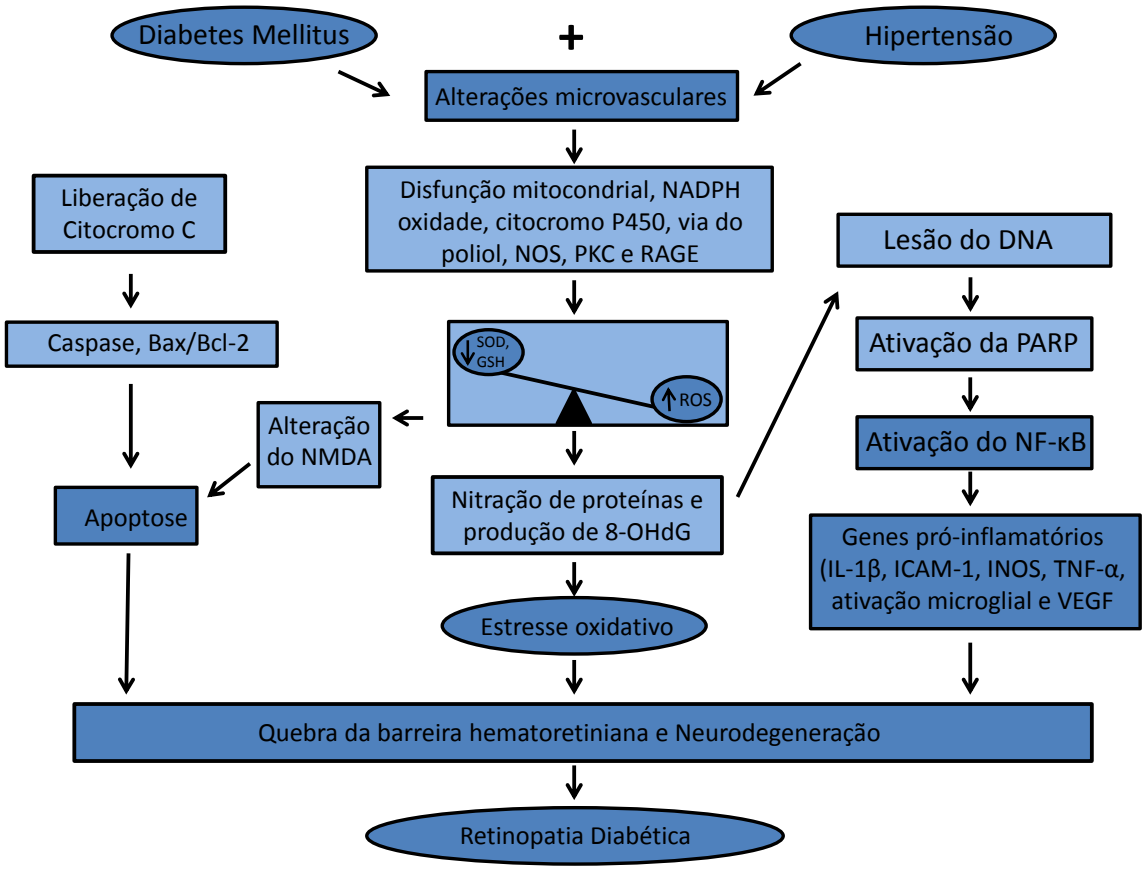

Figura 3. Alterações moleculares da retinopatia diabética (adaptado de Duarte e cols., 2013). Tanto o diabetes como a hipertensão afetam microvasos da retina. Entre as vias bioquímicas são: disfunção mitocondrial, sistema NADPH oxidase, via de poliol e enzima sintetizadora de óxido nítrico. O estresse oxidativo mitocondrial libera o citocromo c, ativando a cascata de caspase e levando à apoptose das células vasculares e neuronais. Além disso, a depleção de glutationa reduzida (GSH) irá prejudicar o ciclo glutamina glutamato, que é o principal mecanismo de citotoxicidade e neurodegeneração da retina. Por outro lado, o stress oxidativo produz nitração de

proteína e os danos do DNA com consequente ativação da via da poli (ADP-ribose) polimerase (PARP), translocação fator nuclear kappa B (NF-κB) e transcrição de vários genes pró-inflamatórios. Como consequência, múltiplas alterações estruturais são detectadas no tecido da retina: capilares acelulares, perda de pericitos e quebra da barreira hematoretiniana (BRB). NMDA: N-Methil-D-aspartato; SOD: superóxido dismutase; ROS: espécies reativas de oxigênio; NO: óxido nítrico; IL-1 β: interleucina-1β; ICAM-1: molécula de adesão intercelular 1; iNOS: óxido nítrico-sintase induzível; TNF-α: fator de necrose tumoral α.

1.1.3.1 – O estresse oxidativo

O estresse oxidativo é definido como o desequilíbrio entre os mecanismos pró-oxidantes e antioxidantes, conceito elaborado pelo pesquisador alemão Helmut Sies. Este sistema é complexo onde, as substâncias consideradas pró-oxidante são capazes de gerar espécies reativas de oxigênio (ROS) e induzir o estresse oxidativo; por outro lado, os antioxidantes previnem a oxidação de substratos, ou seja, evitam a perda de elétrons (Figura 4) (Halliwell e Whiteman, 2004).

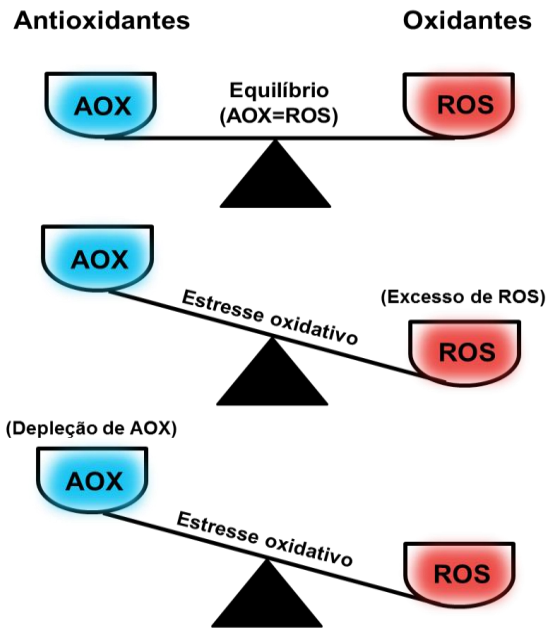

Figura 4. Balança ilustrativa do conceito de estresse oxidativo criada por Helmut Sies e atualmente definido como "um desequilíbrio entre oxidantes e antioxidantes em favor dos oxidantes, conduzindo à lesão celular" (Ohara, 2006).

O estresse oxidativo, consequente da produção exacerbada e/ou pela supressão da ablação de ROS, conduz a lesões celulares e alterações moleculares importantes, como oxidação de biomoléculas, lesão a proteínas e enzimas, mutação e morte celular (Halliwell e Whiteman, 2004). Estas alterações estão implicadas no desenvolvimento de muitas patologias, incluindo a doença de Alzheimer, o diabetes e as suas complicações, tais como a RD (Kowluru e cols., 2007).

A retina é particularmente susceptível ao estresse oxidativo por ter alto consumo de oxigênio, alta proporção de ácidos graxos poliinsaturados e por ser exposta à luz (Jain, 2006). Portanto, o estresse oxidativo é considerado como uma das causas do desenvolvimento e progressão da RD (Kowluru, 2001) com a produção de ROS e/ou diminuição dos sistemas antioxidantes (Budd e cols., 2000). Esse desbalanço oxidativo, leva a inapropriada apoptose, tanto em células vasculares (Danial e Korsmeye, 2004) como em células neurais da retina (Joussen e cols., 2001).

A produção de ânion superóxido na retina se dá em partes pela via mitocôndria, pela NADPH oxidase e pela enzima óxido nítrico sintase (Figura 5). Estudos do nosso grupo demonstraram que a disfunção mitocondrial contribui significativamente na produção de ânion superóxido ($O2^{-}$) na retina (em torno de 60% da produção total) (Silva e cols., 2009). Dados mostram que em condição de diabetes, a NADPH oxidase está ativada e por meio do aumenta da subunidade Nox4 onde ocorre hiperprodução de radicais livres no tecido retiniano (He e cols., 2013). O mesmo tem sido verificado no que se refere a oxido nítrico sintase, onde a hiperativação desta enzima induzida pelo diabetes leva ao estresse oxidativo tecidual da retina (Hao, 2012).

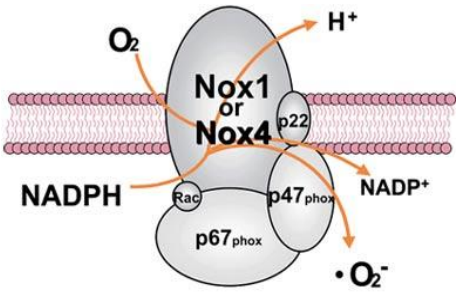

Figura 5. Complexo enzimático NADPH oxidade e a produção de radicais livres (Dusting e cols., 2005).

Juntamente com o aumento de ROS e seus produtos na retina de animais diabéticos, a atividade das enzimas responsáveis pela defesa antioxidante como SOD, glutationa redutase, glutationa peroxidase e catalase também estão diminuídas (Kowluru e cols., 1997; Pinto e cols., 2007). Assim como em modelos animais, pacientes diabéticos também possuem baixos níveis de antioxidantes não enzimáticos como vitamina C, vitamina E e β-caroteno no plasma (Ford e cols., 2003). O aumento de ROS e diminuição na defesa antioxidante, como demonstraram os estudos citados acima, são observados tanto em modelos experimentais, quanto em pacientes diabéticos, o que têm sido associados às complicações microvasculares do DM, incluindo a RD (Dandona e cols., 1996). Portanto o aumento da produção de superóxido associado com diminuição do sistema antioxidante pode estar envolvido no mecanismo das complicações da RD.

Os oxidantes são gerados pelo metabolismo intracelular mitocondrial e peroxissomal, bem como a partir de uma variedade de sistemas de enzimas citosólicas. Além disso, certo número de agentes externos pode desencadear a produção de ROS. Um sofisticado sistema de defesa antioxidante que inclui a catalase, superóxido dismutase (SOD) e da glutationa peroxidase, neutraliza e regula os níveis globais de ROS para manter a homeostasia fisiológica. A diminuição dos radicais livres abaixo dos níveis homeostáticos pode interromper a função fisiológica de oxidantes na proliferação e defesa celular. Da mesma forma, o aumento do ROS também pode ser prejudicial e levar à aceleração no envelhecimento e morte celular. O prejuízo causado pelo aumento de ROS são os danos às proteínas, lipídios e DNA. Além desses efeitos, pode ocorrer a sinalização de vias redox-sensíveis específicas. Uma vez ativadas, essas vias podem ter diversas funções prejudiciais ou potencialmente protetoras (Figura 6).

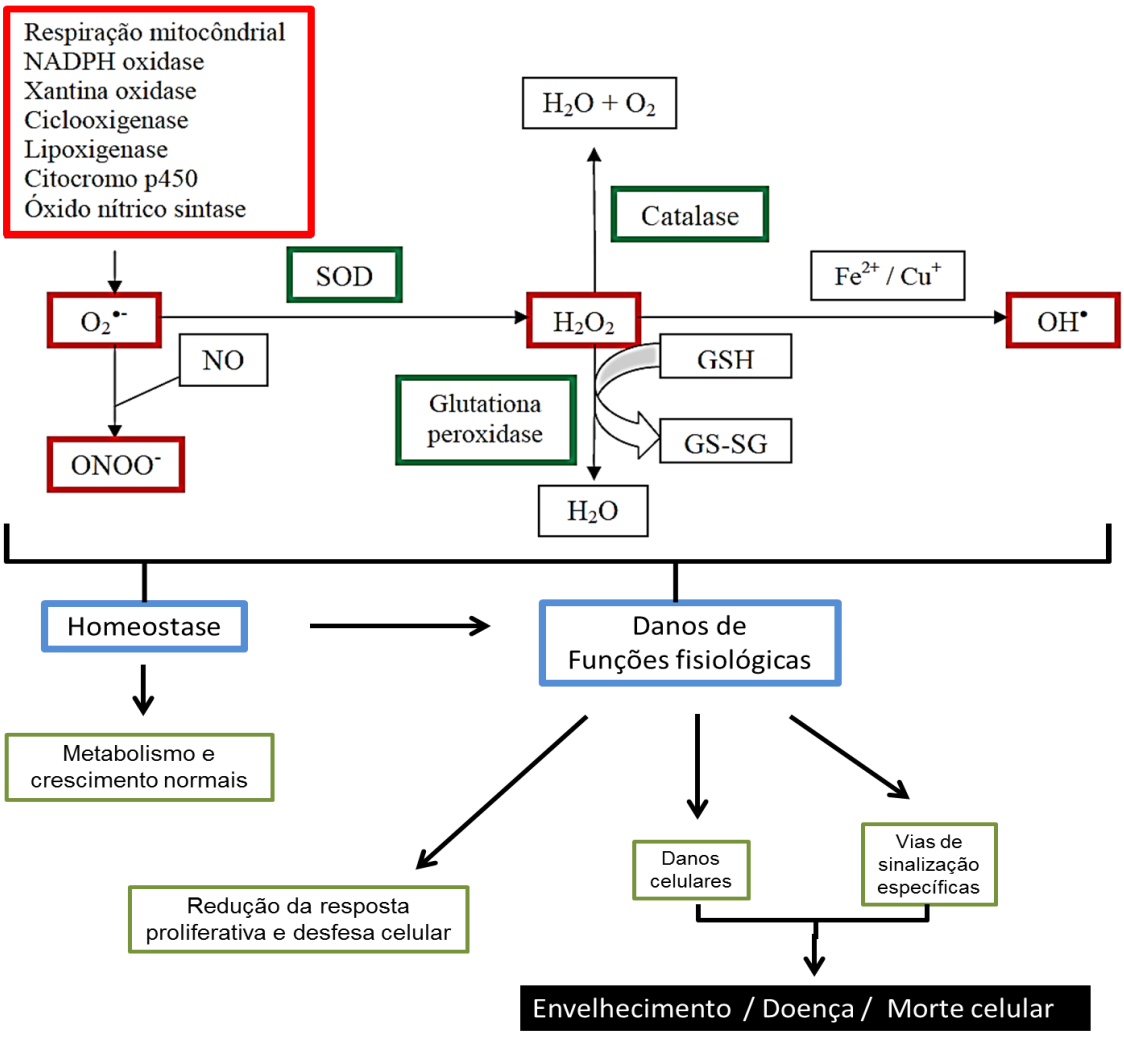

Figura 6. As fontes e as respostas celulares a espécies reativas de oxigênio (adaptado de Finkel e Holbrook, 2000 e Schnachenberg, 2002). superóxido ($O_2^{\bullet-}$), óxido nítrico (NO), peroxinitrito ($ONOO^-$), peróxido de hidrogênio (H_2O_2), ferro (Fe^{2+}), cobre (Cu^{+}), radicais livres hidroxila ($OH^{\bullet}$), glutationa reduzida (GSH), glutationa oxidada.

Estudos do tecido retiniano diabético mostram que o H_2O_2 é instantaneamente convertido via catalase e glutationa peroxidase gerando OH-, o que leva à rápida interação com gorduras insaturadas causando peroxidação lipídica e oxidação do DNA (indicada pela presença do 8-hidrox-2`-deoxiguanosina (8-OHdG) (Sullivan e cols., 2000). Níveis elevados de peroxinitrito também foram encontrados elevados na retina no DM de curta duração e

permanecem elevados até 14 meses de DM em ratos (Kowluru e cols., 2001). Dados obtidos pelo nosso grupo de pesquisa demonstraram que em ratos experimentalmente diabéticos ocorre um aumento da produção de superóxido e uma significativa diminuição nos níveis de glutationa reduzida na retina em comparação com os animais controles. Estes eventos culminaram na toxicidade tecidual caracterizada pela nitração de tirosina e da lesão do DNA, demonstrando o prejuízo do sistema antioxidante nos animais diabéticos (Pinto e cols., 2007). Os animais tratados com um antioxidante natural contendo polifenóis, o composto preveniu a nitração de proteínas e evitou a lesão do DNA, melhorando assim marcadores precoces de RD (Silva e cols., 2013). Esses dados sugerem que a restauração do desbalanço oxidativo pode ser um alvo terapêutico no combate a insultos da retina pelo diabetes.

1.1.3.2 – Poli (ADP-ribose) polimerase (PARP)

Poli ADP-ribose polimerases (PARPs) são enzimas nucleares que catalisam a transferência de múltiplos grupos ADP-ribose da nicotinamida adenina dinucleotídeo (NAD) para as proteínas alvo. Esta transferência é considerada uma modificação pós-tradução que recebe o nome de poli (ADPribosil)ação (Singh e cols., 2014). A família da PARP é composta por 17 membros e cada um deles possui diferentes funções e estruturas na célula. Além da regulação de sobrevivência e morte programada das células, a PARP está associada a outras funções biológicas como a regulação de transcrição, coesão do telômero e formação do fuso mitótico durante a divisão celular, tráfico intracelular e metabolismo energético (Schreiber e cols., 2006).

A isoforma mais caracterizada e estudada é a PARP-1, responsável pela organização espacial e temporal do reparo do DNA. Em resposta à fragmentação do DNA, ela transfere unidades de ADP ribose do NAD^+ para as proteínas nucleares. O NAD^+ é utilizado como substrato para catalisar a ligação covalente das unidades de ADP-ribose do grupo carboxílico dos resíduos do aminoácido Glu de proteínas receptoras que normalmente estão associadas com transações do DNA (heteromodificaçao) ou da própria PARP-1 (automodificaçao). O polímero resultante da ADP-ribose (PAR) pode interagir de forma seletiva, com um número de alvos de

proteínas que estão envolvidas na resposta celular aos danos no DNA. Estes alvos contêm ligações de PAR que muitas vezes confundem-se com um domínio funcional, tais como uma proteína ou um domínio ligado ao DNA e isso explica como ligações de PAR podem alterar as propriedades funcionais dos alvos (Figura 7) (Pleschke e cols., 2000).

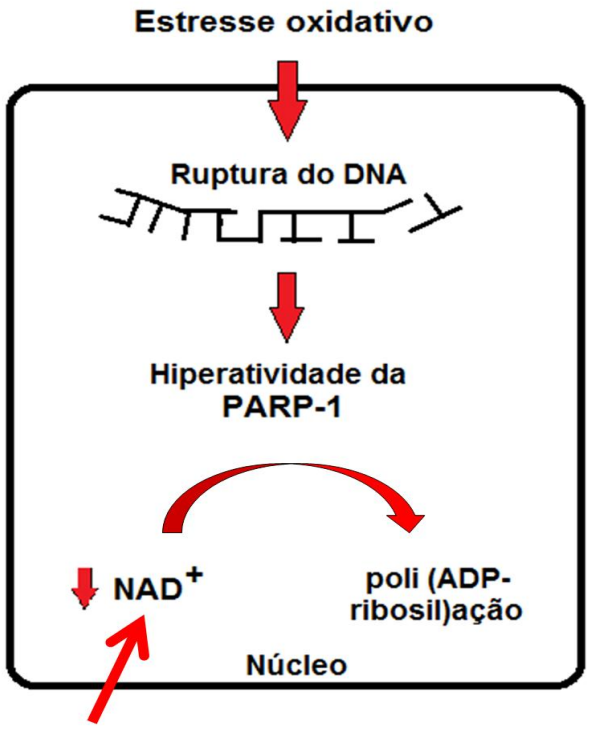

Figura 7. Mecanismo da PARP1 (adaptado de Pellegrini-Giampietro, 2014). Em condição de estresse oxidativo ocorre lesão do DNA, como mecanismo adaptativo, a PARP1 é hiperativada, que por meio da NAD^+ produz poli(ADPribosil)ação a fim de reparar o DNA lesado.

A PARP-1 é também conhecida por desempenhar o papel fundamental em vários distúrbios agudos e crônicos de inflamação (Mabley e cols., 2001), doenças cardiovasculares, câncer e diabetes mellitus (Schreiber e cols., 2006). Em algumas situações, ela funciona como um importante co-ativador de fatores de transcrição como o NF-κB resultando na síntese de mediadores pró-inflamatórios (Hassa e cols., 2003). Entre eles, está a iNOS, que produz óxido

nítrico e, posteriormente, peroxinitritos e radicais hidroxila altamente reativos que, por sua vez, causam danos no material genético na célula-alvo. Em seguida, o resultado da ativação da PARP-1 irá induzir a morte celular independente da caspase, a qual envolve a liberação da proteína indutora de apoptose (AIF), flavoproteína, da mitocôndria (Yu e cols., 2002). Como as caspases não estão envolvidas neste processo, a PARP-1 se mantém intacta para ser ativada pelo DNA fragmentado, o que resulta na síntese excessiva de PAR, esgotamento de NAD^+ e ATP e, finalmente, morte celular.

O desenvolvimento das complicações do DM está associado ao aumento do estresse oxidativo e nitrosativo o que pode danificar o DNA levando à ativação da PARP (Obrosova e cols., 2005; Rosales e cols. 2010). A superativação desta enzima exacerba o estado oxidativo da célula através do seu consumo de NAD. Ela está associada a doenças relacionadas ao diabetes como disfunção endotelial e do miocárdio (Garcia Soriano e cols., 2001; Pacher e cols., 2002), neuropatia autonômica e periférica (Obrosova e cols., 2008) e nefropatia (Szabo e cols., 2006).

O papel da PARP nas complicações oculares dos diabéticos merece uma avaliação profunda, considerando que a PARP-1 é expressa abundantemente no cristalino (Tamada e cols., 2000) e na retina (Obrosova e cols., 2006). Sua ativação contribui para a formação de pericitos fantasmas e capilares acelulares, (Zheng e cols., 2004), aumento da adesão de leucócitos nas células endoteliais (Sugawara e cols., 2004) e produção de VEGF (Obrosova e cols., 2004) e angiogênese (Tentori e cols., 2007). Na RD, dentre as características iniciais, observa-se o aumento da produção de fatores vasoativos, como a endotelina 1 (ET-1) e o aumento da síntese de proteínas de matriz extracelular (ECM) como a fibronectina (FN) (Xu e cols., 2008). Este aumento de fatores vasoativos pode ser explicado pela ativação do NF-κB pela PARP (Hassa e cols., 2003). PARP modula a atividade NF-κB através da histona acetiltransferase (HAT). A p300 desempenha papel fundamental na diferenciação e na regulação de diversos fatores de transcrição (Goodman e Smolik, 2000). A expressão de mRNA da p300 é significativamente reduzida em células de murinos deficientes de PARP (Hassa e cols., 2003). A alta concentração de glicose faz com que a atividade da p300 aumente nas células endoteliais, no coração e na retina de animais diabéticos (Kaur e cols., 2006). A ativação epigenética do p300 pode afetar vários fatores de

transcrição. Foi descrito também que a ativação da PARP está implicada na manifestação da ativação glial (acúmulo de GFAP), apoptose na retina neural (Drel e cols., 2009) e formação de catarata em ratos diabéticos (Drel e cols., 2009).

Estudos farmacológicos têm demonstrado os benefícios terapêuticos de várias classes de inibidores de PARP em diferentes modelos experimentais de inflamação, doenças neurodegenerativas e vasculares (Jagtap e Szabo, 2005; Rosales MA e cols. 2010). No entanto, estudos são necessários a fim de verificar a eficácia deste tratamento em humanos.

1.1.3.3 – Fator nuclear kappa B (NF-κB)

O NF-κB é um fator de transcrição nuclear heterodímero constituído de duas subunidades: p65 (também chamada RelA) e p50 (Baeuerle e Baltimore, 1996; Glezera e cols., 2000). O NF-κB é um fator nuclear que possui a capacidade de ligar-se a uma sequência de 10 pares de bases na região promotora do gene que codifica a cadeia leve k, que está envolvida na transcrição de importantes citocinas pró-inflamatórias envolvidas no câncer e doenças neurodegenerativas tais como o Alzheimer e a RD (Sen e Baltimore, 1986).

O NF-κB é descrito em diversas células que compõem os organismos complexos e apresenta uma gama de ações superiores a todos os fatores de transcrição até então caracterizados. Isso se dá devido aos estímulos de modulação deste fator, como os inúmeros genes por ele regulado. Dentre esses estímulos estão os neurotransmissores (tais como o glutamato), neurotrofinas, proteínas neurotóxicas (como a b-amilóide), VEGF, citocinas (interleucina-1 e fator de necrose tumoral), glicocorticóides, ésteres de forbol, peptídeo natriurético atrial e produtos de reações de enzimas como a óxido nítrico sintase induzível. Independente do estímulo parece haver a participação de espécies reativas de oxigênio e o aumento de cálcio intracelular para a ativação do NF-κB. Estas funções foram caracterizadas principalmente em células neurais e gliais (O'Neill e Kaltschmidt, 1997).

A de ativação da via do NF-κB inicia-se no citoplasma pela fosforilação e a degradação do IκB (proteína inibitória que impede a translocação do NF-κB para o núcleo) (Sen e Baltimore, 1986; Baldwin, 1996). Vários estímulos levam à fosforilação do IκB, que posteriormente recebe a adição de ubiquitina, pela ação da ubiquitina ligase, sendo em seguida degradada pelo complexo proteossoma 26S (Siebenlist, 1997). O desmembramento do complexo IκB/NF-κB permite a translocação p65-NF-κB para o núcleo (Baldwin, 1996). Os primeiros estudos sobre a transcrição do NF-κB mostraram que apenas a translocação deste fator para o núcleo era capaz de ativar a transcrição, no entanto dados atuais mostram que no núcleo, o NF-κB é acetilado (Ito e cols., 2001), o que permite a ligação em genes específico da cromatina que apresentam a sequência regulatória GGGACTTTCC junto à região promotora, levando a um aumento na expressão do gene alvo (Figura 8) (Hiroi e cols., 2003).

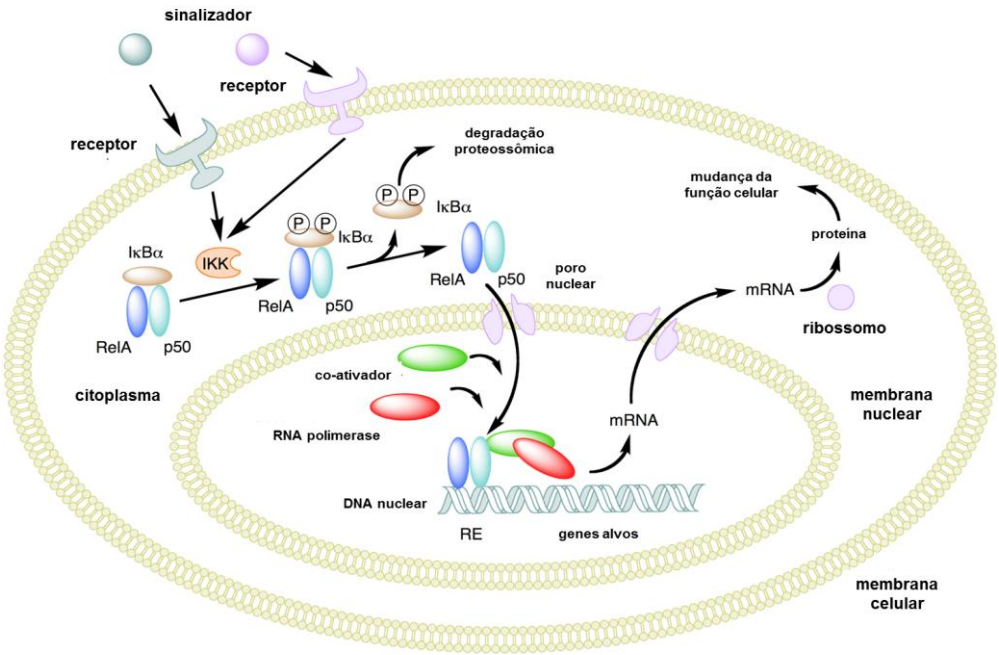

Figura 8. Sinalização do NF-κB (adaptado de Gilmore TD, 2006). Sob estímulos distintos via receptor, a cascata do NF-κB iniciase no citoplasma pela fosforilação e a degradação do I*κ*B. Posteriormente, o complexo p65-NF-κB é translocado para o núcleo e por meio de co-ativadores a transcrição é ativada, o que permite a ligação junto à região promotora, levando a um aumento na expressão do gene alvo.

A acetilação do NF-κB ocorre na subunidade p65 pela ação de proteínas acetiltrasferases, como p300/CBP. O principal alvo da acetilação do p65 são as lisinas 218, 221 e 310, o que regula a atividade transcricional do NF-κB. A acetilação da lisina 218 e 221 aumenta a ligação no DNA e prejudica a ação do IκB, enquanto que a acetilação da lisina 310 é necessária a todas as atividades transcricionais do complexo RelA/NF-κB. Por sua vez, o RelA pode ser desacetilado por histonas desacetilases. Portanto, o mecanismo de acetilação/desacetilação serve como um interruptor molecular intranuclear para promover ambos os efeitos regulatórios positivos e negativos sobre a ação transcricional do NF-κB (Figura 9) (Greene e Chen, 2004).

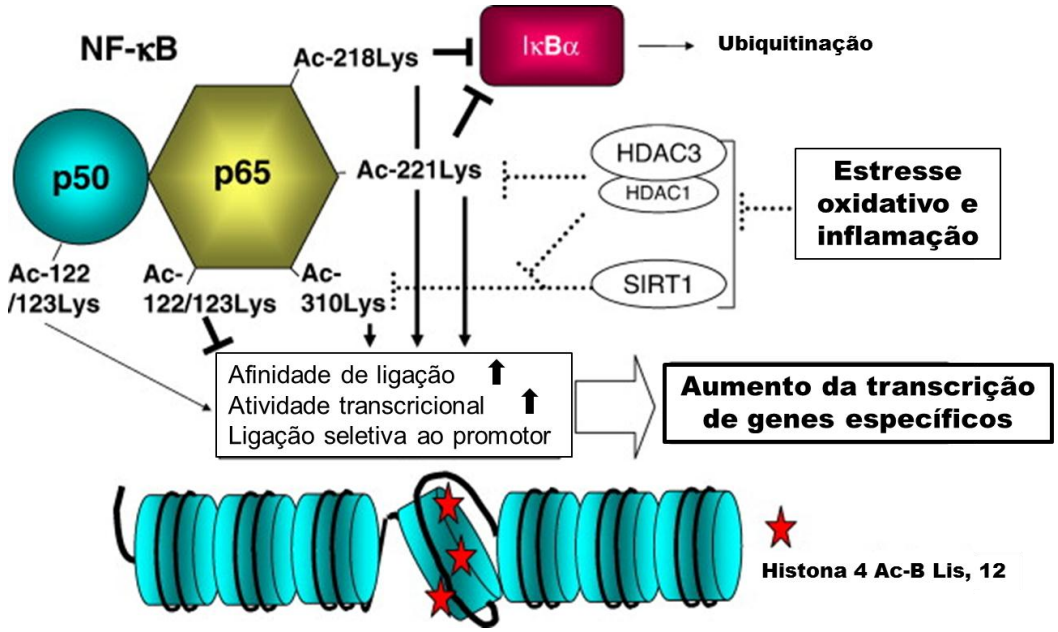

Figura 9. Sítios de modificação e modulação do NF-κB. Lisinas tais como, 122 e 123 do p50 e 218, 221 e 310 do p65, quando acetiladas, levam ao aumento da transcrição gênica. Ao mesmo tempo, histonas desacetilases como a SIRT1 podem modular estes sítios de modificação (Greene e Chen, 2004).

O desenvolvimento das complicações do DM está associado ao aumento de NF-κB, que leva à produção e secreção de citocinas pró-inflamatórias e consequentemente migração e infiltração de macrófagos no tecido alvo (Moura e cols., 2014). Na RD, este processo gera inflamação exacerbada levando à progressão da doença. O NF-κB é sensível ao estresse oxidativo e responsável pela transcrição de importantes genes pró inflamatórios no tecido retiniano, principalmente na micróglia e em células de Müller da retina (Duarte e cols, 2013). Estudos mostram o aumento da fosforilação do complexo p65 no tecido retiniano e consequentemente hiperexpressão de VEGF, das molécula de adesão vascular (VCAM-1) e intracelular-1 (ICAM-1) e das proteínas pró-inflamatórias dependentes do NF-κB (Kaštelan e cols, 2013). Ambas as moléculas, VCAM-1 e ICAM-1, promoverem a quimiotaxia de leucócitos para as paredes vasculares e a sua migração para tecidos da retina (Chibber e cols., 2007; Joussen e cols., 2002), enquanto que o VEGF, um mediador inflamatório, aumenta a permeabilidade vascular da retina interna (Adamis, 2002; Zhang e cols., 2011).

Estas alterações, na fase precoce da RD, estão fortemente relacionadas à reatividade de células gliais, caracterizada por aumento da expressão de GFAP (Adamis AP, 2002) e morte de células neurais por apoptose (Jung KI e cols. 2013). Posteriormente, os fatores acima descritos comprometem na barreira hematoretiniana (Chibber e cols., 2007; Joussen e cols., 2002).

1.2 – SIRT1

1.2.1 - Função desacetilase da SIRT1

As Sirtuínas (SIRTs, *silent information regulator*) são da classe das histonas desacetilases que atuam em resposta a vários processos fisiopatológicos, tais como inflamação, senescência celular, apoptose, metabolismo e regulação do ciclo celular. Existem 7 tipos de sirtuínas em mamíferos (1-7), sendo que cada uma é caracterizada pela localização e função celular (North e cols., 2004). A principal e mais estudada é a Sirtuína 1, que se encontra

principalmente no núcleo e está envolvida na regulação de diversos processo fisiológicos/metabólicos celulares (Tabela 1).

Tabela 1. Família das Sirtuínas, classe das histonas desacetilases.

Classe	Subclasse	Espécie		Localização	Função
		Camundongo	Humano		
I	A	*Sirt1*	*SIRT1*	Núcleo e citoplasma	Metabolismo e inflamação
	B	*Sirt2*	*SIRT2*	Citoplasma	Ciclo celular e tumorigênese
		Sirt3	*SIRT3*	Mitocôndria	Metabolismo
II		*Sirt4*	*SIRT4*	Mitocôndria	Secreção de insulina
III		*Sirt5*	*SIRT5*	Mitocôndria	Detoxificação da amônia
V	A	*Sirt6*	*SIRT6*	Núcleo	Reparo do DNA, metabolismo e secreção TNF
	B	*Sirt7*	*SIRT7*	Núcleo	rDNA e transcrição

A SIRT1 foi inicialmente verificada como um promotor de longevidade associado à restrição calórica. No entanto, dados recentes mostram que a SIRT1 está envolvida na regulação de vários processos fisiopatológicos em diferentes órgãos (Lavu e cols., 2008). Além disso, a SIRT-1 tem sido relacionada a importantes funções moleculares na regulação de várias doenças como Alzheimer e doença pulmonar obstrutiva crônica, bem como longevidade (Figura 10) (Elliott e cols., 2008; Michan e cols., 2007; Westphal e cols., 2007).

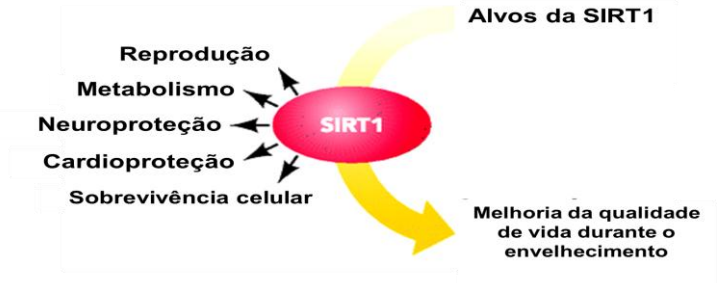

Figura 10. Atuação da SIRT1 frente aos mecanismos fisiológicos celulares (adaptado de The Life, 2014).

A atividade enzimática da SIRT1 é caracterizada por ser dependente da NAD^+, que atua como cofator da SIRT1 (Bonda e cols., 2011). No resíduo de lisina em proteínas alvo, a SIRT1 induz hidrólise de NAD^+ gerando nicotinamida e metabólito (Figura 11) (Guarente, 2000).

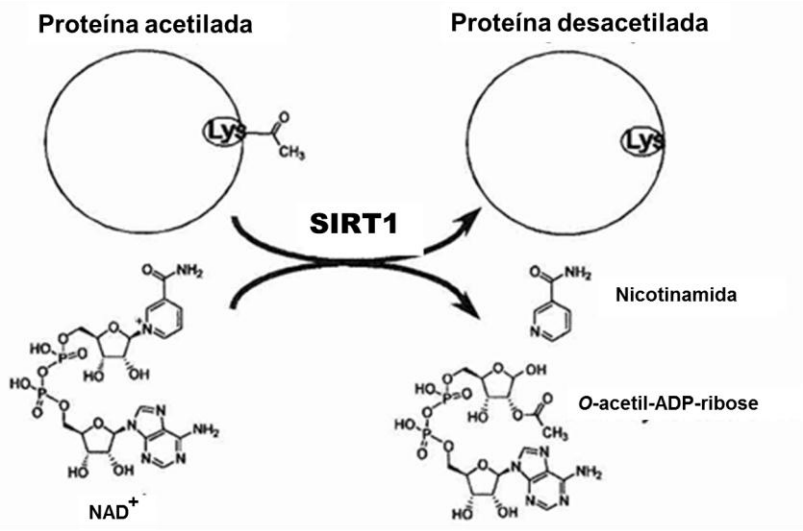

Figura 11. Atividade de desacetilação da SIRT1. O processo enzimático da SIRT1 se dá com o auxílio do cofator NAD^+, onde SIRT1 + NAD^+ + substrato acetilado => SIRT1 + Nicotinamida + *O*-acetil-ADP-ribose + substrato desacetilado (Guarente, 2000).

A SIRT1 atua em alguns locus de cromatina desacetilando lisinas de histonas (Michan, 2007; Zhang e Kraus, 2009). Além disso, pode suprimir a inflamação através da desacetilação da p65 na subunidade do NF-κB (Yeung cols., 2004), reduzir a apoptose através da desacetilação da p53, Ku70, e FOXO3a (Bi B cols., 2007; Brunet e cols, 2004; Luo e cols., 2001), e inibir a senescência através da desacetilação de FOXO3a, FOXO4, e p53 (Michan e cols., 2007; Salminen e cols., 2008), que são características de várias doenças inflamatórias crônicas, assim como a RD (Figura 12).

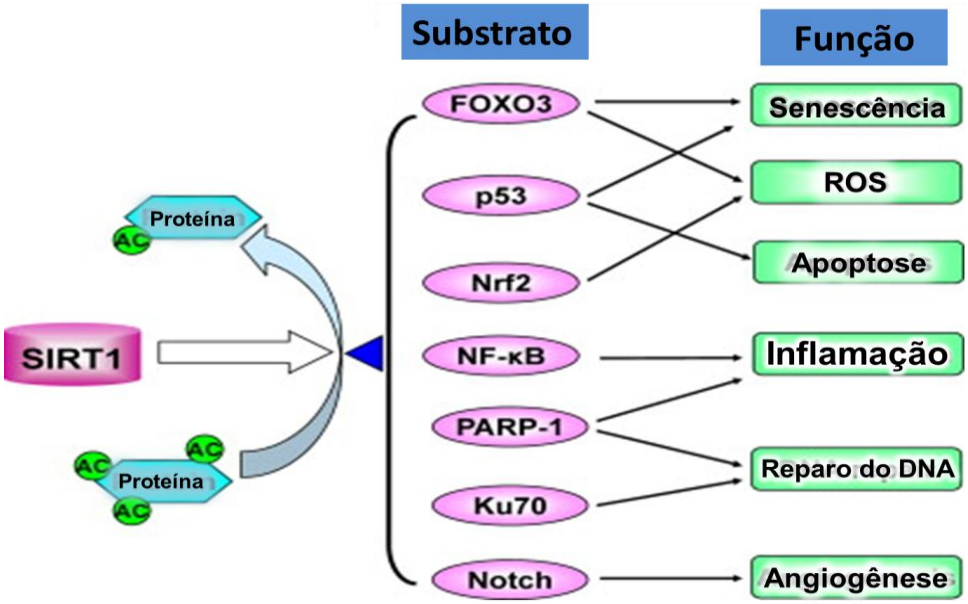

Figura 12. Alvos e funções moduladas pela via da SIRT1 (Yao H e Rahman I, 2014).

1.2.2- A modulação da SIRT1 em resposta a estímulos oxidantes

Estudos mostram que o estresse oxidativo pode modular a atividade e expressão de algumas proteínas importantes no sistema celular (Caito e cols., 2010; Grimsrud e cols., 2008; Ghezzi e Bonetto, 2003). Desta forma, a regulação da SIRT1 por espécies reativas de oxigênio tem sido amplamente investigada. A modulação da SIRT1 é complexa, isso porque esta enzima depende de diversos processos redox intracelulares (Bordone e cols., 2005). No entanto, sabe-se que o estresse oxidativo tem um papel importante da modulação da expressão e atividade de desacetilação desta enzima (Braidy e cols., 2011).

1.2.2.1 - SIRT1 e a via da PARP1

Inúmeros processos biológicos celulares compartilham de moléculas, a SIRT1 é uma delas. Em resposta ao estresse oxidativo a PARP1 é ativada e a NAD^+ é convertida em poli

(ADP-ribose) como mecanismo de reparação do DNA. Tanto a via da SIRT1 como a via da PARP1 utilizam de NAD^+ como substrato, devido a isso, a via da PARP1 tem grande influência na modulação da atividade da SIRT1 (Figura 13) (Kolthur e cols., 2006).

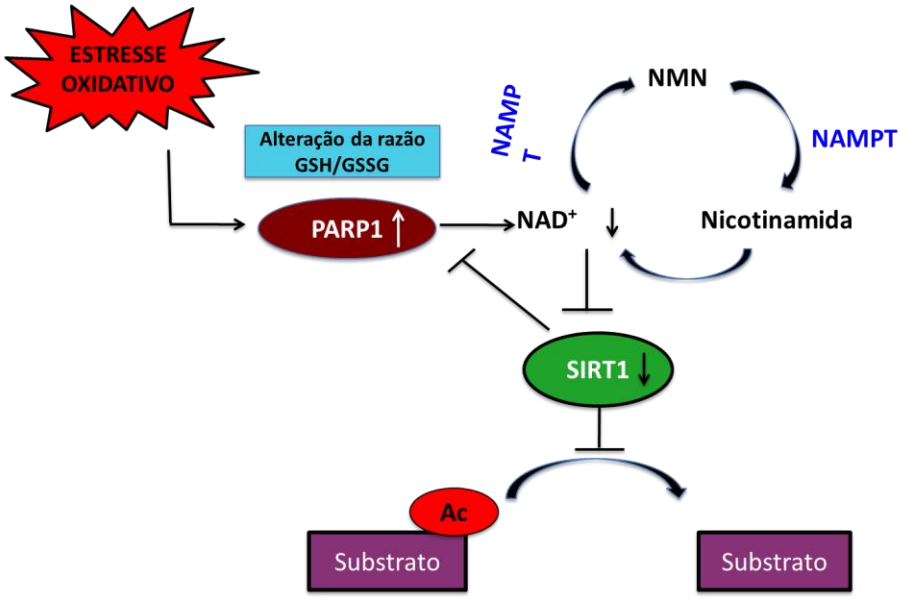

Figura 13. SIRT1 e a via da PARP1. O estresse oxidativo leva na ativação da PARP1 e subsequente depleção da NAD^+, onde a via de regeneração não é capaz de reverter a razão de NAD^+/NADH causando subsequente inativação da atividade desacetilase da SIRT1. Ativação PARP1 também é regulada pelos níveis intracelulares de GSH/GSSG. Além disso, a SIRT1 também é capaz de regular a atividade PARP1 via desacetilação. NMN, Mononucleotídeo de Nicotinamida; NAMPT, nicotinamida fosforibosiltransferase; NMNAT, Ácido nicotínico mononucleotídeo adeniltransferase; PARP1, poli(ADP-ribose) polymerase-1. (Hwang e cols., 2013).

A inibição da SIRT1 pela ativação da via da PARP1 é devido a redução da razão intracelular NAD^+/NADH pelo consumo da NAD^+; este fato tem sido demonstrado em resposta à H_2O_2 em modelo de senescência celular (Furukawa e cols., 2007). No entanto, a exata modulação da via da SIRT1 pela ativação da PARP1 frente a estímulos oxidativos ainda precisa ser estudada, principalmente no que diz respeito a doenças neurodegenerativas como a RD, onde a PARP1 está hiperativa (Hwang e cols., 2013).

Sabe-se que o fumo de cigarro induz a ativação da PARP1 e reduz os níveis de SIRT1 provavelmente devido à depleção intracelular de NAD^+. Um agente anti-inflamatório derivado da metilxantina protege as células pulmonares de camundongos expostos ao fumo contra o esgotamento de NAD^+ via inibição da PARP1, nesta ocasião a atividade da SIRT1 foi restabelecida (Moonen e cols., 2005; Weseler e cols., 2009). Este fenômeno implica o papel de PARP1 na inflamação pulmonar e senescência (Hwang e cols., 2010). Portanto, qualquer alteração nos níveis da atividade da PARP1 e/ou níveis intracelulares de NAD^+, particularmente em resposta a estímulos ambientais oxidantes, pode influenciar a atividade da SIRT1.

1.2.2.2 - Modificação pós-tradução da SIRT1

Dados atuais mostram que a enzima SIRT1 possui 747 aminoácidos, onde formam três regiões: 1) o domínio central, sítio ativo de desacetilação; 2) domínio N-terminal e; 3) domínio C-terminal (Hwang e cols., 2013). Sabe-se que ambos os domínios N- e C- terminal da SIRT1 podem sofrer modificações pós-tradução (Zee e cols. 2010). O domínio catalítico compreende 250 aminoácidos e é altamente conservado entre as espécies. Estes domínios são constituídos por um sítio de ligação ao substrato e um sítio de ligação ao NAD^+ (Sauve e cols., 2006). A região N e C terminais contém elementos reguladores dos domínios de ligação de ativadores da SIRT1. Estudos mostram que a SIRT1 contém 15 sítios de fosforilação (Sasaki e cols, 2008; Zschoernig e cols., 2009 ; Olsen e cols.,2006; Beausoleil e cols., 2004), um de SUMOilação (Yang e cols., 2007), e dois de S-nitrosilação (Kornberg e cols., 2010). Outras modificações pós-tradução, tais como a carbonilação e S-glutationilação são descritas, no entanto, ainda necessários estudos para caracterização da região específica da proteína que ocorre esta modificação (Hwang e cols., 2013).

Estas alterações, provenientes de modificações reversíveis e irreversíveis de proteínas são formadas como resultado de oxidação direta de algumas cadeias de aminoácidos, tais como resíduos de histidina, cisteínas ou lisina. Modificações como formações de pontes disulfeto, S-glutationilação e S-nitrosilação, podem facilmente ser revertidas por algumas enzimas, tais como

tiorredoxina e glutaredoxina (Caito e cols., 2010). No entanto, modificações geradas por carbonização e nitração levam à mudança conformacional da estrutura molecular, inativando e levando à degradação da proteína (Figura 14) (Caito e cols., 2010).

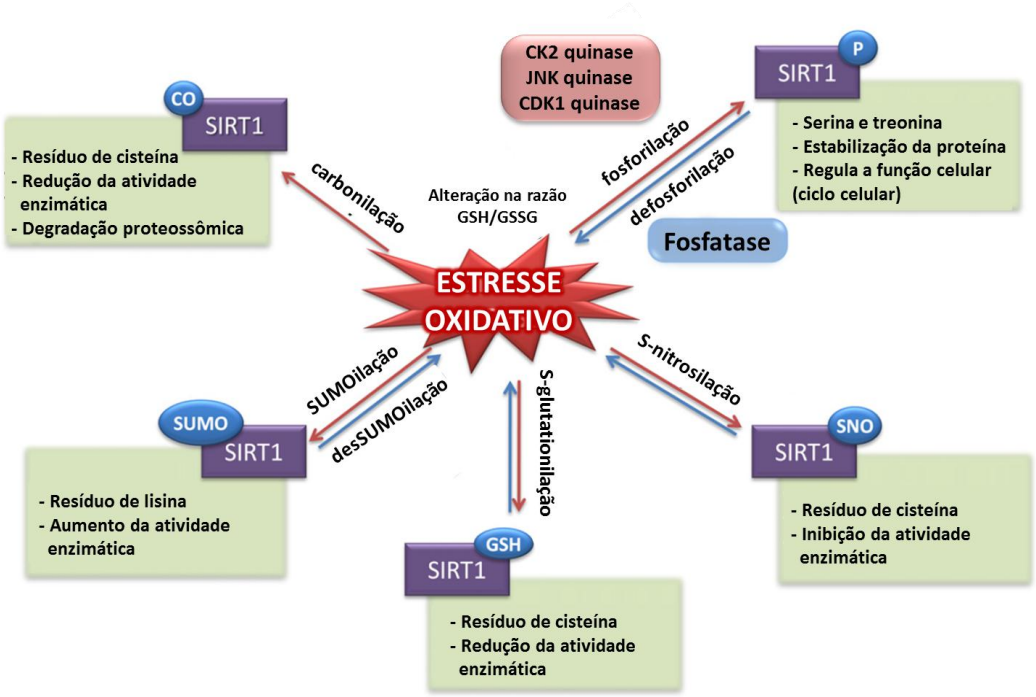

Figura 14. Modificações pós-tradução da SIRT1 (adaptado de Hwang, 2013). Em resposta ao estresse oxidativo, SIRT1 sofre modificações pós-traducionais, tais como fosforilação (P), S-nitrosilação (SNO) S-glutationilação (GSH), carbonilação (CO) e SUMOilação (SUMO). Atividade SIRT1 desacetilase e respostas celulares subsequentes são afetados por estas modificações. Alterações da razão GSH/GSSG intracelulares também pode afetar essas modificações.

A fosforilação da SIRT1 pode ocorrer em vários locais de modificação, das quais sete são localizadas no interior do terminal N e oito no C-terminal e podem modular a atividade desta enzima. A fosforilação da serina 27 aumenta a estabilidade da SIRT1 (Ford e cols, 2008). Da mesma forma, quando a treonina 530 ganha um grupo fosfato, promove a atividade enzimática da SIRT1, enquanto que nas serinas 154, 649, 651, 683 aumenta a afinidade ao substrato. Este efeito foi verificado em células epiteliais em modelo de estresse oxidativo (Caito e cols, 2010).

O SUMO (*small ubiquitin-related modifier proteins*) é uma pequena proteína que tem o efeito oposto da ubiquitina. A ubiquitinação é um processo de modificação que leva a degradação de proteínas, enquanto que SUMOilação induzir a estabilidade de proteínas (Herrmann e cols, 2007). Ambos SUMOilação e ubiquitinação podem ocorrer sob condições de estresse oxidativo, e esses efeitos são regulados pelos níveis de importantes tióis intracelulares. Quando SIRT1 é SUMOilada na lisina 734, sua atividade de desacetilação é aumentada. Interessante que em resposta ao estresse e dano ao DNA, a SUMOilação da SIRT1 apresenta-se reduzida (Yang e cols., 2007).

SIRT1 é uma molécula redox-sensível, portanto tióis intracelulares podem regular o nível de atividade da SIRT1. O tratamento com butionina sulfoximina, um inibidor da biossíntese de GSH, diminuiu ainda mais os níveis de SIRT1 em resposta ao estresse oxidativo, enquanto que o aumento seletivo de tióis intracelulares por GSH monoetilico restabelece a SIRT1 em um modelo de estresse induzido pelo tabaco em células epiteliais do pulmão de ratos (Hwang e cols., 2013). O estudo de Zee (2010) demonstrou que a SIRT1 é alvo da S-glutationilação em resíduos de cisteína (Cis67, Cis268, e Cis623).

A S-nitrosilação é uma ligação covalente de um grupo de óxido nítrico na cisteína tiol/sulfidrilo, o que leva à formação de um derivado de S-nitrosotiol (Shahani e cols, 2011). Kornberg (2010) demonstrou que gliceraldeído-3-fosfato nitrosilado (GAPDH) tem a capacidade de nitrosilar a SIRT1 na cisteína 387 e 390 e inibir a atividade enzimática (Kang e cols., 2011). No entanto, mais estudos são necessários para elucidar este processo.

A carbonilação é uma ligação covalente de grupos carbonilas que ocorre em resíduos de cisteína da SIRT1 resultante de reações oxidativas, que tem como consequência a inativação enzimática e degradação proteossômica da SIRT1. Em modelo *in vivo*, camundongos expostos à fumaça de cigarro apresentaram modificações pós-tradução da SIRT1 por carbonilação e glutationilação. Estas modificações foram atenuadas pelo tratamento antioxidante com N-acetilcisteína (NAC) (Caito e cols., 2010).

1.2.3 - A sinalização da SIRT1 na inflamação via RelA/p65-NF-κB

O NF-κB é um fator óxido sensível e responsável pela transcrição de citocinas pró-inflamatórias, tais como interleucinas (IL), fator de necrose tumoral (TNF-α) e VEGF (Toledano e cols., 1991). Tanto em condições fisiológicas como patológicas, o NF-κB é modulado por diversas modificações pós-tradução. Nesse contexto, recentes estudos mostraram que a forma ativa do NF-κB é caracterizada no núcleo mediante acetilação da lisina 310 na subunidade RelA/p65. Por outro lado, podem ocorrer estímulos de inativação, dirigidos pela SIRT1 (Figura 15) (Chen, 2005).

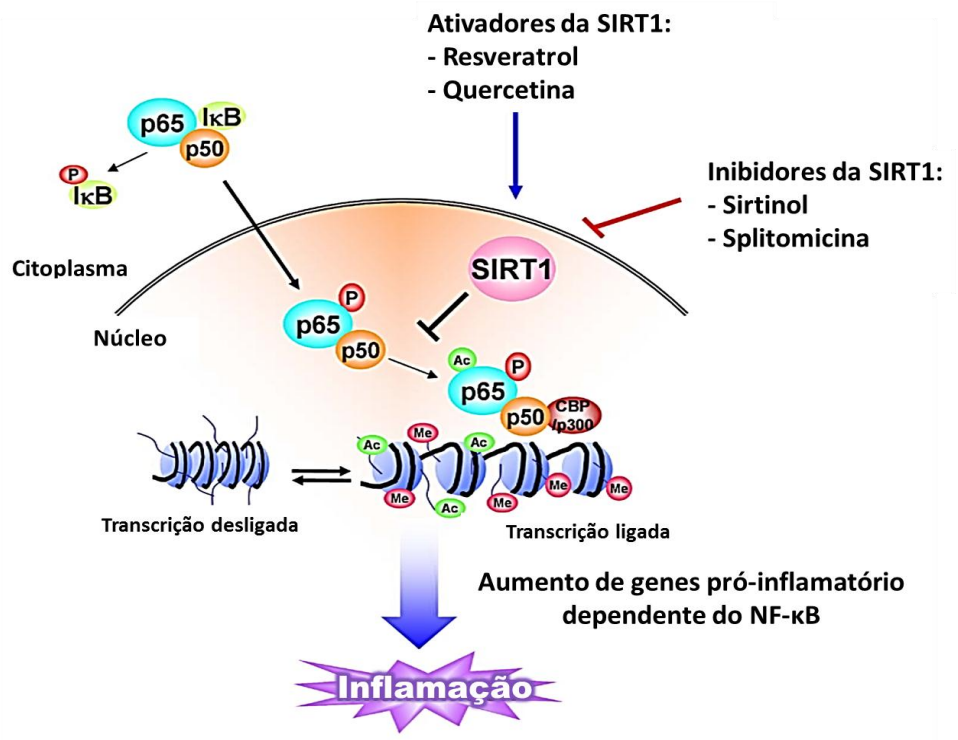

Figura 15. A sinalização da SIRT1 na inflamação via RelA/p65-NF-κB (Hwang e cols., 2013). A sinalização do NF-κB inicia-se no citoplasma, onde por estímulos de fosforilação é translocado para o núcleo, onde é acetilado pelo CBP/p300, resultando na inflamação. A acetilação pode ser modulada pela via da SIRT1, onde, estímulos de ativação levam à desacetilação do p65-NF-κB. Por outro lado, o bloqueio da SIRT1 pode causar hiperacetilação deste fator de transcrição.

O estresse oxidativo mediado pela fumaça do cigarro induz a redução da SIRT1 associada ao aumento da acetilação e ativação de RelA/p65-NF-κB (Yao e cols, 2012; Rajendrasozhan e cols., 2008). Da mesma forma, o silenciamento gênico da SIRT1 leva a hiperacetilação do p65, que resulta na transcrição de citocinas inflamatórias (Rajendrasozhan e cols., 2008). Estes dados foram verificados no tecido pulmonar de rato e em pacientes fumantes com DPOC (Yao e cols, 2012; Rajendrasozhan e cols., 2008, Yang e cols.,2007). Em modelo *in vitro*, o extrato de fumo do cigarro provocou a diminuição, em dose resposta, dos níveis de SIRT1 seguido de redução da atividade da enzima, o que resultou na acetilação do complexo NF-κB em células pulmonares (Rajendrasozhan e cols., 2008, Yang e cols., 2007). O tratamento com SRT1 720, um ativador específico da SIRT1, preveniu a produção de citocinas inflamatórias mediada pela fumaça (Yao e cols, 2012; Rajendrasozhan e cols., 2008). O mesmo foi verificado em modelo de restrição calórica, onde o restabelecimento dos níveis de SIRT1 promoveu redução da inflamação via desacetilação do complexo RelA/p65-NF-κB, resultando no retardo do envelhecimento (Bordone e cols., 2005).

Apenas três estudos publicados recentemente, mostram a importância da via da SIRT1 e do NF-κB na fase precoce da RD. Zheng e cols. (2012) mostrarma em modelo *in vitro*, que células endoteliais expostas à alta dose de glicose apresentam ativação da via da PARP1 e consequente redução da atividade da SIRT1. Em modelo animal, Kubota e cols. (2011) demonstrou a redução da expressão da SIRT1 e aumento da inflamação, caracterizada pelos marcadores VEGF e ICAM-1 em camundongos diabéticos, no entanto, o estudo não verificou os níveis de acetilação do complexo RelA/p65-NF-κB. Mais recentemente, Kowluru e cols. (2014) mostraram que pacientes com RD também apresentam déficit da atividade e expressão gênica da SIRT1. Diante disso, estes estudos mostram a possível participação da via da SIRT1 na patogênese da RD, no entanto, não se sabe ao certo o mecanismo de modulação e/ou atuação da SIRT1 frete aos insultos do DM. Desta forma, tratamentos que modulem ou promovam a ativação a via da SIRT1, podem ser usados com alvo terapêutico no combate as alterações da RD.

1.3 – Polifenóis

Polifenóis são substâncias caracterizadas por possuírem uma ou mais hidroxilas ligadas a um anel aromático (Kirimlioglu e cols., 2006). Os principais grupos de polifenóis são os ácidos fenólicos, tendo como exemplos: o ácido clorogênico, presente no café; os estilbenos como o resveratrol presente nas uvas e vinho; as cumarinas, como as furanocumarinas do aipo; as ligninas, como as lignanas da linhaça; e os flavonoides. Este último grupo é o maior e mais estudado, possuindo mais de 5.000 compostos identificados e estão presentes principalmente em frutas e hortaliças, chás, cacau e soja (Ross e Kasum, 2002). Muitas destas substâncias são classificadas como antioxidantes naturais e possuem propriedades terapêuticas.

As ações fisiológicas exercidas pelos polifenóis já foram relacionadas à prevenção de doenças cardiovasculares, neurodegenerativas, câncer, entre outras (Scalbert e cols., 2005). Os polifenóis têm sido extensivamente estudados devido a seu efeito benéfico à saúde, no combate a formação de radicais livres, bem como na proteção contra danos ao DNA das células (Wollgast e Anklan, 2000). Além do efeito antioxidante, tem sido verificado que os polifenóis também possuem propriedades: anti-inflamatória, anticarcinogênica, anti-aterogênica, antitrombótica, antimicrobiana, analgésica e vasodilatadora, comprovadas em estudos científicos (Wollgast e Anklan, 2000; Gotti e cols., 2006).

1.3.1 - Flavonoides

Os flavonoides são uma subclasse dos compostos polifenólicos biossintetizados que estão presentes na maioria das plantas (Mann, 1987). A principal fonte de flavonoides dos seres humanos é oriunda da dieta (Barnes e cols., 2001), cerca de 1-2g por dia (DeVries e cols., 1997). Em grande parte, a origem desses compostos na dieta se dá pela ingestão de frutos, hortaliças, condimentos e plantas medicinais.

Existem mais de 8 mil flavonoides identificados (Pietta, 2000). A estrutura básica desses compostos consiste em um núcleo comum, constituído de quinze átomos de carbono dispostos

em três anéis (C6-C3-C6), sendo dois fenolicos A e B e um pirano C (Di Carlo e cols., 1999). Existem três requisitos na estrutura química dos flavonoides possivelmente responsáveis pela atividade de neutralização de radicais exercida por esta classe de componentes, sendo eles: 1) o sistema orto-dihidroxi no anel B; 2) a presença da ligação dupla entre C2-C3 conjugada com a função 4-oxo; 3) a presença de um grupo hidroxílico em C3 (Figura 16) (Dornas e cols., 2007).

Os flavonoides são subdivididos em diversas classes onde se encontram as catequinas, caracterizadas principalmente pela capacidade antioxidante (Kähkönen e Heinonen, 2003).

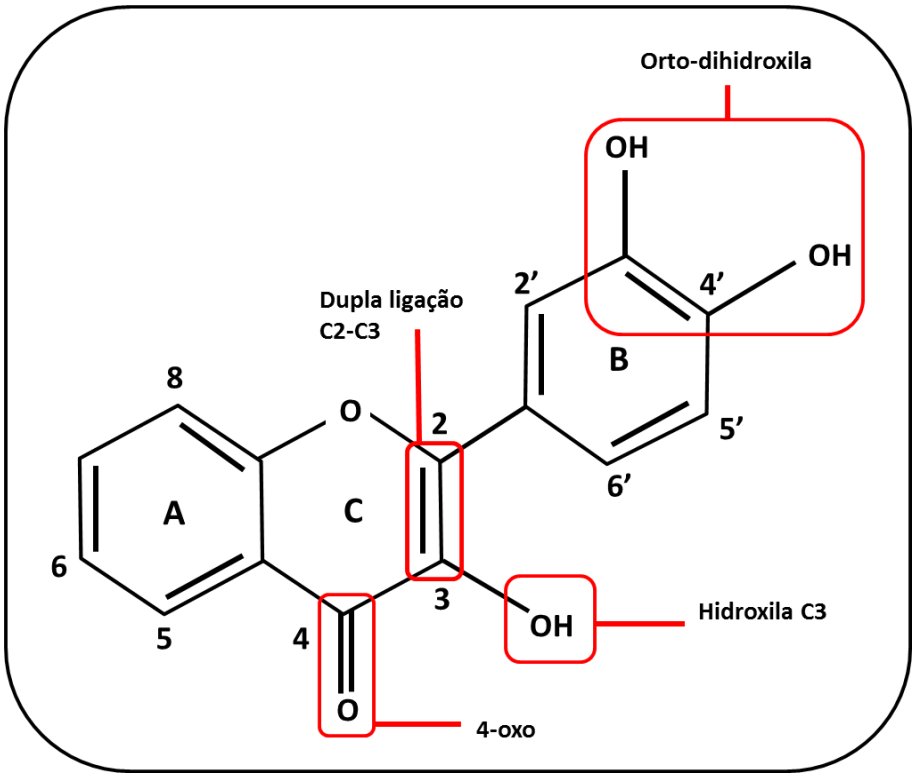

Figura 16. Estrutura molecular básica dos flavonoides e as regiões que favorecem a atividade antioxidante (adaptado de Dornas e cols., 2007)

1.3.1.1 - Catequinas

O grupode das catequinas é caracterizado principalmente por sua estrutura molecular. Estudos indicam que a presença de uma hidroxila no C3 no anel heterocíclico e um catecol no anel B favorecem a atividade antioxidante. A sua composição inclui epicatequina galato (ECG), epigalocatequina (EGC) e epigalocatequina galato (EGCG), epicatequina (EC), sendo que este último é o mais abundante em compostos naturais tais como a cacau, o que merece maior atenção do ponto de vista farmacológico (Figura 17) (Pietta, 2000).

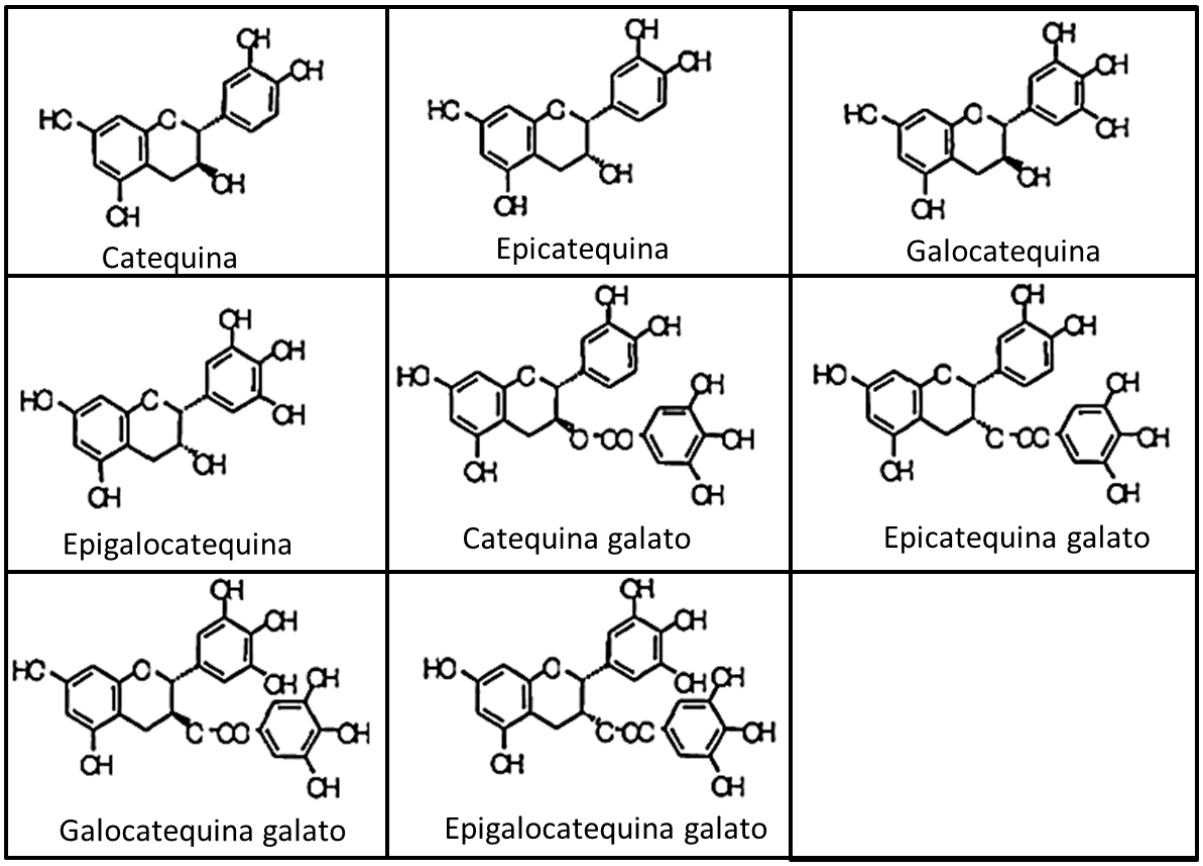

Figura 17. Estrutura molecular das catequinas (Pietta, 2000).

As catequinas são incolores, solúveis na água e não existem referências de contraindicações do seu consumo. Além da atividade antioxidante, tem sido descrito que as catequinas possuem várias outras propriedades, incluindo: anti-inflamatória, anticarcinogênica, antitrombótica, antimicrobiana, analgésica e vasodilatadora. O estudo com uso de catequinas em camundongos diabéticos, inibiu a formação de AGEs, controlou a via inflamatória e preveniu a disfunção renal (Zhu, 2014). Outro dado experimental mostrou que a quercetina e a catequina exibem atividades anti-inflamatórias através da inibição das vias JNK/NF-κB de macrófagos (Liu, 2014). Em modelo *in vitro*, as catequinas foram benéficas na regulação do ciclo celular e danos ao DNA de células cancerosas intestinais, induzindo a apoptose por meio de uma via dependente de p53 e inibição da PI3K/Akt/MAP (Enayat, 2013). Em modelo de hipertensão arterial, o tratamento com epicatequina reduziu a pressão artéria, por aumento de óxido nítrico na vasculatura (Galleano, 2013), além de promover o controle e oxigenação de microvasos no tecido muscular (Copp, 2013).

Pouco se sabe a respeito dos efeitos terapêuticos das catequinas no tratamento de doenças degenerativas mediadas pelo estresse oxidativo (Middleton e cols., 2000, Lopez-Revuelta e cols., 2006). Estudos experimentais mostraram que o uso da catequina melhora a aprendizagem, a memória e a neurogênese em modelo de envelhecimento (Gibbons, 2014). Um estudo multicêntrico, randomizado, duplo-cego realizado por Morillas-Ruiz (2014) mostrou que a suplementação oral de polifenóis (200 ml/dia) em 100 indivíduos, reduziu as concentrações plasmáticas de homocisteína, aminoácido envolvido nas complicações cardíacas e na demência senil como o Alzheimer (Copp, 2013). Recentemente, nosso grupo mostrou o efeito benéfico do chá verde, componente rico em catequinas, no combate às alterações da RD. Neste estudo, revelamos que o chá verde protegeu a retina contra a toxicidade do glutamato por meio de um mecanismo antioxidante; além disso, mostramos em modelo *in vitro* que tanto o chá verde como a (-)-epigalocatequina galato, catequina mais abundante do chá verde, restabeleceu as alterações induzidas de alta glicose em dois tipos celulares da retina: levou a neuroproteção de células de Müller e melhorou a disfunção da barreira retiniana externa em células do epitélio pigmentar da retina (Silva e cols,. 2013).

1.3.2 - *Theobroma cocoa*

Theobroma cocoa, nome científico dado ao cacau, é um fruto com um longo histórico de utilização medicinal (Kwik-Uribe, 2005). Registros mostram que no século XVI na Europa o extrato líquido do cacau era utilizado, como veículo de medicamentos. Além de ser considerado por si só como medicamento, o extrato conjugado a outros suplementos alimentares eram utilizados no tratamento de doenças, como desordens digestivas, dores de cabeça, inflamações e insônias (Kwik-Uribe, 2005).

O cacau é rico em polifenóis do tipo flavonoides. A epicatequina é o principal componente ativo do cacau responsável pelos efeitos benéficos à saúde (Schroeter e cols., 2006). Há um crescente número de estudos clínicos e meta-análise sobre o uso do cacau e seus componentes que fornecem evidências clínicas da relação entre flavonoides do cacau e a melhoria de algumas doenças, principalmente as cardiovasculares (Tokede e cosl., 2011; Desch e cols., 2010; Hooper e cols., 2008; Ried e cols., 2012; Grassi e cols., 2012).

Estudos sugerem que o consumo diário de 200 mg de flavonoides presentes no cacau auxilia na manutenção da vasodilatação do endotélio. Esta quantidade pode ser fornecida pela ingestão de 2,5g de cacau em pó rico em flavonoide ou 10g de chocolate escuro também rico em polifenóis, as quais podem ser consumidos no quadro de uma dieta equilibrada pela população em geral (Davison e cols., 2008; EFSA, 2012 Balzer e cols., 2008; Heiss e cols., 2003, 2005 e 2010; Monahan e cols., 2011).

1.3.2.1 - Efeitos na homeostase

A agregação plaquetária é um mecanismo essencial na homeostase vascular (Buch e cols., 2010). Portanto, vários estudos sustentam evidências sobre a potente atividade de flavonoides presentes no cacau como inibidores da agregação plaquetária (Pearson e cols., 2005; Rein e cols., 2000). Kwil-Uribe (2005) mostrou que o consumo de produtos de cacau com alto

teor de flavonoides e procianidinas diminui a tendência de agregação das plaquetas e, portanto, a formação de coágulos.

Schramm e cols. (2001) relataram que o consumo de chocolate com alto teor de procianidina (147 mg) reduz significativamente os níveis de leucotrieno, um vasoconstritor endógeno, e aumenta os níveis de prostaciclina, um vasodilatador natural. No estudo de Innes e cols. (2003) os resultados foram semelhantes, no entanto, com o consumo de 100g de dois tipos de chocolate: o escuro e o branco. O chocolate escuro reduziu de forma significativa a agregação de plaquetas e o chocolate branco, com teor mínimo de flavonoide, não teve nenhum efeito.

No estudo duplo-cego randomizado de Heptinstall e cols. (2006) com 12 voluntários saudáveis, foi verificada a dose resposta do consumo de bebidas de cacau com diferentes quantidades de flavonoides (baixa, 80 mg e rico, 300, 600 ou 900 mg). O consumo da bebida de cacau contendo 600 e 900 mg flavonoides resultou na inibição significativa de agregação de plaquetas, fato que não foi observado nas doses de 80 e 300 mg. Estes efeitos foram atribuídos aos metabolitos dos flavonoides presentes no cacau, principalmente, 4-O-metil-epicatequina e 3-O-metil-catequina.

1.3.2.2 - Capacidade antioxidante

As espécies reativas de oxigênio têm um papel importante em muitos processos biológicos. Para auxiliar os sistemas antioxidantes de defesa, é desejável a ingestão de substâncias com capacidade antioxidante para combater o excesso de radicais livres (Jacob e Burri, 1996). A atividade antioxidante do cacau foi avaliada em diversos estudos. Sanbongi e cols. (1998) verificaram o efeito do extrato de cacau rico em flavonoides. O estudo *in vitro* mostrou que não apenas catequinas e epicatequinas apresentaram efeito antioxidante, como também quercetina, quercetina-3-glicosídeo, quercetina-3-arabinosídeo e dideoxiclovamida. Outro estudo demonstrou a elevada atividade antioxidante das procianidinas do cacau, (Mao e

cols., 2000). Os mesmos compostos mostraram-se capazes, ainda, de retardar a oxidação lipídica, inibindo totalmente o estresse induzido.

O cacau tem se mostrado eficiente no tratamento clínico. Em estudos realizados em humanos saudáveis, as catequinas foram responsáveis pelo aumento da atividade antioxidante, diminuído o malonaldeído e a peróxidação lipídica no plasma. Além disso, o cacau aumentou a concentração de ascorbato no plasma, reduziu a absorção de ferro não-heme e melhorou a resistência do LDL à oxidação (Williamson e Manach, 2005). O mesmo efeito foi observado em outros estudos com indivíduos saudáveis (Baba e cols., 2007; Rein e cols., 2005; Nanetti e cols. 2012; Fraga e cols., 2005; Sarria e cols., 2012; Roberts e cols., 2009).

No estudo clínico de Davison e cols. (Richelle e cols., 1999), os autores mostram que o consumo agudo de chocolate escuro possui capacidade antioxidante em resposta ao estresse oxidativo, aumentando a atividade da superóxido dismutase e catalase. O efeito foi visto entre 1 a 2 horas após a administração de cacau. A análise do plasma destes pacientes mostrou que após 2 horas, a meia-vida dos flavonoides diminuiu progressivamente até alcançar níveis basais em aproximadamente 6 horas pós-ingestão (Martin e cols., 2010).

Wiswedel e cols. (Wiswedel e cols., 2004) realizaram um estudo randomizado com indivíduos saudáveis que consumiram bebida de cacau contendo duas doses de flavonoides (14 ou 187 mg). Os dados mostraram que o a ingestão de cacau com 187mg de flavonoides, combinada com exercícios físicos, reduziu as concentrações de isoprostana, um biomarcador de peroxidação lipídica, por outro lado, o cacau com dose baixa de flavonoides não apresentou efeito sobre este marcador. Em um estudo cross-over, observou que a ingestão aguda de 40 g de chocolate escuro diminuiu os níveis plaquetários de isoprostanos em fumantes por redução de Nox2, uma proteína envolvido da produção de radicais livres (Carnevale e cols., 2012).

1.3.2.3 - Atividade anti-inflamatória

Estudos mostram que o cacau também possui atividade anti-inflamatória. Em modelo *in vitro*, o tratamento com flavonoides do cacau gerou redução da produção de compostos pró-inflamatórios. Rein e cols. (2000) mostraram ainda que os flavonoides tem a capacidade de modular etapas importantes na regulação da produção de moléculas pró-inflamatórias. Interessante que o grau de condensação (monômeros, dímeros, trímeros, etc.) dos flavonoides interferiu em suas atividades. Também em modelo *in vivo*, Osakabe (1998) verificou que a ingestão de flavonoides e procianidinas favoreceu a produção de compostos anti-inflamatórios. Foi demonstrada ainda propriedade anti-inflamatória por meio da redução de lesões gástricas induzidas pelo consumo de álcool.

Os polifenóis presentes no cacau também estão relacionado à diminuição da inflamação através de vários mecanismos: inibir a ativação de células T, a ativação de células B policlonais e reduz a expressão e secreção de interleucina-2 (IL-2) por células T (Sanbongi e cols., 1987). Além disso, as procianidinas do chocolate também exercem efeitos anti-inflamatórios, diminuindo as expressão de diversas citocinas, como por exemplo, IL-5, TNF-α e TGF-β. Por outro lado, Muniyappa e cols. (2008) não viram nenhum efeito sobre os níveis de ICAM e IL-6 em pacientes hipertensos após a ingestão diária de 900 mg de cacau rico em flavonoides por 14 dias. Do mesmo modo, Grassi e cols. (2005) descobriram que o consumo de 88 mg de chocolate escuro rico em flavonoides consumidos por 15 dias não afetou a proteína reativa-C (RPC) ou ICAM. A discrepância dos resultados pode ser devido a diferença na dose de cacau ou tipo de chocolate administrado, ou ainda ao estado de saúde dos indivíduos estudados.

Um recente estudo randomizado sugere que a ingestão de polifenóis do cacau pode modular mediadores inflamatórios em pacientes com alto risco de doenças cardiovasculares através da avaliação de biomarcadores inflamatórios celulares e séricos relacionados à aterosclerose (Monagas e cols., 2009). Neste estudo, os pacientes receberam 40 g de cacau/dia durante quatro semanas. Não foram encontradas diferenças significativas na expressão de moléculas de adesão nas superfícies de linfócitos T. Entretanto, a expressão de monócitos CD40

e CD36 foi significativamente menor após a ingestão de cacau. Outro ensaio clínico com 18 voluntários saudáveis verificou que o cacau diminuiu significativamente a ativação do NF-kB em relação aos níveis basais 6 h após a administração oral. Apesar destas evidências, estudos de longo prazo são necessários para confirmar a propriedade anti-inflamatória do cacau.

1.3.2.4 - Efeitos no sistema vascular

O consumo diário de cacau está associado à redução de 50% da mortalidade entre pacientes cardiopatas (Buijsse e cols., 2006), o que sugere que os polifenóis do cacau também conferem proteção cardiovascular, independente da redução da pressão arterial, possivelmente devido ao óxido nítrico (Heiss e cols., 2006; Taubert e cols., 2007).

A associação epidemiológica entre o consumo de cacau e baixa incidência de hipertensão é consistente com os resultados de vários estudos de intervenção em curto prazo. Grassi e cols. (2005) observaram que a ingestão de chocolate escuro esteve associado à diminuição da pressão arterial sistólica (PAS) em indivíduos saudáveis, e este efeito também foi observado em pacientes hipertensos (Grassi e cols., 2005), bem como pacientes com pré-hipertensão ou no estágio I de hipertensão (Grassi e cols., 2008). Taubert e cols. (2008) estudaram os efeitos de baixas doses de chocolate amargo rico em polifenóis em pacientes com hipertensão; durante 18 semanas, os autores observaram que o consumo diário de pequenas quantidades de chocolate escuro reduziu a média da PAS de -2,9 ± 1,6 mm Hg e a pressão arterial média diastólica em -1,9 ± 1,0 mmHg e melhorou a formação do vasodilatador NO. Faridi e cols. (2007) observaram que a ingestão aguda de chocolate escuro melhora a função endotelial e reduz a PAS em adultos com sobrepeso.

Evidências sobre os efeitos do cacau sobre o sistema vascular e a influência da idade, duração e dose administrada de polifenóis/cacau foi estatisticamente analisado em recente metanálise (Hooper e cols., 2012). Em relação à idade, o efeito do cacau na pressão arterial pode ser mais acentuado em indivíduos mais jovens, devido à reatividade vascular. Quanto ao tempo

de estudo, os protocolos mais curtos parecem ser mais eficazes na diminuição da PAS dose-dependente, enquanto que em estudos de longo prazo têm relatado dados controversos. Um ponto preocupante foi a dose, pois a ingestão de grandes quantidades (1 barra equivalente a 100g/dia) está relacionada a alguns efeitos colaterais indesejados, tais como o ganho de peso. Além disso, tem sido sugerido que outros compostos presentes no cacau, tais como a teobromina, também poderiam ser responsáveis por vasoatividade e, portanto, a redução da pressão arterial (Kelly e cols, 2005).

1.3.2.5 - Efeitos no Diabetes e suas complicações

Os efeitos benéficos dos flavonoides sobre a homeostase da glicose têm sido verificados por evidências epidemiológicas e clínicas. Hanhineva e cols. (2010) mostraram que diferentes polifenóis, como os flavonoides e proantocianidinas, atuaram no metabolismo de carboidratos. O estudo mostrou que os polifenóis do cacau atenuaram a resposta glicêmica pós-prandial e em jejum durante a hiperglicemia, melhorando a secreção e sensibilidade aguda da insulina. Os possíveis mecanismos de ação incluem a inibição de carboidratos da digestão e absorção da glicose no intestino, a estimulação da secreção de insulina pelas células pancreáticas, a modulação da libertação de glicose do fígado, a ativação de receptores de insulina e a absorção de glicose nos tecidos sensíveis à insulina, possivelmente através de vias de sinalização intracelular (Hanhineva e cols., 2010).

Não existem estudos epidemiológicos que avaliaram a associação entre o consumo de cacau ou chocolate e diabetes. No entanto, estudos clínicos com pacientes hipertensos, a administração de 100 g/dia de chocolate amargo (contendo 88 mg flavonoides) durante 15 dias diminuiu a resistência à insulina tanto em indivíduos saudáveis quanto hipertensos, com (Grassi e cols., 2005b) ou sem (Grassi e cols., 2005) tolerância à glicose. Por outro lado, outro estudo não conseguiu encontrar alterações na sensibilidade à insulina após 2 semanas de administração de uma bebida de cacau fornecendo um dose diária de 900 mg de flavonoides a indivíduos com

hipertensão (Muniyappa e cols., 2008). Em pacientes com sobrepeso e obesidade, a ingestão de cacau rico em flavonoides mostrou melhora na resistência à insulina observada pela redução da glicemia de jejum; curiosamente, os efeitos benéficos foram observados apenas nas doses de 50 a 100 mg/epicatequina/dia, mas não em uma dose superior (Davison e cols., 2008).

Pouco se sabe a respeito do uso do cacau e seus compostos no tratamento das complicações do diabetes. Dados experimentais em modelo de nefropatia diabética mostraram que o cacau atua restabelecendo a AMPK no tecido renal por redução da sinalização da Nox4/Fator de transformação do crescimento beta-1 (TGFβ-1) (Papadimitriou e cols., 2014). O mesmo estudo verificou redução de acúmulo de matris extracelular em ratos diabéticos tratados com cacau. Na retina, estudos preliminares com células de linhagem do epitélio pigmentado da retina humana (ARPE-19), mostraram que a condição de alta glicose (30mM) induz o estresse nitrosativo, gerando S-nitrosilação da caveolina-1 (uma proteína de membrana importante no fluxo celular), e esta modificação gerou endocitose e degradação de proteínas de junção envolvidas na permeabilidade paracelular, alterando as características da barreira celular. O tratamento com 100 ng/ml de extrato de cacau enriquecido com polifenóis ou (-)- epicatequina, 12 ng/ml referente a 12% do extrato total, restabeleceram estas alterações restaurando as proteínas de junção intercelular da ARPE-19 (Rosales e cols., 2014). Neste sentido, devido à eficaz atuação do cacau e seus compostos frente à inflamação e estresse oxidativo/nitrosativo, a administração oral de cacau enriquecido com polifenóis pode conferir neuroproteção retiniana em pacientes com diabetes.

1.4 - Terapia celular

Atualmente, com o avanço do conhecimento e das técnicas moleculares, a ciência conseguiu gerar informações importantes para o diagnóstico e tratamento de algumas doenças. Novos tratamentos foram criados utilizando as células-tronco (CT), através da terapia celular. As

CT são células indiferenciadas definidas pela capacidade de auto renovação e são capazes de se dividirem indefinidamente (Gnecchi e cols., 2008).

Estudos têm demonstrado que certas células da medula podem auxiliar na reparação de tecidos com lesão em uma grande variedade de doenças cardiovasculares (Alaiti e cols., 2010). Embora ainda não se saibam os mecanismos pelos quais as células exerçam seus efeitos; vários estudos têm documentado a retenção de parte destas células nos órgãos-alvo, mantendo melhoras estruturais e funcionais (Hofmann e cols., 2005; Gnecchi e cols., 2008). De acordo com Gnecchi e cols (2008), as células progenitoras endoteliais da medula podem atuar através de fatores parácrinos locais e não por incorporação de estruturas pré-existentes.

Asahara e cols (1999) descreveram pela primeira vez, há pouco mais de 10 anos, as células progenitoras endoteliais (EPCs) derivadas da medula óssea circulantes no sangue. Desde então, inúmeros estudos demonstraram que as EPCs circulantes atuam na reparação de vasos sanguíneos danificados e na neovascularização de lesões isquêmicas (Kaushal e cols., 2001; Kong e cols., 2004), sugerindo que essas células apresentam importante função na patogênese da aterosclerose e doenças cardiovasculares.

Existe uma variedade de EPCs originárias da medula, uma dela é a *Early Outgrowth* (EOC). Consideradas células progenitoras endoteliais, estas expressam uma variedade de marcadores de membrana (Asahara e cols. 1997, Hur J e cols., 2004).

1.4.1 – Células *Early Outgrowth* (EOCs)

As EOCs são um tipo raro de células progenitoras de origem endotelial definidas principalmente pela identificação de seus marcadores de superfície celular (Hur J e cols., 2004). As EOCs são particularmente importantes no que diz respeito à produção e secreção de fatores antifibróticos (Yuen e cols., 2010) e anti-oxidativo (Yang Z e cols., 2010). Este tipo celular é produzido *in vitro* a partir de células da medula óssea pluripotentes e são consideradas um tipo de célula endotelial devido à técnica de cultivo e diferenciação. Sob estes estímulos, as células

passam a expressar marcadores de superfície celular característicos, como CD34 e VEGFR2 (Hur e cols., 2004). Apesar de se assemelhar a células endoteliais maduras, EOCs são secretoras, tendem a não integrar as estruturas pré-existentes e exibem um fenótipo parecido a monócitos imaturos (Figura 18) (Hur e cols., 2004; Krenninge e cols., 2009).

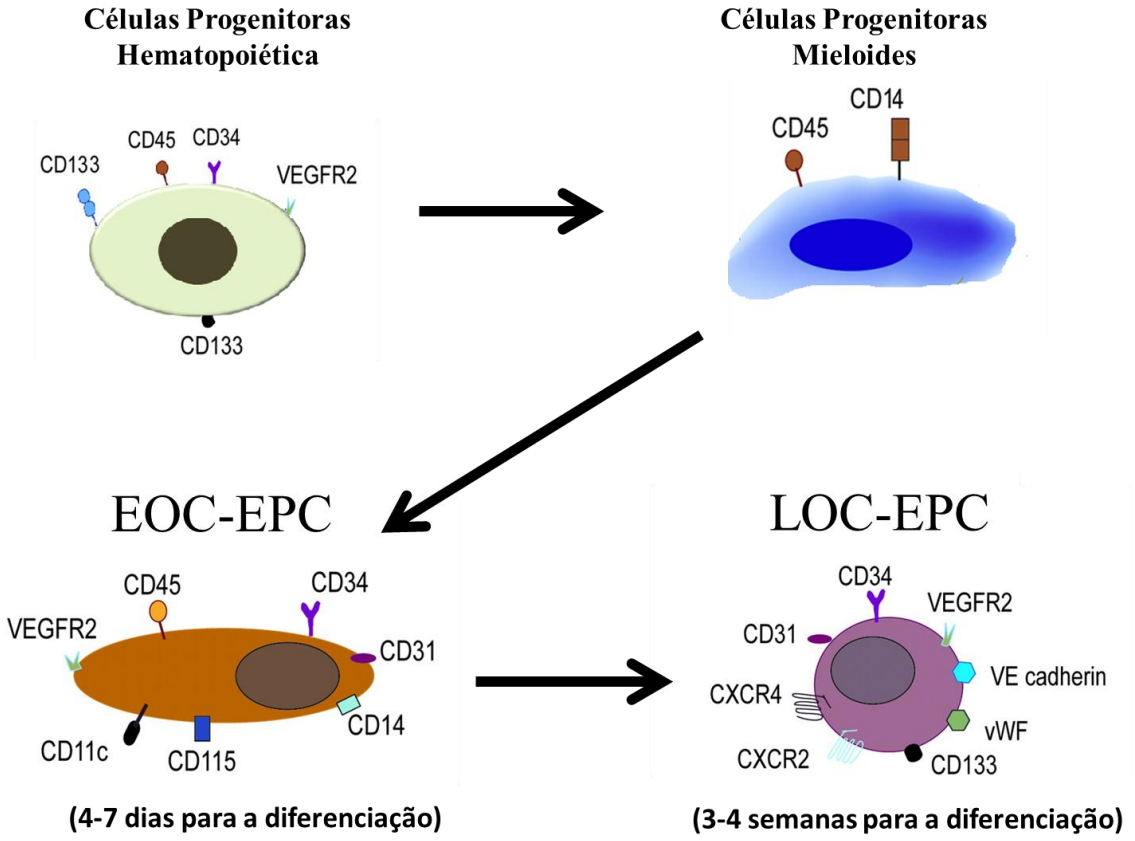

Figura 18. Origem e diferenciação das células *early outgrowth* (adaptado de Jones CP, Rankin, 2011). Sob estímulos com fatores de crescimento contidos em meio de células endoteliais, as células progenitoras hematopoiéticas da medula diferenciam-se em progenitora mielóides e posterirormente em EOC, expressando os marcadores de membrana CD43 e VEGFR2. O processo de diferenciação ocorre *in vitro* de 4 a 7 dias pós-extração. Além disso, é possível gerar o modelo tardio, de 3 a 4 semanas de diferenciação, onde as células ganham o marcador CD133. EOC-EPC: células progenitoras endoteliais *early outgrowth*; LOC-EPC: células progenitoras endoteliais *late outgrowth.*

As EOCs tem a capacidade de diferenciação e/ou fusão em células do parênquima maduro, e destacam-se pelas funções parácrinas (Gnecchi e cols., 2008). Embora a maioria dos estudos tenha abordado a atividade pró-angiogênica das EOCs, este tipo celular tem a capacidade de secretar fatores antifibróticos (Yuen e cols., 2013) e antioxidantes, atenuando a apoptose de células endoteliais induzida pelo H_2O_2 (Yang e cols., 2010). No entanto, os mecanismos pelos quais as células progenitoras EOCs medeiam a reparação e regeneração de órgãos, permanecem especulativos.

Estudo desenvolvido no Canadá por Zhang e cols. (2012), descreve o tratamento bem sucedido da nefropatia diabética usando uma única infusão de EOCs em modelo experimental de DM tipo 2 (db/db). A terapia celular com EOCs preveniu alterações estruturais do rim, tais como a deposição de matriz extracelular e a apoptose de células do epitélio tubular, o que levou à redução de albuminúria, uma característica da lesão renal diabética. Embora as EOCs tenham promovido esses efeitos, foi observada a retenção mínima destas células no tecido renal dos animais diabéticos. Achados semelhantes foram demonstrados nos animais diabéticos tratados com meio condicionado das EOCs, sugerindo que os efeitos foram mediados por liberação de fatores. Por outro lado, foi observada a presença abundante das EOCs no fígado, pulmão, baço, e medula óssea, sugerindo um mecanismo de atividade parácrina pela secreção de fatores renoprotetores a distancia (Zhang et al, 2012).

A falta de retenção celular nos órgãos lesionados também foi descrita por outros trabalhos (Yuen et al, 2013). No entanto, Gilbert e cols caracterizaram a capacidade secretora das EOCs. A análise do meio condicionado das EOCs detectou onze tipos de citocinas secretadas por estas células, dentre elas estão: interferon-γ, interleucina-1a e inibidor de metaloproteinase-1 (Zhang e cols., 2012). Além disso, o grupo documentou quase duzentos tipos de proteínas detectadas por mapeamento proteômico do meio condicionado das EOCs. O fato que chamou a atenção foi a presença de proteínas que regulam as funções celulares implicadas na fibrose e também proteínas envolvidas na respostas ao stress oxidativo, tais como, glutationa-S-transferase, peroxiredoxina, superóxido dismutase, tiorredoxina, e hemeoxigenase-1 (Yuen e cols., 2013).

Estes resultados indicam que EOCs exibem uma grande capacidade na secreção de fatores solúveis com potente atividade antioxidante, quando injetado por via intravenosa.

Na retina, avanços de estudos com células-tronco proporcionam grande expectativa para uso destas células na regeneração dos tecidos oculares (Siqueira, 2009). Recentemente, tem sido testado o uso da terapia celular no tratamento de doenças degenerativas da retina tais como retinopatia diabética e degeneração macular com a utilização de uma variedade de tipos celulares, incluindo: células pluripotentes embrionárias progenitoras do epitélio pigmentado da retina (Buchholz e cols., 2009); células mesenquimais da medula óssea ou derivadas do condão umbilical (Schwartz e cols., 2012); células fetais progenitoras neuronais e retinianas (Aftab e cols., 2009); e, células tronco adulta do epitélio pigmentado da retina (Inoue e cols., 2007; Siqueira e cols., 2011). Entre os muitos tipos de células propostas, as EPCs circulantes no sangue derivadas de medula têm sido estudadas em grande parte dos ensaios clínicos (Tateishi-Yuyama e cols., 2002; Mund e cols., 2009). No entanto, os mecanismos pelos quais essas células exercem seus efeitos benéficos ainda não são claros.

2 – OBJETIVOS

2.1 - Objetivo geral

- Investigar os mecanismos de regulação da neurodegeneração da retina diabética através da modulação da atividade da SIRT1.

2.1 - Objetivos específicos

2.2.1 - Artigo I

- Investigar os possíveis efeitos protetores da administração oral de cacau enriquecido com polifenóis na RD em modelo de diabetes tipo 1.
- Verificar os mecanismos neuroprotetores do cacau enriquecido com polifenóis na RD.
- Elucidar o mecanismo da sinalização do GFAP em células de Müller.
- Acessar a participação e a modulação da atividade da SIRT1 na RD.

2.2.2 - Artigo II

- Investigar o possível efeito terapêutico de células da medula óssea derivadas de animais saudáveis (Dock7 m +/+ Leprdb db/m) e diabéticos (BKS.Cg-Dock7 m +/+ Leprdb/J, db/db) na RD em modelo de diabetes tipo 2.
- Verificar os mecanismos neuroprotetores das células *EOCs* na RD.

3 - ARTIGO I

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Polyphenol-enriched cocoa protects the diabetic retina from glial reaction through the sirtuin pathway☆

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Abstract

Cocoa is rich in flavonoids, which are potent antioxidants with established benefits for cardiovascular health but unproven effects on neurodegeneration. Sirtuins (SIRTs), which make up a family of deacetylases, are thought to be sensitive to oxidation. In this study, the possible protective effects of cocoa in the diabetic retina were assessed. Rat Müller cells (rMCs) exposed to normal or high glucose (HG) or H_2O_2 were submitted to cocoa treatment in the presence or absence of SIRT-1 inhibitor and small interfering RNA The experimental animal study was conducted in streptozotocin-induced diabetic rats randomized to receive low-, intermediate-, or high-polyphenol cocoa treatments via daily gavage for 16 weeks (i.e., 0.12, 2.9 or 22.9 mg/kg/day of polyphenols). The rMCs exposed to HG or H_2O_2 exhibited increased glial fibrillary acidic protein (GFAP) and acetyl-RelA/p65 and decreased SIRT1 activity/expression. These effects were cancelled out by cocoa, which decreased reactive oxygen species production and PARP-1 activity, augmented the intracellular pool of NAD^+, and improved SIRT1 activity. The rat diabetic retinas displayed the early markers of retinopathy accompanied by markedly impaired electroretinogram. The presence of diabetes activated PARP-1 and lowered NAD^+ levels, resulting in SIRT1 impairment. This augmented acetyl RelA/p65 had the effect of up-regulated GFAP. Oral administration of polyphenol cocoa restored the above alterations in a dose-dependent manner. This study reveals that cocoa enriched with polyphenol improves the retinal SIRT-1 pathway, thereby protecting the retina from diabetic milieu insult.

Keywords: Diabetic retinopathy; Cocoa; GFAP; SIRT1; NF-kB; NAD^+; Neurodegeneration

1. Introduction

Diabetic retinopathy (DR) is a potentially devastating disease and is the most common cause of blindness among working-age people in the world [1]. It is a multifactorial progressive disease affecting neuro and glial cells and vascular elements of the retina [2–4].

Retinal Müller glia, blood vessels and neuronal cells interact closely to maintain retinal tissue homeostasis and cell survival [5]. Müller cells are the primary glial type in the retina. Müller cells activate under stress and produce proinflammatory cytokines and growth factors to restore tissue homeostasis [6]. However, in chronic diseases such as DR, retinal gliosis is prolonged, causing sustained inflammation, cell injury or death, and worsening disease. When exposed to an insult such as high glucose, Müller cells become active, thus up-regulating glial fibrillary acidic protein (GFAP) and increasing retinal glial reactions [5]. As upstream stimuli for glial reaction, recent reports have demonstrated that oxidative stress and redox-sensitive transcription factor nuclear factor NF-kB signaling contribute to the pathogenesis of DR [7].

Silent information regulator 1 (SIRT1) proteins, known as sirtuins, were identified as genetic silencing factors [8] and were later found to prolong the lifespan in yeast [9]. In addition, NAD^+-dependent deacetylation by sirtuins was linked with caloric restriction and aging in several organisms [10]. The functions attributed to sirtuins are mediated by deacetylation of histones (known as epigenetic chromatin remodeling), transcriptors factors, coactivator such as p53, Forkhead box O (FOXO) and NF-kB [11–14]. Several lines of evidence indicate that SIRT1 are responsible for redox modulation since their activity relies on intracellular NAD^+ [10,15]. As sirtuins require NAD^+ and are therefore sensitive to redox changes, hydrolyzing one NAD^+ generating nicotinamide and 2′-*O*-acetyl-ADP-ribose will affect the NAD^+ salvage pathway because this is a NAD^+-dependent reaction [16].

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0955-2863/

NF-kB is a key redox-sensitive transcription factor responsible for proinflammatory gene transcription [17]. In retinal tissue, the major sources of this proinflammatory transcriptor are microglial and Müller cells. The gene transcribed by NFkB will modulate tumor necrosis factor (TNF)-α, interleukin-8 and interleukin-6 expression in reactive cells, thus aiding the initiation of inflammatory response under pathological conditions. In normal and pathological conditions, NF-kB undergoes posttranslational modifications, including phosphorylation and acetylation, to control its nuclear action [18]. It has been shown that histone acetyltransferases p300 and CREB-binding protein (CBP) acetylases the RelA/p65 subunit of NF-kB at Lys218, Lys221 and Lys310 [19], thus modulating NF-kB. This includes transcriptional activation, DNA binding and assembly with its inhibitor IκBα [20]. SIRT1 interacts with RelA/p65 at Lys310 and deacetylates it, thus inhibiting the transcription capacity of NF-kB [21].

Cocoa (*Theobroma cacao*) and cocoa-derived products, such as chocolate with 70% or more cocoa content, have gained attention because of evidence that they lower blood pressure and improve endothelial function [22–24]. Because major polyphenols are lost during the classical technique of extraction, attempts have been developed to prepare cocoa-based functional food-employing methods such as ACTICOA [25], where higher amounts and bioavailability of polyphenols are achieved. Previously, the efficacy of ACTICOA as a potent chemopreventive agent against malignant cell *in vivo* and *in vitro* by targeting the Kras/Akt/NF-kB signaling was clearly demonstrated [26]. More recently, the protective effects of catechin and epicatechin were demonstrated in experimental models of Alzheimer's [27] and Parkinson's disease [28]. Some of the protective effects of epicatechin in the diabetic retina were demonstrated by Al-Gayyar and colleagues [29]. The authors revealed that epicatechin decreased proNGF levels and the nitration of tyrosine of the TrKA-Y490 survival receptor, thus decreasing ganglion cell death. The effect of cocoa enriched with polyphenol in diabetic retinopathy is unknown.

This present study aimed to assess the signaling of GFAP in Müller cells, a crucial event for neurodegeneration in diabetic retina, and to verify the efficacy of oral administration of cocoa enriched with increased content of polyphenols (ACTICOA). It was hypothesized that the activation of the NF-kB complex in RelA/p65 subunit by acetylation on Lys310 modulates GFAP expression and SIRT1 is acting as a protective agent deacetylating it. As a result, the retinal glial reaction is mitigated. This hypothesis was tested in *in vitro* and confirmed in *in vivo* studies.

2. Methods and materials

2.1. In vitro studies

2.1.1. Transformed rat retinal Müller cell line (rMC-1)

Vijay J. Sarthy (Northwestern University, Evanston, IL, USA) provided rMC-1 for this study. The rMC-1 was grown in Dulbecco's modified Eagle medium. The cells were maintained in a humidified incubator at 37 °C and 5% CO_2. The rMC-1 cultures at 70% of confluence were serum starved by reducing the fetal bovine serum concentration to 1% and then were exposed for 24 h to 5.5 mM D-glucose (NG); 25 mM D-glucose (HG); HG plus 100 ng/mL, 1 μg/mL or 10 μg/mL of polyphenol-enriched cocoa (cocoa contenting 60% of polyphenol) (HG+CC); HG plus (−)-epicatechin (Sigma, St. Louis, MO, USA) (HG+EP); or different treatments as specified. Distillated water was used as a vehicle for cocoa or epicatechin treatments. The cells were also treated with hydrogen

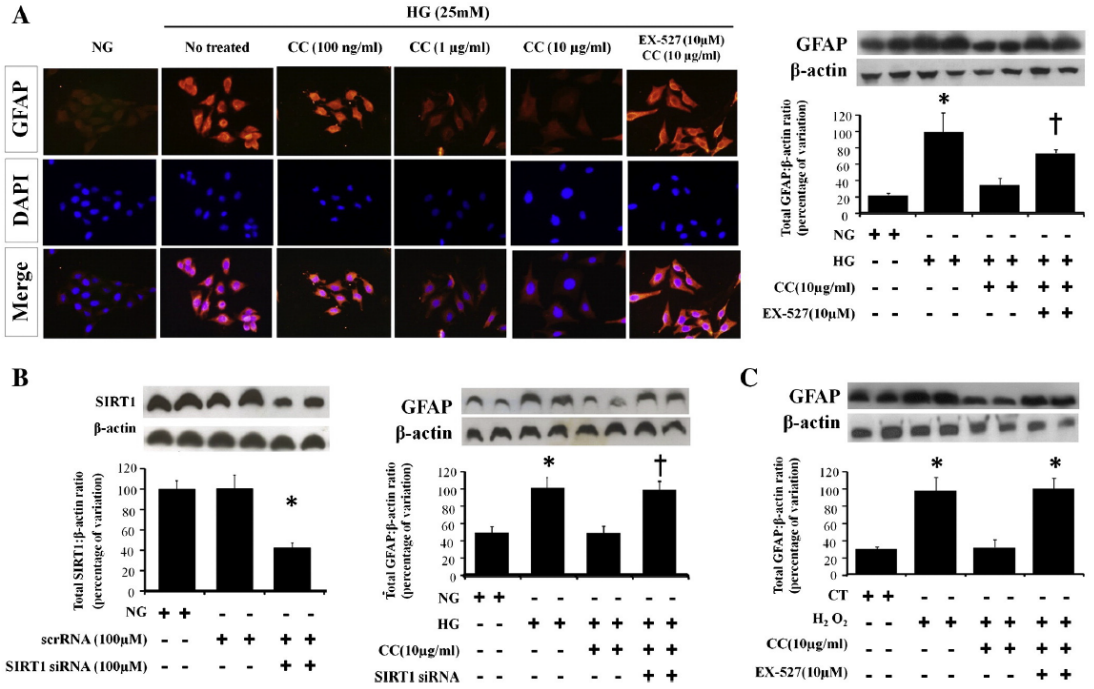

Fig. 1. GFAP was down-regulated by polyphenol-enriched cocoa via SIRT1 in rMC-1 exposed to HG or H_2O_2. (A) A representative photomicrograph of glial reactivity as revealed by GFAP immunofluoresecence in rMC-1 cultured for 24 h in HG in the presence or not of cocoa in different doses. Magnification: ×630. Western blot analysis of the GFAP in total cell lysates; *P=.001, †P=.04. (B) Western blot analysis of the SIRT1 in total cell lysates. The efficiency of SIRT1 siRNA was about 60% of reduction (P<.0001). Western blot analysis of the GFAP in total cell lysates. The siRNA abolished the cocoa effect; *P=.0005, †P<.0001. (C) Western blot analysis of the GFAP in total cell lysates; *P<.0001. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean±S.D. of band densities expressed as the percentage of variation from at least three independent experiments. CC: polyphenol-enriched cocoa; scrRNA: scrambled; siRNA: small interfering RNA (100 μM); EX-527, SIRT1 blocker (10 μM).

peroxide (H_2O_2, 10 μM) as an oxidative stress inducer. *We used distilled water as a vehicle for both.*

2.1.2. Pharmacological treatments and transient transfection with small interfering RNAs (siRNAs)

The cytotoxicity of the treatments and vehicle on rMCs was determined by a thiazolyl blue tetrazolium bromide colorimetric assay [30]. Concentrations that caused less than 10% of cell toxicity were chosen for the experimental treatments.

The siRNA duplexes and scrambled siRNA corresponding to rat Sirt-1 were obtained from Invitrogen (Santa Cruz Biotechnology). The transient transfection of siRNAs was carried out using Lipofectamine Transfection Reagent (Life Technologies, Carlsbad, CA, USA) [31].

2.1.3. Immunofluorescence in Müller cells

The immunofluorescence was performed as previously described [32]. The cells were incubated with anti-GFAP or anti-SIRT1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by the appropriate secondary antibody. Afterward, the sections were rinsed and cover-slipped with Vectashield antifade medium containing 4′,6′-diamino-2-phenylindole used for nuclei staining (Vector Laboratories, Burlingame, CA, USA). The slides were examined under a scanning microscope (CLSM, LSM510; Zeiss, Jena, Thuringia, Germany). Digital images were captured using specific software (AxioVision, Zeiss).

2.1.4. Western blot

Retinal protein extraction or total rMC-1 lysate was incubated with anti-GFAP, anti-SIRT1, antiacetylated RelA/p65-NF-kB and p65 subunit NF-kB complex (Santa Cruz); antinitrotyrosine (Upstate USA, Inc., Lake Placid, NY, USA) and anti-PAR ribosylated proteins (Trevigen Inc., Gaithersburg, MD, USA). Immunoreactive bands were visualized with the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, IL, USA). Exposed films were scanned with a densitometer (Bio-Rad) and analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems Bio-Rad (Hercules, CA, USA).

2.1.5. Immunoprecipitation studies

Immunoprecipitation was performed as before with minor modifications [33]. Using Protein A/G agarose beads, 500 μg of total protein diluted in extraction buffer was immunoprecipitated with rabbit anti-p65 NF-kB (Santa Cruz). The samples were submitted to Western blotting against acetyl lysine residues (anti-acetyl-lys-310; Cell Signaling Technology, Danvers, MA, USA) or anti-p65 subunit.

2.1.6. SIRT1 activity

The SIRT1 activity was measured by a fluorescent activity assay/drug discovery kit designed to measure the lysyl deacetylase (Enzo Life Sciences, Farmingdale, NY, USA). NAD+-dependent deacetylation of the substrate by recombinant human SIRT1 sensitized it to Developer II, which then generated a fluorophore; the fluorophore was excited with 360-nm light, and the emitted light (460 nm) was detected on a Synergy Mx fluorometric plate reader (BioTek, Winooski, VT, USA).

2.1.7. 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA)

Intracellular reactive oxygen species (ROS) levels were measured by H_2DCF-DA. Relative fluorescence was measured using a Synergy Mx fluorescence plate reader at excitation and emission wavelengths of 485 and 528 nm, respectively. The values were corrected by the number of cells.

2.1.8. NAD^+/NADH ratio levels

The NAD^+/NADH Quantitation Colorimetric Kit (BioVision, Inc., Milpitas, CA, USA) provides detection of the intracellular nucleotides NAD^+ and NADH. The NAD Cycling Enzyme Mix in the kit specifically recognizes NAD^+/NADH in an enzyme cycling reaction. The colorimetric kit was read in spectrometer at 450-nm absorbance.

2.1.9. Estimation of catechins in polyphenol-enriched cocoa

The quantification of components in polyphenol-enriched cocoa was assessed by high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA), as previously reported [34].

2.1.10. Animal experiments

This study protocol was approved by the local committee for ethics in animal research (CEEA/IB/Unicamp, protocol number 1834-1). Experimental diabetes was

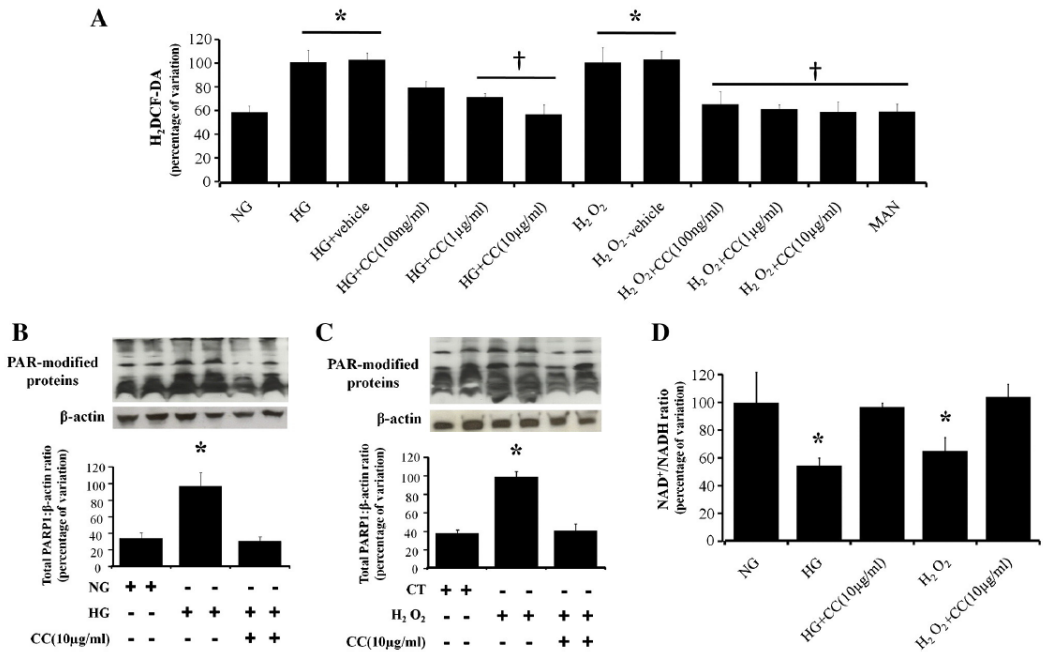

Fig. 2. Polyphenol-enriched cocoa decreases PAR-modified proteins and reestablishes NAD^+/NADH levels by reduction of oxidative status in rMC-1. (A) The quantification of total intracellular ROS levels in rMC-1 cultured for 24 h by H_2DCF-DA. The values are expressed by mean±S.D. and expressed as percentages of fluorescence units. Values were corrected by the number of cells at the end of each treatment; *P<.0001, †P>.6. (B and C) Representative Western blots for PAR-modified proteins in total cell lysates in cells under HG or H_2O_2 and treated with cocoa (100 ng); *P<.0001. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean±S.D. of band densities expressed as the percentage of variation from at least three independent experiments. (D) Intracellular NAD^+/NADH levels in rMC-1 cultured for 24 h in HG or H_2O_2. The NAD^+/NADH ratios are means±S.D. of absorbance units; *P<.0001 vs. other conditions. CC: polyphenol-enriched cocoa; Vehicle: DMSO (0.1%); MAN: mannitol (25 mM).

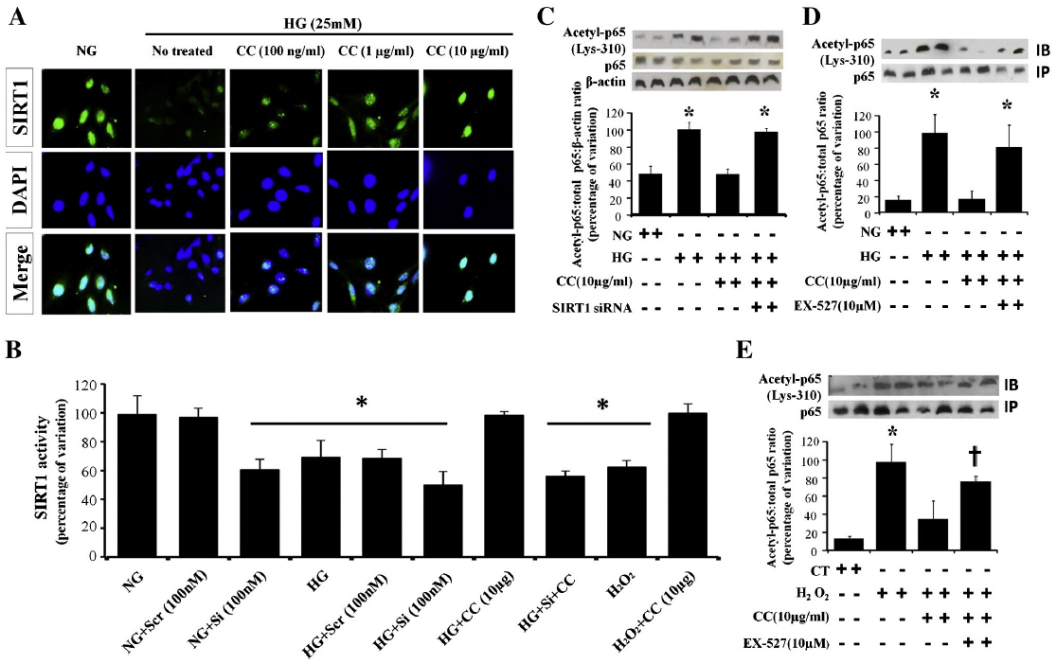

Fig. 3. The polyphenol-enriched cocoa improved SIRT1 pathway in rMCs-1 exposed to HG or H_2O_2. (A) Immunofluorescence for SIRT1 in rMC-1 cultured for 24 h in HG. Magnification: ×1000. (B) SIRT1 activity in rMC-1 cultured for 24 h with different treatments by fluorescent method. The mean±S.D. expressesthe percentage of fluorescence units; *P<.0001. (C) Representative Western blots for acetyl-Lys310-RelA/p65 in rMCs-1 lysates exposed to HG and treated with cocoa in the presence or not of SIRT1 siRNA; *P<.0001. (D) Immunoprecipitation for acetyl-Lys310-RelA/p65 in rMCs-1 lysates exposed to HG and treated with cocoa in the presence or not of EX-527; *P<.002. (E) Similarly, immunoprecipitation for acetyl-Lys310-RelA/p65 in total rMCs-1 lysates exposed to HG and treated with cocoa in the presence or not of EX-527; *P=.0002, †P=.003. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for total p65 subunit. The bars represent mean±S.D. of band densities expressed as the percentage of variation from at least three independent experiments. CC: polyphenol-enriched cocoa; siRNA: small interfering RNA (100 µM); EX-527, SIRT1 blocker (10 µM).

induced in 12-week-old hypertensive male SHR by intravenous injection of streptozotocin (60 mg/kg in 0.5-M sodium citrate buffer, pH4.5; Sigma); the control animals received vehicle alone. Blood glucose levels were measured using an enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany) 72 h after the injection. Values ≥15 mmol/L were considered diabetic. The animals were randomized to receive one of the following treatments: 24 mg/kg/day of cocoa with 0.5% of polyphenols (low dose, i.e., 0.12 mg polyphenols/kg/day), 24 mg/kg/day of cocoa enriched with 60% of polyphenols (intermediate dose, i.e., 2.9 mg polyphenols/kg/day), 190 mg/kg/day of cocoa enriched with 60% (high dose, i.e., 22.8 mg polyphenols/kg/day) (*Barry Callebaut*, Louviers, France) or water by gavage daily for 16 weeks (Supplementary Fig. 2). Similarly, nondiabetic rats were randomized to the same above treatment regimen. After treatment, the animals were submitted to electroretinogram (ERG) and then euthanized; the eye globes and retinas were collected. During the study, the diabetic rats received 2 U of insulin (human insulin HI-0310; Eli Lilly and Company, Indianapolis, IN, USA), three times per week, subcutaneously. The control rats received the vehicle only. Systolic blood pressure was obtained by tail-cuff plethysmography (MK III Physiograph; Narco Bio-Systems, Houston, TX, USA).

2.1.11. Immunohistochemistry in retinal tissues

The retinal sections were incubated with goat polyclonal anti-GFAP (Santa Cruz), rabbit polyclonal antioccludin (Invitrogen Corp., Camarillo, CA, USA) or rabbit polyclonal antinitrotyrosine (Upstate Cell Signaling) overnight at 4 °C. The slides were then incubated with the appropriate secondary antibodies. The analyses were performed using the Leica Application Suite (Leica Microsystems, Wetzlar, Germany) in nine nonconsecutive retinal sections divided among three slides per animal per group under high-powered microscopic viewing (Zeiss).

2.1.12. Full-flash ERG recording

Retinal function was measured at the end of the study using the UTAS-E3000 system (LKC Technologies Inc., Gaithersburg, MD, USA) as previously described [35]. The pupils were dilated with tropicamide (Mydriacyl 0.5%; Allergan, Irvine, CA, USA). General anesthesia was induced with ketamine and xylazine (75 and 7.5 mg/kg, respectively) under dim red illumination (λmax=650 nm). The measurements were taken after overnight dark adaptation. An intensity-response series was recorded using a series of ganzfeld flashes with intensities ranging from −3.60 to 2.40 log cd-s/m^2 luminance. Recordings were amplified and digitized using a 24-bit A/D converter band passed from 0.3 to 300 Hz with a 50-Hz notch filter.

2.1.13. Liquid chromatography (LC)/mass spectrometry (MS) and tandem mass spectrometry (MS/MS) assays

The sample preparation was performed as previously reported [36,37]. Plasma or retinal samples were drawn into EDTA-containing vials, supplemented with ascorbate (1 mg/ml) and snap-frozen in liquid nitrogen. For HPLC analysis, samples were defrosted on ice and prepared by using a three-step procedure: (a) Samples were mixed with twice their volume of acidified methanol (100% vol/vol, −20 °C, internal standard A=3′EthylEC) and centrifuged at 17,000×g for 15 min at 4 °C. (b) The supernatant was collected, and the pellet was resuspended in methanol as detailed for step 1. This resuspension was centrifuged, the supernatant was collected, and the pellet was washed in methanol (50% vol/vol, internal standard A). After centrifugation, the supernatant was collected and combined with the supernates from steps 1 and 2. This mixture was centrifuged (as above), and the solvents were removed by using a rotary evaporation system at 4 °C.

2.1.14. Statistical analysis

The results were expressed as the mean±S.D. Comparisons between groups were done using analysis of variance followed by Fisher's protected least significant difference test. For correlation analyses, the Pearson correlation coefficient test was used. The analyses were performed using Statview Software (SAS Institute Inc., Cary, NC, USA). Significance was determined at P<.05.

3. Results

3.1. In vitro studies

3.1.1. Polyphenol-enriched cocoa provides protection from glial reaction via SIRT1 in rMC-1

The rMC-1 exposed to HG medium displayed a marked increase in GFAP immunolabeling. The treatment with cocoa prevented the glial

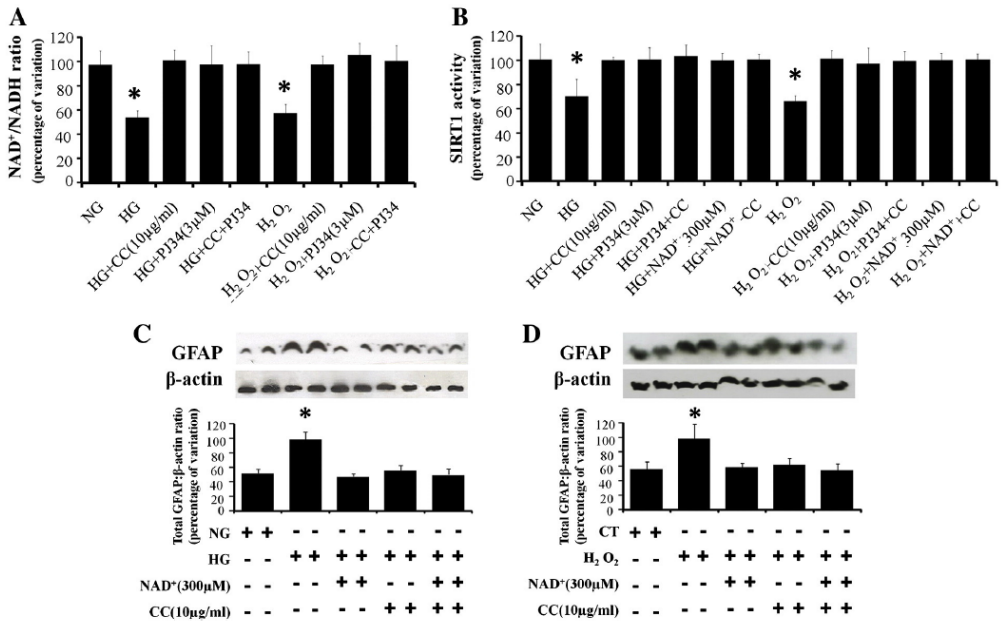

Fig. 4. PARP blocker or NAD^+ treatment increases SIRT1 activity and prevents glial reaction in rMC-1 under HG or H_2O_2 condition. (A) NAD^+/NADH ratio levels in total rMCs lysates cultured for 24 h; *P<.0001. (B) SIRT1 activity evaluated by fluorometric assay in total rMCs lysates; *P<.0001. The values are expressed in mean±S.D. of percentage of absorbance units. (C and D) Representative Western blots for GFAP expression in total cell lysates under HG or H_2O_2 treated with cocoa or NAD^+ supplementation. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean±S.D. of band densities expressed as the percentage of variation from at least three independent experiments; *P<.0001 and *P<.0005, respectively. CC: polyphenol-enriched cocoa; NAD^+: exogenous NAD^+ (300 µM); PJ-34: PARP blocker (3 µM).

reaction in a dose-dependent manner, and the presence of EX-527, a SIRT1-specific blocker, or SIRT1 siRNA abolished the observed effects (Fig. 1A and B, respectively). Similarly, the rMCs exposed for 24 h to H_2O_2, a potent oxidizer, and treated with cocoa had a marked prevention of glial reaction in a dose-dependent manner, and the presence of EX-527 abolished the effects (Supplementary Figs. 2 and 1C). These findings suggest that the insults of HG and H_2O_2 induce GFAP up-regulation in rMCs, and the protective effects of cocoa treatment are at least partly mediated by SIRT1 pathway.

3.1.2. Polyphenol-enriched cocoa decreases PAR-modified proteins and reestablishes NAD^+/NADH ratio by redox balance in rMC-1

In HG conditions, there was a significant increase in total intracellular ROS (P=.0001); the treatment with cocoa decreased it in a dose–response manner, leading to normal levels (P>.6 vs. NG condition). Similar effects were observed in cells under H_2O_2 conditions (Fig. 2A). PARP1 is involved in the regulation of poly(ADP)-ribosylation using NAD^+ as a donor of ADP-ribose units. Therefore, PARP1 has profound effects on SIRT1 activity since both enzymes require NAD^+ as a substrate [38]. The PARP-1 activity was assessed through quantification of PAR-modified proteins in rMCs. Either in HG or in H_2O_2 conditions, there were marked increases in PAR-modified proteins (P<.0001), which were abolished by cocoa treatment (Fig. 2B, C). Consequently, the intracellular NAD^+/NADH ratio was decreased in HG and H_2O_2 conditions (P<.0001) and restored to normal levels in the presence of cocoa (Fig. 2D). This data set indicates that, in HG/H_2O_2 conditions, the intracellular NAD^+/NADH ratio is decreased due to PARP-1 activation. The high-dose cocoa treatment prevented this detriment, which lessened PARP-1 activation and ameliorated NAD^+ levels.

3.1.3. Polyphenol-enriched cocoa improves SIRT1 activity, inducing deacetylation of Lys310-RelA/p65 in rMC-1

By immunofluorescence assay, SIRT1, which is a nuclear factor when in active form, is highly expressed in normal conditions. The presence of HG or H_2O_2 induced a marked decrease in its nuclear expression, accompanied by an increased translocation to cytosol; the treatments with cocoa reestablished the nuclear positiveness in a dose-dependent manner in both HG (Fig. 3A) and H_2O_2 (Supplementary Fig. 3A). SIRT1 activity evaluated through a fluorometric assay revealed that rMC-1 cells exposed to the HG or H_2O_2 media have lower SIRT1 activity (P<.0001). The presence of cocoa prevented this abnormality in both conditions (Fig. 3B), and the cotreatment with siRNA diminished SIRT1 activity by 60% compared to normal conditions. The combination of cocoa and siRNA in cells exposed to HG completely abolished the effect observed with cocoa alone (Fig. 3B) (P<.0001). Similar effects were observed in cells under HG or H_2O_2 in the presence of SIRT1 blocker EX-527 treated with cocoa or not (Supplementary Fig. 3B).

To address the SIRT1 activity, one target of SIRT1 deacetylation, the Lys310-RelA/p65 complex, was assessed. Cells exposed to HG or H_2O_2 media displayed a sixfold increment of Lys310-RelA/p65 acetylation, and the treatment with cocoa reduced it to normal levels (Fig. 3C–E). The presence of siRNA or SIRT1 inhibitor completely abolished this effect. This set of observations indicated that the marked reduction in SIRT1 activity in rMCs observed in both HG and H_2O_2 conditions led to

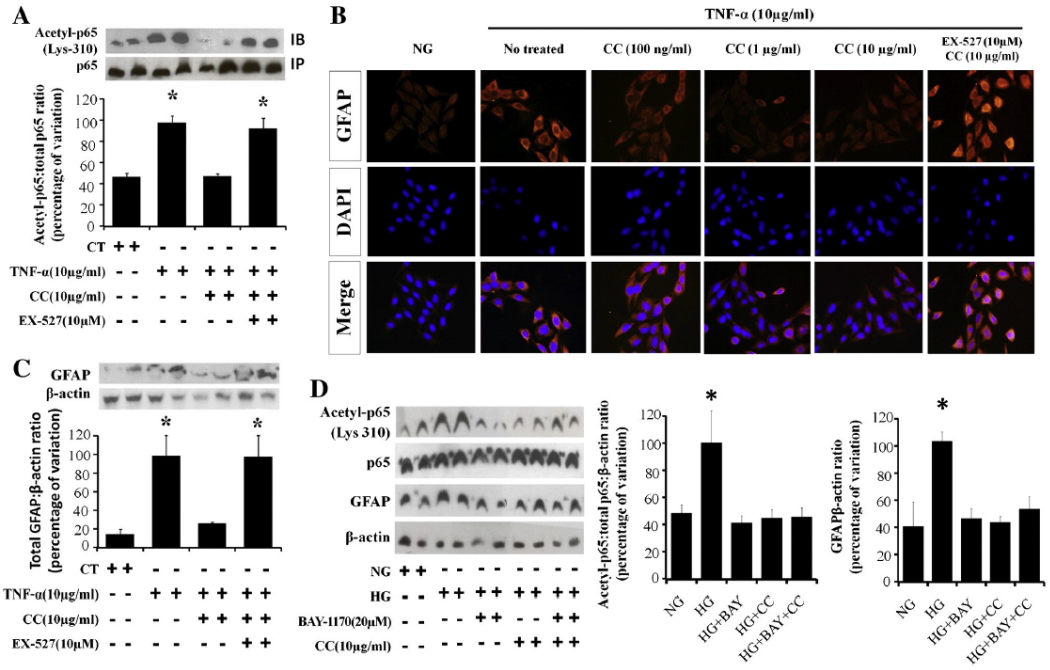

Fig. 5. GFAP was reduced by polyphenol-enriched cocoa through SIRT1 via deacetylation of p65-lys310. (A) Protein extract immunoprecipitated with anti acetyl-Lys310-p65 subunit in rMC-1 cultured for 24 h with TNF-α (positive control for p65 subunit acetylation) and treated with cocoa and/or EX-527. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for total p65 subunit; *P<.0001. (B) Immunofluorescence for GFAP in rMC-1. The cells were exposed to TNF-α in the presence of cocoa and EX-527. (C) Western blots for GFAP in total cell lysates; the cells were treated with cocoa and/or EX-527. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for total p65 subunit; *P<.02. (D) A representative Western blot of total cell lysates under NG, HG in the presence or not of BAY-1170 (NF B inhibitor) and/or cocoa against acetyl-Lys310-p65, total p65 subunit and GFAP. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin; *P<.0001. The bars represent mean±S.D. of band densities expressed as the percentage of variation in at least three independent experiments. TNF-α: exogenous tumor necrosis factor-α (10 μg/ml); CC: polyphenol-enriched cocoa; BAY-1170: NFkB blocker (20 μM).

an increase of acetyl-Lys310-RelA/p65, and this cascade of events was prevented by cocoa treatment.

3.1.4. PARP blocker or NAD$^+$ supplementation increases SIRT1 activity and prevents glial reaction in rMC-1 under oxidative imbalance

SIRT1, poly(ADP)-ribose polymerase-1 is a NAD$^+$-dependent enzyme. Based on this, the cell was treated with PJ-34, a specific blocker of PARP1 that competes with NAD$^+$ to inhibit at the catalytic site of PARP1 [39]. The efficiency of PARP1 blockage in rMCs is demonstrated in Supplementary Fig. 4. The cells exposed to HG or H_2O_2 and treated with PJ-34 revealed PAR-modified proteins restored to normal levels (P<.001). Similarly, in cells exposed to HG or H_2O_2 conditions, the presence of PJ-34 prevented the decrease in the NAD$^+$/NADH ratio similar to cocoa alone or in combination (P<.0001). This indicates that HG or H_2O_2 conditions induce a significant reduction in the intracellular NAD$^+$ pool (Fig. 4A).

Concordant with NAD$^+$/NADH ratios in the tested conditions, the SIRT1 activity decreased its levels in HG or H_2O_2 conditions; the treatment with cocoa in combination or not with PJ34 restored this effect in both conditions. The exogenous administration of NAD$^+$ in cells under HG or H_2O_2 equally restored SIRT1 activity, and the cotreatment with cocoa did not add further effect (Fig. 4B). To exclude possible antioxidant properties of both PJ-34 and NAD$^+$ supplementation, the total intracellular ROS was measured. In rMC-1 exposed to HG or H_2O_2, the treatment with PJ34 or NAD$^+$ did not reduce ROS production in these cells (Supplementary Fig. 4), demonstrating their direct effect on SIRT1-deacetylase activity independent of ROS production.

The GFAP expression was assessed to demonstrate the sequence of events from the NAD$^+$ intracellular pool leading to proper SIRT1 activity that protects the rMCs from glial activation. The cells exposed to HG or H_2O_2 conditions exhibited significant increases in GFAP protein expression, which reverted to normal levels in the presence of NAD$^+$ supplementation, cocoa or both (P<.0001) (Fig. 4C, D). Taken together, these data suggest that cocoa, through PARP1 reduction, ameliorates NAD$^+$ availability and increases SIRT1 activity, thus reducing glial reaction.

3.1.5. Polyphenol-enriched cocoa down-regulates GFAP in rMC-1 through deacetylation of Lys310-RelA/p65 via SIRT1 pathway

To study the mechanism whereby cocoa down-regulates GFAP expression, the rMC-1 was treated with TNF-α or BAY-1170. It is well known that TNF-α activates NF-kB by acetylation of Lys310 in RelA/p65 subunit, the same acetylation site of SIRT1 on NF-kB [40]. Based on this evidence, the cells were treated with TNF-α, and the acetylation on Lys310-RelA/p65 was verified. TNF-α increased the Lys310-p65 acetylation, and the treatment with cocoa was effective in reducing it (P<.0001). The cotreatment with SIRT1 inhibitor (EX-527) abolished the effects of cocoa on the rMCs exposed to TNF-α (Fig. 5A).

To investigate whether TNF-α treatment through acetylation on Lys310-RelA/p65 induces GFAP up-regulation, an immunostaining assay was conducted. The GFAP immunostaining markedly increased

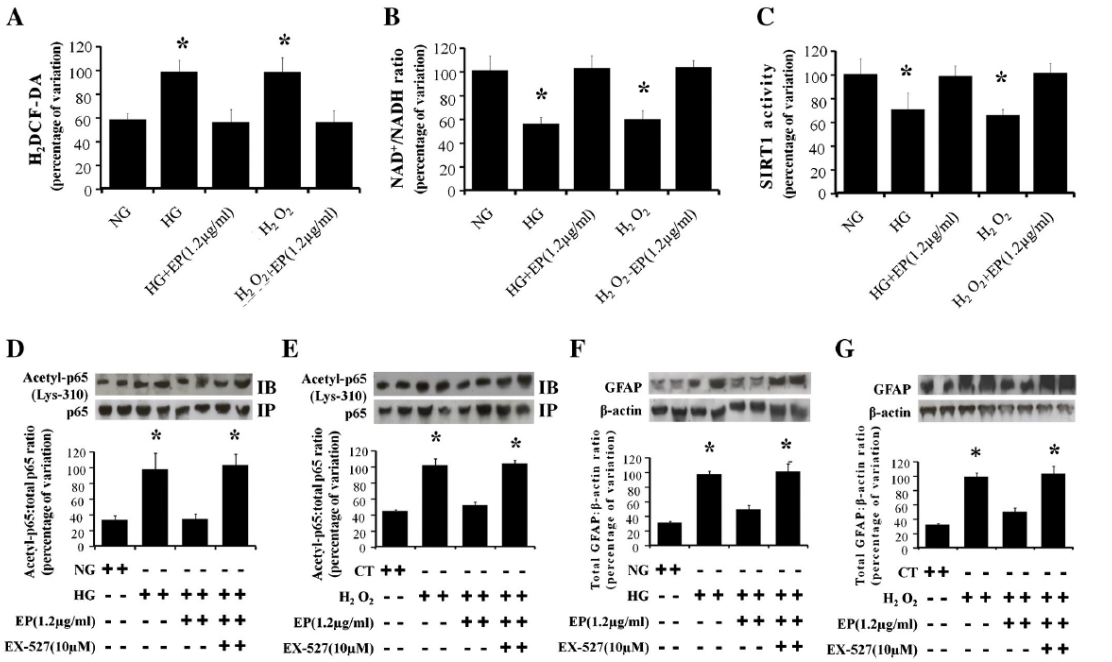

Fig. 6. The effect of (−)-epicatechin in rMCs is through SIRT-1 activation. (A) Total intracellular ROS levels quantified by H_2DCF-DA in rMC-1 cultured for 24 h. The values are means± S.D. of fluorescence units; *P<.0001. (B) NAD^+/NADH ratio in rMC-1 cultured for 24 h in total cell lysates; P<.0001. (C) SIRT1 activity in rMC-1 cultured for 24 h in total cell lysates; *P<.001. The values are means±S.D. and expressed as percentages of fluorescence units. (D and E) Immunoprecipitation of retinal proteins with anti-acetyl-Lys310-p65 subunit; *P<.0001. (F and G) Representative Western blots for GFAP in total cell lysate; *P<.001. EP: (−)-epicatechin (1.2 µg/ml); EX-527, SIRT1 blocker (10 µM).

in cells under TNF-α, which was prevented by cocoa treatments in a dose-dependent manner (Fig. 5B). The presence of EX-527 prevented the protective effects of cocoa treatment. These findings were confirmed through Western blot (Fig. 5C). To confirm the modulation of Lys310-RelA/p65 acetylation on glial reaction, the cells were treated with a blocker of NF-kB under HG BAY-1170 blocks NF-kB signaling pathway by inhibition of IkB-k phosphorylation [41]. As expected, the BAY-1170 was able to significantly reduce the acetylation of p65 subunit and the GFAP up-regulation in HG condition (Fig. 5D) (P<.0001). The combined treatments did not confer additional effect compared to treatments alone. These results indicate that cocoa down-regulates GFAP in rMC-1 through deacetylation of Lys310 RelA/p65 subunit- NF-kB complex via SIRT1 pathway.

3.1.6. The (−)-epicatechin effect in rMCs

Ultra-performance liquid chromatography analyses of the cocoa extract showed that epicatechin is the more abundant catechin present in cocoa extract used in this work (Supplementary Fig. 1B).

To assure whether (−)-epicatechin is responsible in part for the effects observed in this study, the cells were exposed to (−)-epicatechin in the same concentrations found in the cocoa extract used herein. Like the cocoa-extract findings, the presence of (−)-epicatechin in rMCs exposed to HG or H_2O_2 decreased ROS production (P<.0001) (Fig. 6A). The treatment with (−)-epicatechin also increased the intracellular pool of NAD^+ (P<.0001) (Fig. 6B) and restored SIRT1 activity (P<.0009) (Fig. 6C). The treatment with (−)-epicatechin was able to reduce acetyl-Lys310-p65 (P<.0003) (Fig. 6D and E, respectively) and to prevent glial reaction by down-regulation of GFAP expression (P<.001) in cells under HG or H_2O_2 conditions (Fig. 6F and G, respectively). These observations suggest that the effects of cocoa herein described may be attributed, in part, to its (−)-epicatechin content.

3.1.7. Animal studies

The final body weights were lower and blood glucose levels higher in diabetic rats compared to nondiabetic control groups (P<.001) (Supplementary Table 1). None of the treatment regimens altered body weight or blood glucose levels.

3.1.8. Polyphenol-enriched cocoa protects retinal function in diabetic rats in a dose-dependent manner

To determine whether polyphenol-enriched cocoa is capable of preventing visual disturbances, retinal function was estimated through full-flash ERG. Diabetic rats showed a significant decrease in the amplitude of the *b*-waves accompanied by an increase in implicit time responses (P<.001) (Fig. 7A). The low-dose treatment did not improve these parameters; however, intermediate and high doses protected the retinal function in a dose-dependent manner, suggesting that polyphenol cocoa is effective in promoting improvement of inner retinal function in SHR diabetic rats.

3.1.9. Oral administration of cocoa ameliorates early markers and oxidative damage in diabetic rat retinas in a dose-dependent manner

The presence of early diabetes exacerbates GFAP immunolabeling throughout the retinal layers (P<.0001) (Fig. 7B). In a dose-dependent manner, the oral treatment with intermediate- and high-dose cocoa regimens prevented glial reactions in diabetic retina.

Nitrotyrosine expression was assessed as a marker of oxidative stress tissue damage. There was a marked increase in retinal tissue in diabetic compared to nondiabetic control animals (P<.0001) (Fig. 7C).

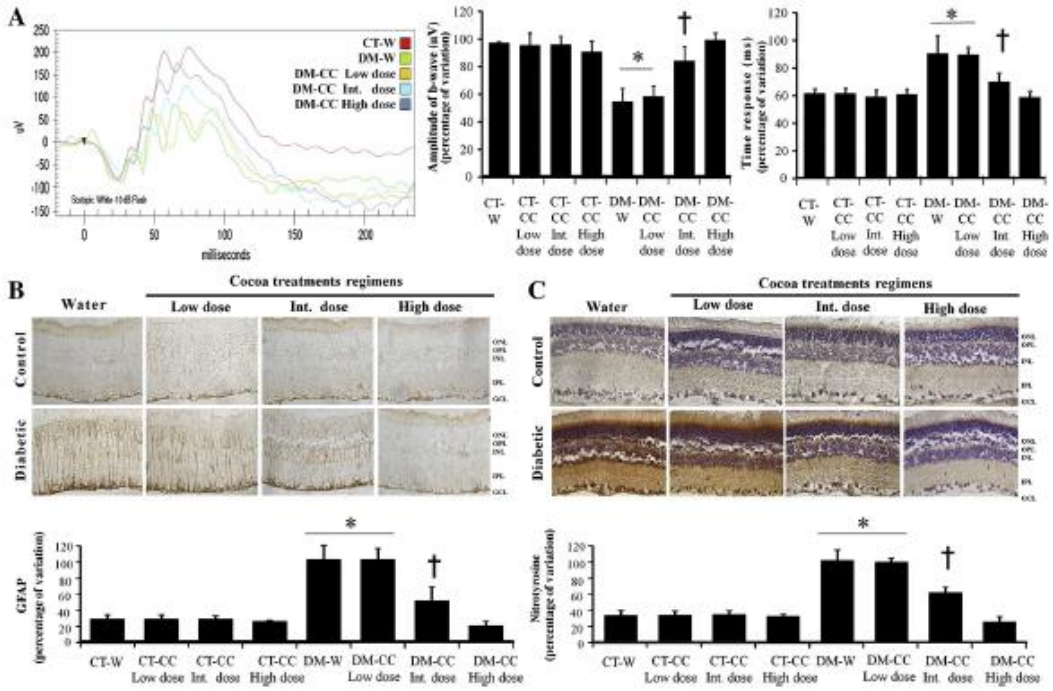

Fig. 7. Polyphenol-enriched cocoa protects in a dose–response the retinal function, ameliorates the early marker of DR and prevents oxidative stress in diabetic rats. (A) Representative waveforms for *b*-waves in the studied rats. The *b*-wave is a positive deflection generated in part by the Müller and mainly by the bipolar cell potentials. The figure shows representative full-flash ERG waveforms at −20dB for a nondiabetic (red), diabetic (green) and diabetic treated with cocoa by the following treatment regimen: low dose (yellow), intermediate dose (light blue) and high dose (dark blue). The bars represent with all groups the mean amplitude and implicit time of *b*-waves, expressed in microvolts (μV) and milliseconds (ms); *$P<.0001$ and †$P<.001$ vs. CT-W. (B) A representative photomicrograph of glial reactivity revealed by GFAP immunolabeling in rat retinas. Magnification: ×400. The bars represent mean±S.D. for the percentage of GFAP-positive retinal cells per mm^2 of retina; *$P<.0001$, †$P=.007$. (C) A representative photomicrograph of immunolocalization of nitrotyrosine (NT) in retinal tissue. NT is a stable product formed from the reaction of peroxynitrate with tyrosine residues and is accepted as an index of nitrosative damage. The bars represent mean±S.D. for the percentage of nitrotyrosine-positive retinal cells/mm^2 of retina; *$P<.0003$, †$P=.02$. CT-W: nondiabetic treated with water; CT-CC Low dose: nondiabetic treated with low dose of cocoa (approximately 0.12 mg of polyphenols/kg/day); CT-CC Int dose: nondiabetic treated with intermediate dose of cocoa (approximately 2.9 mg of polyphenols/kg/day); CT-CC High dose: nondiabetic treated with high dose of cocoa (approximately 22.8 mg of polyphenols/kg/day); DM-W: diabetic treated with water; DM-CC Low dose: diabetic treated with low dose of cocoa (approximately 0.12 mg of polyphenols/kg/day); DM-CC Int dose: diabetic treated with intermediate dose of cocoa (approximately 2.9 mg of polyphenols/kg/day); DM-CC High dose: diabetic treated with high dose of cocoa (approximately 22.8 mg of polyphenols/kg/day).

Cocoa completely restored retinal nitrosative status to nondiabetic levels in rats in the high-dose regimen group. These findings suggest that cocoa exerts antioxidant effects in the early phases of DR, thus indicating a possible neuroprotective effect in diabetic retina.

3.1.10. Polyphenol-enriched cocoa improves SIRT1 activity inducing deacetylation of Lys310-RelA/p65 through PARP1 reduction in diabetic rats

To verify whether the beneficial effects of cocoa are mediated by the SIRT1 pathway in diabetic retina, SIRT1 activity was assessed. The SIRT1 activity was decreased in retinal tissue from diabetic rats ($P<.0001$) (Fig. 8A). Oral treatments with higher-dose polyphenol cocoa preparations prevented SIRT1 down-regulation in a dose-response manner, thus improving its activity. Diabetic rats also showed a significant reduction in NAD^+ intracellular levels in retinal tissue, which were restored by oral supplementation of higher-dose cocoa treatments ($P<.001$) (Fig. 8B).

In total retinal lysates, the PAR-modified proteins were markedly increased in diabetic rats ($P<.001$), and the oral administration of cocoa prevented this increase (Fig. 8C). Finally, this study addressed the acetylation of Lys310-RelA/p65. Diabetes increased the acetylation of Lys310-p65 in retinal tissue ($P<.0003$), and oral administration with cocoa in its intermediate- and high-dose regimens reduced this (Fig. 8C).

This sequence of events, as demonstrated by *in vitro* studies, indicates that intermediate and high doses of polyphenol cocoa are effective in improving SIRT1 activity through modulation of PARP-1 activity, thus increasing NAD^+ availability. This leads to deacetylation of Lys310-p65, which in turn down-regulates GFAP expression in the retinal tissue of diabetic rats. Of interest, besides the concentration of polyphenols, the other cocoa compounds such as xanthines are present in the same concentration in both low- and intermediate-dose regimens. This indicates that it is possible that the polyphenol cocoa content is the main compound which provides the above-described affect. At the high-dose regimen, 17.8 mg/kg/day of xanthines was offered. For this reason, we cannot rule out the contribution of other cocoa compounds on these protective effects at the high-dose regimen.

3.1.11. Epicatechin levels in the plasma and retinal tissue

Since epicatechin was found to be responsible at least in part for the observed effects, we verified the content of epicatechin in plasma and possibly in retinal tissue. Nondiabetic control animals ($n=10$) were treated with the high-dose regimen for 20 days. At the end of the treatment, the animals were anesthetized and perfused with

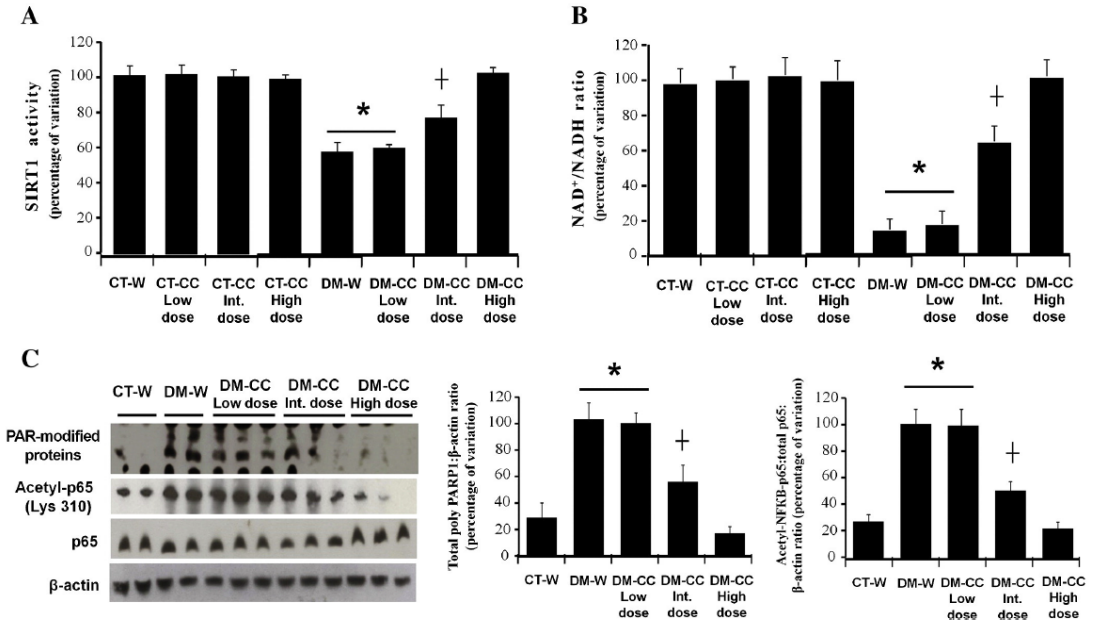

Fig. 8. The polyphenol-enriched cocoa improved in a dose–response manner SIRT1, inducing deacetylation of Lys 310-p65 through reduction in PARP activity in diabetic rats. (A) SIRT1 activity in total retinal lysates measured through fluorimetric assay. The values are means±S.D. expressed as percentages of fluorescence units. The presence of diabetes reduced to 62.5% the SIRT1 activity, and the cocoa treatment fully restored it to normal levels in a dose–response manner; *$P<.0001$, †$P=.007$. (B) NAD^+/NADH ratio in total retinal lysates. The NAD^+/NADH ratios are means±S.D. and expressed as percentages of absorbance units; *$P<.0001$, †$P=.001$ (C) Representative Western blots for PAR-modified proteins in total retinal lysates. In diabetic rats, the amount of PAR modified proteins was increased compared with that in nondiabetic rats, and the oral administration of cocoa prevented this effect at intermediate- and high-dose regimens; *$P<.0001$, †$P=.002$. Western blot analysis of acetyl-Lys310-p65 subunit-NF B complex in total retinal lysates. The presence of DM induced acetylation of p65 subunit, and cocoa prevented it partially in intermediate-dose and completely in higher-dose regimen; *$P<.0002$, †$P=.005$. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean±S.D. of band densities expressed as the percentage of variation from at least three independent experiments.

phosphate-buffered saline buffer, the retinas collected, and the serum separated by centrifugation. The samples were submitted to LC-MS and LC-MS/MS analysis (see methods at Supplemental Material). The epicathechin content measured in plasma sample was 422.84 μg of epicatechin/ml and 4.42 μg of epicatechin/mg of retinal tissue (Table 1 and Supplementary Fig. 5). This is the first evidence that epicatechin can cross the blood retinal barrier and be found in the retinal tissues of animals treated with oral administration of cocoa extract.

4. Discussion

The current study aimed to investigate whether cocoa could protect the diabetic retina from glial reaction and whether the SIRT1 pathway could be involved. Glial reaction, a marker of retinal neurodegeneration present in early DR, is characterized by overexpression of GFAP; nevertheless, the mechanism underlying this process is not yet fully identified. Further, this research explored the mechanisms by which increased polyphenol contents in cocoa extracts could mitigate GFAP up-regulation, both *in vitro* and *in vivo*. It was demonstrated that, in rMCs exposed to HG and in diabetic retina, PARP-1 is activated due to oxidative imbalance, thus consuming the intracellular NAD^+ and diminishing SIRT1 activity. As a result, Lys310-RelA/p65 subunit NF-kB complex is acetylated. As NF-kB is a key redox-sensitive transcription factor, it transcribes the proinflammatory gene of GFAP. This is the first demonstration that GFAP up-regulation depends on the acetyl Lys310-RelA/p65 through SIRT1 activity. The treatment with polyphenol-enriched cocoa reduced reactive oxygen species formation, thus inhibiting the poly(ADP)-ribosylation of nuclear proteins by PARP-1. Consequently, the NAD^+/NADH ratio was improved, and SIRT1 became activated, deacetylating Lys310-RelA/p65 and inhibiting its transactivation capacity. In the animal study, increased polyphenol-enriched cocoa extracts conferred neuroprotection in diabetic retinas in a dose-dependent manner. This effect, estimated by GFAP expression (the major end point in this study), is partly dependent on polyphenols, mainly epicatechin compound. The epicatechin molecule was detected in retinal tissue by LC/MS and MS/MS methods. This dose of ACTICOA was already applied in a previous experimental study [42].

The ability of DNA binding and further transcriptional activity of NFkB is dependent on the acetylation of its lysine residues [43,44]. The activation of the p65 subunit occurs posttranslationally, including phosphorylation and acetylation. Acetylation of the lysine residues in

Table 1
Analyses of epicatechin in plasma and retinal tissue from rats.

		Epicatechin (μg)	
Samples	*n*	Mean	S.E.M.
Plasma (ml)	10	422.84	76.36
Retina (mg)	5	4.42	0.459

The peak area of plasma or retina sample was calculated, resulting in the amount of epicatechin (μg) per milliliter (ml) of plasma or per milligram (mg) of retinal tissue. The amount of epicatechin per mg of tissue was calculated based on the weight of frozen retina.

p65 increases its DNA binding activity, coactivator recruitment and GFAP transcription. On the other hand, the deacetylation of NF-kB subunit p65 leads to a decrease in its transcription activity, thereby reducing production of proinflammatory cytokines and antiapoptotic genes [21]. The RelA/p65-NF-kB subunit has been identified as one of many substrates for SIRT1 [45], including E2F, p53 and FOXO [46–48]. In the present study, the interaction between acetyl-Lys310-RelA/p65 and GFAP is demonstrated through treatment with BAY, a blocker of the NF-kB signaling pathway, reducing GFAP expression on rMCs.

The regulation of SIRT1 by oxidative stress is complex, involving direct and indirect redox sensitive mechanisms through its post-translational modifications. Based on the data collected, the presence of HG or H_2O_2 increased GFAP expression and PAR-modified proteins accompanied by SIRT1 expression and activity impairment in rMCs. Consequently, acetyl-Lys310-RelA/p65 NF-kB complex was increased and the NAD^+/NADH ratio impaired. The treatment with polyphenol-enriched cocoa reversed GFAP and SIRT1 expressions in a dose-dependent manner and restored the intracellular NAD^+/NADH ratio, a redox sensitive cofactor, and the PAR-modified protein levels. The interaction between SIRT1 and PARP1 activity that has already been demonstrated in other situations [49] is herein disclosed. The SIRT1 siRNA or cotreatment with EX-527 is an important tool and enables better understanding of the cross talk between SIRT1 activity and GFAP expression. The presence of siRNA or EX-527 abolished the all-protective effect observed with cocoa treatment in rMCs under HG, suggesting that SIRT1 is directly or indirectly regulating GFAP expression in these cells.

In summary, oral administration of polyphenol-enriched cocoa extracts conferred retinal neuroprotection in a dose-dependent manner. The down-regulation of histone deacetylase SIRT1 abrogating the effect of cocoa on GFAP up-regulation and on Lys310-RelA/p65 acetylation by silencing or blockage was clearly demonstrated herein *in vivo* and *in vitro*. As GFAP (one indirect SIRT1 target) is altered in diabetic retinal neurodegeneration, compounds, such as cocoa, that modulate the activity of sirtuins are promising therapeutic strategies for future clinical trials.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2014.09.003.

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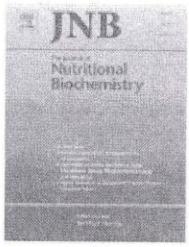
References

[1] Klein R, Klein BE, Moss SE, Wong TY. The relationship of retinopathy in persons without diabetes to the 15-year incidence of diabetes and hypertension: Beaver Dam Eye Study. Trans Am Ophthalmol Soc 2006;104:98–107.
[2] Asnaghi V, Gerhardinger C, Hoehn T, Adeboje A, Lorenzi M. A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. Diabetes 2003;52:506–11.
[3] Duarte DA, Silva KC, Rosales MA, Lopes de Faria JB, Lopes de Faria JM. The concomitance of hypertension and diabetes exacerbating retinopathy: the role of inflammation and oxidative stress. Curr Clin Pharmacol 2013;8:266–77.
[4] Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 1998;102:783–91.
[5] Bringmann A, Iandiev I, Pannicke T, Wurm A, Hollborn M, Wiedemann P, et al. Cellular signaling and factors involved in Müller cell gliosis: neuroprotective and detrimental effects. Prog Retin Eye Res 2009;28:423–51.
[6] Vincent JA, Mohr S. Inhibition of caspase-1/interleukin-1β signaling prevents degeneration of retinal capillaries in diabetes and galactosemia. Diabetes 2007;56:224–30.
[7] Nagai N, Izumi-Nagai K, Oike Y, Koto T, Satofuka S, Ozawa Y, et al. Suppression of diabetesinduced retinal inflammation by blocking the angiotensin II type 1 receptor or its downstream nuclear factor-kappaB pathway. Invest Ophthalmol Vis Sci 2007;48:4342–50.
[8] Rine J, Strathern JN, Hicks JB, Herskowitz I. A suppressor of mating-type locus mutations in *Saccharomyces cerevisiae*: evidence for and identification of cryptic mating-type loci. Genetics 1979;93:877–901.
[9] Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev 1999;13:2570–80.
[10] Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. Science 2000;289:2126–8.
[11] Chung S, Yao H, Caito S, Hwang JW, Arunachalam G, Rahman I. Regulation of SIRT1 incellular functions: role of polyphenols. Arch Biochem Biophys 2010;501:79–90.
[12] Rahman I, Kinnula VL, Gorbunova V, Yao H. SIRT1 as atherapeutic target in inflammaging of the pulmonary disease. Prev Med 2012;54:S20–8.
[13] Yao H, Rahman I. Perspectives on translational and therapeutic spects of SIRT1 in inflammaging and senescence. Biochem Pharmacol 2012;84:1332–9.
[14] Yao H, Chung S, Hwang JW, Rajendrasozhan S, Sundar IK, Dean DA, et al. SIRT1 protects against emphysema via FOXO3-mediated reduction of pre-mature senescence in mice. J Clin Invest 2012;122:2032–45.
[15] Dioum EM, Chen R, Alexander MS, Zhang Q, Hogg RT, Gerard RD, et al. Regulation of hypoxia-inducible factor 2 alpha signaling by the stress-responsive deacetylase sirtuin1. Science 2009;324:1289–93.
[16] Bordone L, Guarente L. Calorie restriction, SIRT1and metabolism: understanding longevity. Nat Rev Mol Cell Biol 2005;6:298–305.
[17] Toledano MB, Leonard WJ. Modulation of transcription factor NF-kappa B binding activity by oxidation–reduction in vitro. Proc Natl Acad Sci 1991;88:4328–32.
[18] Chen LF, Williams SA, Mu Y, Nakano H, Duerr JM, Buckbinder L, et al. NF-kappaB RelA phosphorylation regulates RelA acetylation. Mol Cell Biol 2005;25:7966-797.
[19] Chen LF, Mu Y, Greene WC. Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB. EMBO J 2002;21:6539–48.
[20] Lanzillotta A, Sarnico I, Ingrassia R, Boroni F, Branca C, Benarese M, et al. The acetylation of RelA in Lys310 dictates the NF-kappaB-dependent response in post-ischemic injury. Cell Death Dis 2010;1:e107.
[21] Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J 2004;23:2369–80.
[22] Grassi D, Necozione S, Lippi C, Croce G, Valeri L, Pasqualetti P, et al. Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. Hypertension 2005;46:398–405.
[23] Taubert D, Roesen R, Lehmann C, Jung N, Schömig E. Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: a randomized controlled trial. JAMA 2007;298:49–60.
[24] Desch S, Schmidt J, Kobler D, Sonnabend M, Eitel I, Sareban M, et al. Effect of cocoa products on blood pressure: systematic review and meta-analysis. Am J Hypertens 2010;23:97–103.
[25] Rimbach G, Melchin M, Moehring J, Wagner AE. Polyphenols from cocoa and vascular health: a critical review. Int J Mol Sci 2009;10:4290–309.
[26] Siddique HR, Liao DJ, Mishra SK, Schuster T, Wang L, Matter B, et al. Epicatechin-rich cocoa polyphenol inhibits kras-activated pancreatic ductal carcinoma cell growth in vitro and in a mouse model. Int J Cancer 2012;131:1720–31.
[27] Ejaz Ahmed M, Khan MM, Javed H, Vaibhav K, Khan A, Tabassum R, et al. Amelioration of cognitive impairment and neurodegeneration by catechin hydrate in rat model of streptozotocin-induced experimental dementia of Alzheimer's type. Neurochem Int 2013;62:492–501.
[28] Kang KS, Yamabe N, Wen Y, Fukui M, Zhu BT. Beneficial effects of natural phenolics on levodopa methylation and oxidative neurodegeneration. Brain Res 2013;1497:1–14.
[29] Al-Gayyar MM, Matragoon S, Pillai BA, Ali TK, Abdelsaid MA, El-Remessy AB. Epicatechin blocks pro-nerve growth factor (proNGF)-mediated retinal neurodegeneration via inhibition of p75 neurotrophin receptor expression in a rat model of diabetes. Diabetologia 2011;54:669–80.
[30] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[31] Xu Y, Nie L, Yin YG, Tang JL, Zhou JY, Li DD, et al. Resveratrol protects against hyperglycemia-induced oxidative damage to mitochondria by activating SIRT-1 in rat mesangial cells. Toxicol Appl Pharmacol 2012;259:395–401.
[32] Silva KC, Rosales MA, Hamassaki DE, Saito KC, Faria AM, Ribeiro PA, et al. Green tea is neuroprotective in diabetic retinopathy. Invest Ophthalmol Vis Sci 2013;54:1325–36.
[33] Amadio M, Scapagnini G, Laforenza U, Intrieri M, Romeo L, Govoni S, et al. Post-transcriptional regulation of HSP70 expression following oxidative stress in SH-SY5Y cells: the potential involvement of the RNA-binding protein HuR. Curr Pharm Des 2008;14:2651–8.
[34] Goto T, Yoshida Y, Kiso M, Nagashima H. Simultaneous analysis of individual catechins and caffeine in green tea. J Chromatogr 1996;749:295–9.
[35] Bui BV, Armitage JA, Tolcos M, Cooper ME, Vingrys AJ. ACE inhibition salvages the visual loss caused by diabetes. Diabetologia 2003;46:401–8.
[36] Paudel L, Wyzgoski FJ, Scheerens JC, Chanon AM, Reese RN, Smiljanic D, et al. Nonanthocyanin secondary metabolites of black raspberry (*Rubus occidentalis* L.) fruits: identification by HPLC-DAD, NMR, HPLC-ESI-MS, and ESI-MS/MS analyses. J Agric Food Chem 2013;61:12032–43.
[37] Qiao Shi, Shi Rui, Liu Man, Zhang Chong, Yang Wei, Shi Xiaowei, et al. Simultaneous quantification of flavonoids and phenolic acids in Herba Scutellariae barbatae and its confused plants by high performance liquid chromatographytandem mass spectrometry. Food Chem 2011;129:1297–304.

[38] KolthurSeetharam U, Dantzer F, McBurney MW, de Murcia G, Sassone-Corsi P. Control of AIF-mediated cell death by the functional inter play of SIRT1 and PARP-1 inresponse to DNA damage. Cell Cycle 2006;5:873–7.
[39] Madison DL, Stauffer D, Lundblad JR. The PARP inhibitor PJ34 causes a PARP1-independent, p21 dependent mitotic arrest. DNA Repair (Amst) 2011;10: 1003–13.
[40] Rothgiesser KM, Erener S, Waibel S, Lüscher B, Hottiger MO. SIRT2 regulates NF-κB dependent gene expression through deacetylation of p65 Lys310. J Cell Sci 2010; 123:4251–8.
[41] Dai Y, Rahmani M, Dent P, Grant S. Blockade of histone deacetylase inhibitor-induced RelA/p65 acetylation and NF-κB activation potentiates apoptosis in leukemia cells through a process mediated by oxidative damage, XIAP downregulation, and c-Jun N-terminal kinase 1 activation. Mol Cell Biol 2005; 25:5429–44.
[42] Bisson JF, Hidalgo S, Rozan P, Messaoudi M. Preventive effects of ACTICOA powder, a cocoa polyphenolic extract, on experimentally induced prostate hyperplasia in Wistar-Unilever rats. J Med Food 2007;10:622–7.
[43] Chen LF. Duration of nuclear NF-κB action regulated by reversible acetylation. Science 2001;293:1653–7.
[44] Kiernan R, Brès V, Ng RW, Coudart MP, El Messaoudi S, Sardet C, et al. Post-activation turn-off of NF-κB-dependent transcription is regulated by acetylation of p65. J Biol Chem 2003;278:2758–66.
[45] Chen LF, Fischle W, Verdin E, Greene WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science 2001;293:1653–7.
[46] Martínez-Balbás MA, Bauer UM, Nielsen SJ, Brehm A, Kouzarides T. Regulation of E2F1 activity by acetylation. EMBO J 2000;19:662–71.
[47] Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 1997;90:595–606.
[48] Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004; 26:2011–5.
[49] Zheng Z, Chen H, Li J, Li T, Zheng B, Zheng Y, et al. Sirtuin 1-mediated cellular metabolic memory of high glucose via the LKB1/AMPK/ROS pathway and therapeutic effects of metformin. Diabetes 2012;61:217–28.



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CLOSE WINDOW

4 – ARTIGO II

Early-Outgrowth Bone Marrow Cells prevent SIRT1 post-translational modification in retina from an experimental model of type 2 Diabetes

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ABSTRACT

The cellular therapy has been studied in cardiovascular disease, but has been little investigated in eye diseases. Sirtuins (SIRTs), a family of deacetylases, is thought to be oxidative sensitive. In this study, we hypothetised that cell therapy using early outgrowth cells (EOC) can protect the diabetic retina through antioxidant means thus improving SIRT1 pathway. We investigated the possible therapeutic effect of cells derived from health (db/m) and diabetic (db/db) animals on diabetic retinopathy (DR). The db/db mice with 8 weeks of age were randomized to receive a unique intravenous injection of PBS or $0{,}5x10^5$ db/m EOCs or $0{,}5x10^5$ db/db EOCs. Four weeks later, the animals were euthanized and the eyes enucleated. For *in vitro* study, EOCs conditioned medium (EOC-CM) was generated from db/m and db/db EOCs cultures. The rat Müller cells (rMCs) were exposed for 24h to normal (NG) or high glucose (HG) combined or not with db/m or db/db EOC-CMs. In diabetic rats, there was an increase of DR and oxidative damage markers, accompanied by decrease in SIRT1 protein followed by lysine-310-p65-NFκB acetylation. The treatment with cells from db/m significantly reduced all the above-mentioned, but interestingly the treatment with cells from db/db mice fully restored the above alterations to normal levels. rMCs exposed to HG displayed GFAP and VEGF expression upregulated, accompanied by increase in Nox4 expression and ROS levels, and acetyl-lysine-310-p65-NFκB. SIRT1 protein expression and activity were markedly reduced in diabetic milieu conditions. The treatment with both EOC-CMs prevented all these abnormalities, but db/db EOC-CM fully restored to NG conditions. This study demonstrates that endocrine capacity of EOCs is effective in improving retinal SIRT1 pathway thus protecting the retina from diabetic milieu insult.

Keywords: diabetes mellitus, diabetic retinopathy, cell Therapy, sirtuin, posttranslational modification

INTRODUCTION

Diabetic retinopathy (DR) remains the leading cause of blindness in young adults affecting over 90% patients with 20 years of diabetes. With the incidence of diabetes increasing at an alarming rate, the number of people with DR is expected to grow from 126.6 million in 2010 to 191.0 million by 2030 (Zheng et al, 2012). The known pathways including oxidative stress, increased formation of advanced glycation end products, and activation of protein kinase-C, polyol production, and hexosamine pathways (Kitada et al, 2010; Santos et al, 2011; Frank et al, 2004) seems to cross-talk. For this reason, new strategies to prevent this devastating complicatios in deserved.

Recently, cellular therapies for degenerative retinal diseases as diabetic retinopathy and aged macular degeneration have been raised using a variety of cell types, including embryonic or induced pluripotent stem cell–derived RPE, bone marrow– or umbilical cord–derived mesenchymal stem cells, fetal neural or retinal progenitor cells, and adult RPE stem cells–derived RPE (Buchholz DE et al, 2009; Schwartz SD et al, 2012, Aftab U et al, 2009; Inoue Y et al, 2007; Siqueira RC et al. 2011). Among many proposed cell types, the circulating or bone-marrow (BM)-derived endothelial progenitor cells (EPCs) largely made it into clinical trials (*Tateishi-Yuyama et al, 2002;* Mund et al, 2009). The mechanisms by which they exert their beneficial effect remains unclear and several studies have documented only minimal retention of administered cells within organs that have nevertheless sustained functional and structural improvements (Hofmann et al, 2005; Gnecchi et al, 2008). The so called early-outgrowth cells (EOCs), defined by identification of their cell surface markers (Hur J et al, 2004) have been taken attention regard to their secretory feature releasing antifibrotic (Yuen et al, 2010) and anti-oxidative stress factors (Yang Z et al, 2010). Gilbert and colleagues have described a successful treatment for diabetic nephropathy using a single infusion of EOCs in experimental model of type 2 DM mice (db/db) (Zhang et al, 2012). The cellular therapy used in that study mitigated structural changes such as extracellular matrix deposition and tubular epithelial cell apoptosis leading to reduction on albuminuria. Although these impressive effects observed, only minimal retention of administered cells was noted in the kidneys of diabetic animals. Similar findings were demonstrated in db/db mice treated with conditioned medium from EOCs, suggesting that the effects were mediated through released factors (Zhang et al, 2012). This lack of cell retention within injured organs was lately explained in other work from the same group where not only in the plasma from EOCs treated rats but also in those treated with EOC conditioned medium, antifibrotic factors were present attenuating the TGF-b pathway (Yuen et al, 2013). Mass spectrometric analysis of EOC conditioned medium (EOC-CM) identified proteins that regulate cellular functions implicated in fibrosis and also proteins that are part of stress response such as heat shock protein-19, glutathione S transferase, peroxiredoxin, superoxide dismutase, thioredoxin and hemo oxygenase1 (Yuen et al, 2013). These results indicate that EOCs display a high capacity in secreting soluble factor(s) with potent antioxidant activity that when injected intravenously replicate the salutary effects of the cells themselves.

Sirtuins (SIRTs) are a family of deacetylases which require NAD^+ as cofactor for the deaecetylation reaction. SIRT1 has been interestingly recognized to play roles in gene silencing, apoptosis, senescence and aging (Chung et al, 2010; Rahman et al, 2012.; Yao et al, 2012; Yao et al, 2012). As NAD^+ dependent, Silent information regulator 1 (SIRT1) acts in some locus removing the acetyl group from the histone chromatin (Zhang T, 2009), but it can also suppress other proteins by deacetylation, including the NFκB-p65 subunit protein (Yang H, 20012). By another way, under certain stimuli, such as reactive oxygen species, SIRT1 is inactivated thus suppressing its deacetylase activity (Braidy, 2011; Furukawa,A, 2007). This redox-sensitive particularity of SIRT1 is explained due to reduction on substrate NAD^+ (Kolthur-Seetharam U, 2006), or by post-translational modification of SIRT1 protein. (Caito S, 2010). Irreversible oxidants/electrophiles covalently modifications, such as carbonylation and tyrosine nitration, can lead to loss of protein function, protein aggregation folding, or degradation (Ghezzi et al, 2003).

In light of these evidences, the aims of this present study were to investigate whether cellular therapy using either intravenously injected EOCs or their conditioned media from cultured EOCs derived from db/m or db/db mice protect the retina from db/db mice from the diabetic environment effect. Since in previous work the db/m and db/db mice used in this present study demonstrated absence of EOCs in ocular tissues (Zhang Y et al, 2012), the endocrine mode of action of EOCs was addressed *in vitro* setting. Rat Muller cells (rMCs) were exposed to high glucose (HG) and treated with conditioned media from db/m (db/m EOC-CM) and db/db (db/db EOC-CM) EOCs. Herein, we show that intravenous infusion of EOCs attenuated DR and oxidative markers, ameliorated SIRT1 expression, followed by increase on Lys-310-p65-NFκB acetylation in diabetic retina. More intriguingly, EOCs from db/db mice were more efficient that EOCs from db/m in protect the deleterious effects of diabetes in retinal

tissue. Similarly, in rMCs exposed to HG, the db/db EOC-CM was more capable in downregulating ROS production and preventing SIRT1 lost, thus led to establishment of deacetylation of Lys-310-p65-NFκB and prevention of the early DR markers.

METHODS

Animal model and experimental design

The retinal tissues and extracts were derived from the same animals from which the kidney findings were published in a previous paper (Zhang Y et al, 2012). Thirty-six 6-week-old male diabetic db/db (BKS.Cg-Dock7 m +/+ Leprdb/J) and 12 age-matched db/m (Dock7 m +/+ Leprdb; heterozygote from the same colony) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). At 8 weeks of age, the db/db mice were randomized to receive a tail-vein injection of medium only (Dulbecco's PBS; DPBS; n = 12), or 5 x 10^5 EOCs derived from db/m mice (n = 12), or 5 x 10^5 EOCs derived from db/db mice. db/m mice served as nondiabetic controls. Mice were housed in a temperature-controlled room (22°C) with a 12-h:12-h light:dark cycle with free access to food and water. All animal studies were approved by the St. Michael's Hospital Animal Ethics Committee in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publ. no. 85-23, revised 1996). Four weeks after treatment, the animals were killed, samples were collected, and retinal tissues were harvested.

Bone marrow harvesting and cell culture

EOCs were cultured as previously described (Zhao et al, 2005;Yuen et al, 2010). In brief, bone marrow cells were collected from the femora and tibiae of 3- to 4-week-old male db/m or db/db mice and cultured in endothelial growth medium-2 (EGM-2, Lonza, Walkersville, MD) at 37°C with 5% CO2 for 7–10 days to produce EOCs. Adherent CMCs adopted a typical cobblestone appearance in culture, and displayed positivity for a range of endothelial cell markers including all $CD34^+$ and $VEGFR2^+$ by flow cytometry (FACSCalibur) using CellQuest data acquisition and analysis software (Zhang Y et al, 2012) . No cell surface expression of either CD133 or the monocyte marker CD11b was noted among EOCs, and although the vast majority of cells were $Ly6C^-$, a small proportion (0.42%) was strongly positive for this cell surface marker (Zhang Y et al, 2012)

Cell infusion

As previously described (Zhang at el, 2012) EOCs were washed with DPBS to remove all medium components. Viable cells were analyzed by trypan blue exclusion and counted by a hemocytometer. Cells were resuspended in DPBS at a final concentration of 2 x 10^6 EOCs/mL. Eight-week-old db/db mice received an infusion of 5 x 10^5 db/m EOCs, 5 x 10^5 db/db EOCs, or DPBS by tail vein injection.

Immunohistochemistry in Retinal Tissues

4μm retinal sections were incubated with antibody against GFAP (DakoCytomation, São Paulo, Brazil), anti-VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-nitrotyrosine (Upstate Cell Signaling Solutions, Lake Placid, NY, USA), anti-8-hydroxy-deoxiguanosine (8-OHdG) (JaICA, **Nikken Seil Co**. Ltd, Shizuoka, Japan), or anti-SIRT1 (Cell Signaling Technology, Inc. Danvers, MA, USA) overnight at 4°C. The slides were then incubated with the appropriate secondary antibodies. The analyses were performed by using the Leica Application Suite (Leica Microsystems, Wetzlar, Germany) in nine nonconsecutive retinal sections divided among three slides per animal per group under high-powered microscopic viewing (×400) (Zeiss, Jena, Germany).

In Vitro Studies

Transformed Rat Retinal Müller Cell Line (rMC-1)

rMC-1 was kindly provided by Vijay J. Sarthy, PhD (Northwestern University, Evanston, IL). rMC-1 was grown in Dulbecco's modified Eagle's medium containing 5 mM of glucose, 10% fetal bovine serum, 24 mmol/L of NaHCO3, 10 mmol/L of HEPES, and 10.000 U/L of penicillin/streptomycin. Cells were maintained in a humidified incubator at 37 °C and 5% CO_2. rMC-1 cell cultures at 70% of confluence were serum starved by reducing the FBS concentration to 1%, then were exposed for 24 with: DMEM with 5.5 mmol/L glucose (NG), DMEM with 25 mmol/L glucose (HG), DMEM with 25 mmol/L glucose plus 10% of 10-times concentrated db/m EOC-conditioned medium (HG + db/m CM), DMEM with 25 mmol/L glucose plus 10% of 10-times concentrated db/db EOC-conditioned medium (HG + db/db CM), or with different treatments as specified.

The citotoxicity of the treatments on rMCs was determined by a thiazolyl blue tetrazolium bromide (MTT) colorimetric assay (Mosmann T, 1983). Concentrations that cause less than 10% of cell toxicity were chosen for the experimental treatments (Supplemental Figure 1).

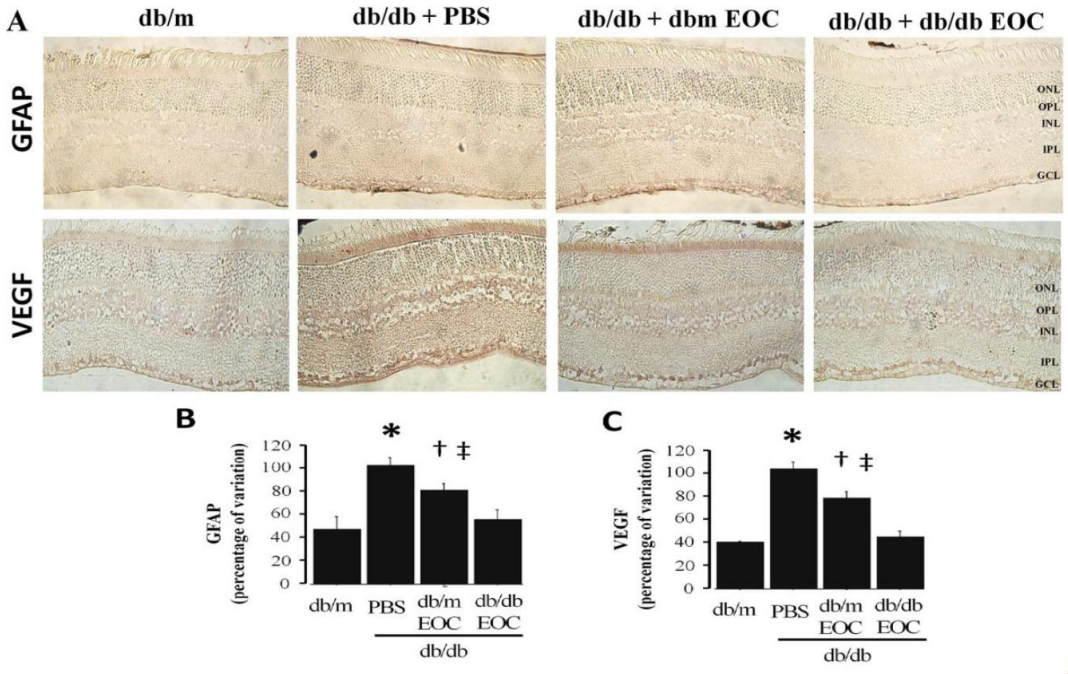

Figure 1. Diabetic retinopathy is ameliorated by EOC intravenous administration. (A) A representative photomicrograph of glial reactivity revealed by GFAP immunolabeling and VEGF in mice retinas tissue. In db/m retinas, there is a light retinal glial reaction mainly in astrocytes at ganglion cell layer. After 12 weeks of diabetes, marked staining is observed in astrocytes which characterizes the glial reaction. In EOC db/m treated mice, the glial reaction is significantly reduced and further decriment is observed in retina from db/db EOC treated mice; magnification X400. Similarly, VEGF signal is faint throuthgout all retinal layers. (B) and (C) Semiquantitative analyses of GFAP and VEGF immunolabelings expressed by mean ±SD of percentage positiveness per mm^2 of retina, respectively. *P<0.0001 vs db/m and db/db+db/db EOC; †P<0.02 vs db/db+PBS; and ‡P<0.04 vs db/m and db/db+db/db EOC.

EOC-conditioned medium generation

EOC-conditioned medium was generated as previously described (Yuen DA, 2010). Subconfluent EOCs were cultivated serum-free endothelial cell basal medium-2 (EBM-2; Lonza) for 24h. The medium was then collected and used for in vitro experiments. Serum-free EBM-2 medium served as a control.

Transient transfection with siRNAs

The small interfering RNA (siRNA) duplexes and scrambled siRNA corresponding to rat Sirt-1 (Santa Cruz Biotechnology) and rat NOX-4 (Dharmacon, CO, USA). The transient transfection of siRNAs was carried out using lipofectamine transfection reagent (Carlsbad, CA, USA) (Xu Y, 2012).

Immunofluorescence in Müller cells

The immunofluorescence was performed as previously described (Silva KC, 2013). The cells were incubated with anti-GFAP, anti-VEGF, or anti-SIRT1 antibody (Santa Cruz) followed by the appropriate secondary antibody (Santa Cruz). Afterwards, the sections were rinsed and cover-slipped with Vectashield antifading medium containing 4′,6′-diamino-2-phenylindole used for nuclei staining (Vector Laboratories, Burlingame, Calif., USA). The slides were examined under scanning microscope (CLSM, LSM510 Zeiss, Jena, Thuringia, Germany) with appropriate emission filters for FITC and rhodamine. Digital images were captured using specific software (Axionvision; Zeiss). The negative controls consisted in omitting the primary antibody.

Western blot

Retinal protein extraction or total rMC-1 lysate were incubated with anti GFAP (Santa Cruz), anti-nitrotyrosine (Upstate), anti-SIRT1 (Santa Cruz), anti-acetylated-lys310-RelA/p65-NF-κB (Assay Biotechnology, Sunnyvale, CA, USA), anti-p65 subunit NF-κB complex (Santa Cruz) and anti-Nox4 (Santa Cruz). Immunoreactive

bands were visualized with the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, IL, USA). Exposed films were scanned with a densitometer (Bio-Rad) and analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems Bio Rad (Hercules, CA, USA).

SIRT1 Activity

The SIRT1 activity was measured by a Fluorescent Activity Assay/Drug Discovery Kit designed to measure the lysyl deacetylase (Enzo Life Sciences, Farmingdale, NY, USA). NAD+-dependent deacetylation of the substrate by recombinant human SIRT1 sensitized it to Developer II, which then generated a fluorophore; the fluorophore was excited with 360nm light and the emitted light (460nm) was detected on a fluorometric plate reader (SynergyMx, Biotek,Winooski, VT, USA).

2',7'-Dichlorodihydrofluorescein diacetate (H_2DCF-DA)

Intracellular ROS levels were measured by H_2DCF-DA. Relative fluorescence was measured using a fluorescence plate reader (SynergyMx, Biotek) at excitation and emission wavelengths of 485 and 528nm, respectively. The relative fluorescence values were corrected by the number of cells in each treatment.

Statistical Analysis

The results were expressed as the means± SD. Comparisons between groups were done using ANOVA followed by Fisher's protected least-significant difference test. The analyses were performed using StatView software (SAS Institute Inc., Cary, NC, USA). $P<0.05$ was considered significant.

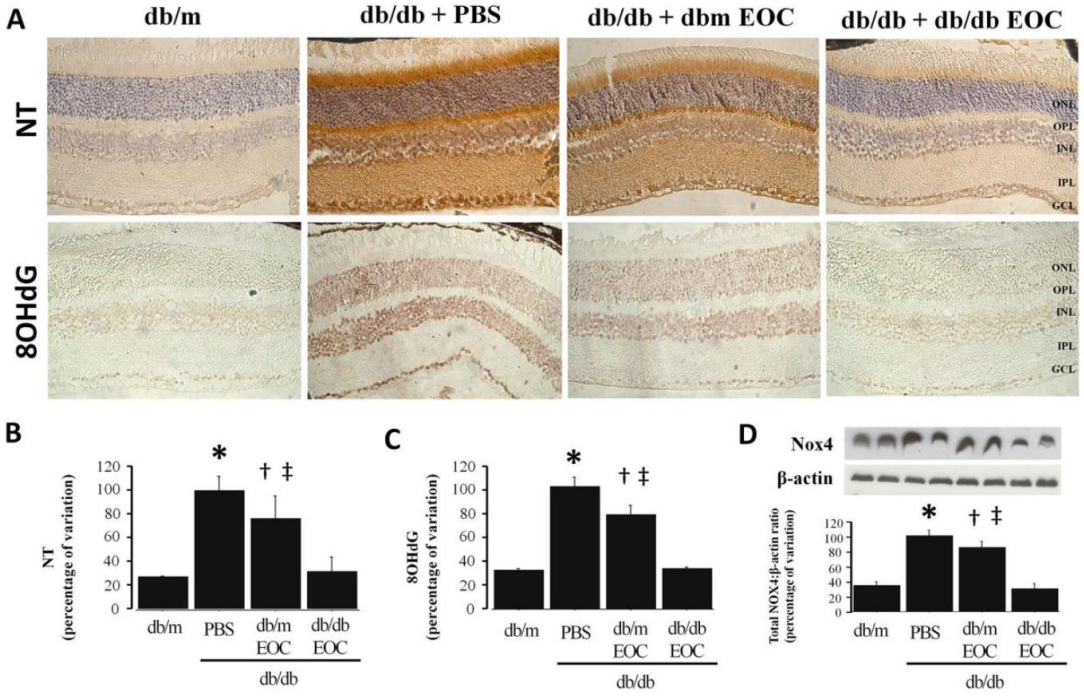

Figure 2. Intravenous injection of EOCs prevented oxidative retinal damage in db/db mice. (A) A representative photomicrograph of immunolocalization of nitrotyrosine (NT) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in retinal tissue. NT is a stable product formed from the reaction of peroxynitrate with tyrosine residues and is accepted as an index of nitrosative damage. 8-OHdG is a product of DNA oxidative damage in its guanine base by hydroxyl radical. (B) and (C) Semiquantitative analyses of NT and 8-OHdG immunolabelings expressed by mean ±SD of percentage positiveness per mm^2 of retina, respectively. (D) Representative Western blot analysis of the NOX4 in total retinal lysates of the mice retinal tissue. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. *$P<0.0001$ vs db/m and db/db+db/db EOC; †$P<0.03$ vs db/db+PBS; and ‡$P<0.0004$ vs db/m and db/db+db/db EOC.

RESULTS

Animal Experiments

The characteristics of the db/m and db/db mice with and without EOC infusion were previously presented (Zhang Y, 2012). db/db mice developed hyperglycemia and increased body weight in comparison with db/m mice. The EOC intravenous infusion didn't affect any of these parameters. All four groups had normal systolic blood pressure during the study.

EOC intravenous administration ameliorated the early markers of DR in db/db mice

To investigate the effect of EOC administration on the diabetic retina, we assessed two markers for DR, glial fibrilary acidic protein (GFAP) and vascular endothelial growth factor (VEGF) (Fig 1). The db/db non treated mice expressed higher GFAP immunolabeling in retinal ganglion cell layer compared with db/m mice (P<0.0001) (Fig 1 A,B). Similarly, there was increase of the VEGF immunoreactivity, as compared with db/m mice (P<0.0001) (Fig 1 A,C). Intravenous injection of EOC either from db/m or db/db mice prevented these alterations in diabetic retina in a different extent. The EOCs from db/m mice partially prevented the above mentioned alterations (P<0.02) but EOCs from db/db totally prevented (P<0.0001) them leading to normal levels (P=0.004 for db/m EOC vs db/db EOC treated mice for GFAP expression and P=0.0001 for db/m EOC vs db/db EOC treated mice for VEGF expression).

EOC intravenous administration prevents retinal oxidative stress through Nox4 inhibition in db/db mice

As an index of nitrosative and oxidative stress tissue damage, nitrotyrosine (NT) and 8-hydroxydeoxyguanosine (8-OHdG) expression were assessed respectively (Fig 2). There was a marked increase NT immunoreactivity in retinal tissue from db/db mice as compared to db/m control animals (P<0.0001) (Fig 2 A,B). Similarly, there was increase of 8-OHdG immunolabeling in db/db mice compared with db/m (P<0.0001) (Fig 2 A,C). EOC intravenous infusion from either db/m or db/db donor mice prevented these alterations in diabetic retina into different extent for NT (P<0.02 for db/m EOC treated mice and P<0.0001 for db/db EOC treated mice compared with non-treated db/db mice; P=0.0004 for db/m EOC vs db/db treated mice) and 8-OHdG (P<0.0001 for db/m EOC treated mice and db/db db/m EOC treated mice compared with non-treated db/db mice; P<0.0001 for db/m EOC vs db/db EOC treated mice).

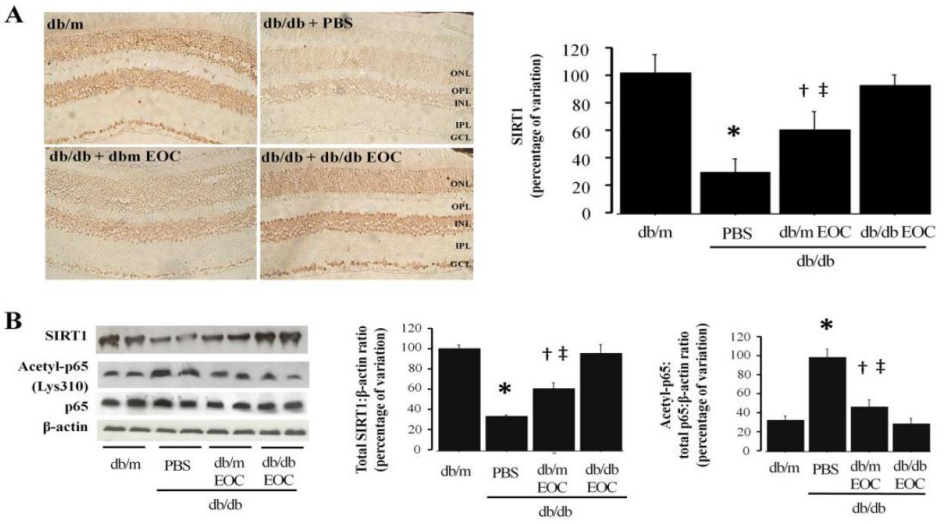

Figure 3. Intravenous EOC treatments improve retinal Sirt1 deacetylase actcity in db/db mice. (A) A representative photomicrograph of immunolocalization of Sirt1 in retinal tissue. Semiquantitative analyses of SIRT1 immunolabelings expressed by mean ±SD of percentage positiveness per mm^2 of retina. (B) Representative Western blots in total retinal lysates for Sirt1 and acetyl-Lys310-p65 subunit-NFKB of mice retinal tissue. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for total p65-NFkB subunit or for β-actin. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. *P<0.0001 vs db/m and db/db+db/db EOC; †P<0.009 vs db/db+PBS; and ‡P<0.01 vs db/m and db/db+db/db EOC.

To investigate the mechanism by which EOC intravenous administration prevents retinal oxidative stress, we assessed Nox4 expression, a subunit of NADPH oxidase (Fig 2 D). Diabetic animals display higher expression of Nox4, as compared with db/m mice, which was partially reduced by db/m EOC infusion (P=0.02) and completely prevented by EOC from db/db mice (P<0.0001) (Fig 2D). These findings indicate that EOC administration exerts antioxidant effects in the early phases of DR through NADPH oxidase pathway.

EOC treatment improves SIRT1 pathway inducing deacetylation of Lys310-RelA/p65 in db/db mice

Since oxidative stress markers were ameliorate in db/db mice treated with either db/m and db/db EOCs and that SIRT1 is oxidative sensitive pathway, we addressed the SIRT1 pathway modulation. SIRT1 immunoreactivity was decreased in retinal tissue from db/db mice compared with db/m (P<0.000, Fig 3A) and db/m and db/db EOC infusion prevented SIRT1 downregulatio into a different extent (P=0.0004 vs db/m EOC treated mice and db/db and P<0.0001 vs db/db EOC treated mice, P<0.0001 for db/m EOC vs db/db EOC treated mice). Similar findings were demonstrated by western blot assay (Fig 3 B).

To further address the SIRT1 activity, we assessed one target of SIRT1 deacetylase. Acetyl-Lys310-RelA/p65 NFκB subunit markedly increased in db/db mice as compared to db/m control animals (P<0.0001) (Fig 3 B). This SIRT1 acetylase activity was prevented by EOC administration under the same fashion as previous findings in which db/m EOCs partially reduced acetyl-Lys310-RelA/p65 NFκB subunit and db/db EOCs completely reduced to normal levels (P<0.0001) (Fig 3 B).

From this set of observations, we demonstrated for the first time in this animal study that EOC intravenous therapy protects the retinal tissue from diabetes related damage through antioxidant pathway through SIRT1 activation. In addition, the antioxidant capacity of db/db EOCs is higher than db/m EOCs. In order to better understand the underlay mechanism, we conducted *in vitro* experiments using rat retinal Muller cells.

In Vitro Studies

EOC-conditioned medium prevents VEGF upregulation and Glial Reaction in rMC-1 via SIRT1

To investigate whether rMC-1 exposed to HG medium displayed the same markers present in diabetic retina and whether the presence of EOC-CM could abrogate these alterations, GFAP and VEGF immunolabeling assays were performed. (Fig 4 A,B). The treatment with EOC-CMs prevented the glial reaction and VEGF upregulation (P<0.0001). In the same fashion observed in vivo study, the db/db EOC-CM was more efficient that db/m EOC-CM (P=0.004 for db/m EOC-CM vs db/db EOC-CM for GFAP expression and P=0.02 for db/m EOC-CM vs db/db EOC-CM for VEGF expression). To verify if SIRT1 is play a role in these parameters, SIRT1 small interfering RNA or SIRT1 specific inhibitor EX-527, were applied. The presence of siRNA or EX-527 abolished the protective effects observed in rMC exposed to HG and treated with EOC-CM (Fig 4 C and Supplemental Fig 2). This is an indicative that SIRT1 is pivotal in its mechanism of action and the protective effects of EOC-CMs are at least partly mediated by SIRT1 pathway.

EOC-CM reduces ROS production in diabetic milieu conditions via Nox4 dowregulation

Under HG condition, there was a significant increase in Nox4 expression (P<0.0001) (Fig 5 A); the treatment with db/m EOC-CM partially decreased its expression and db/db EOC-CM was able to reduce it to normal levels (P<0.0001) (P=0.004 for db/m EOC-CM vs db/db EOC-CM). In accordance, the total ROS in rMC exposed to HG was significantly reduced by db/m EOC-CM (P=0.002) and normalized by db/db EOC-CM (P<0.0001) (Fig 5 B). These data indicate that EOC-CM reduces ROS production induced by HG condition through downregulation of Nox4-NADPH-oxidase subunit.

EOC-CM improves SIRT1 activity leading to Lys310-RelA/p65 NFκB subunit deacetylation in rMC-1

By immunofluorescence assay, SIRT1, which is a nuclear factor when in its active form, is highly expressed in normal condition. rMCs exposed to HG induced a marked decrease in its nuclear expression, accompanied by an increased translocation to cytosol; the treatments with EOC-CM restablished the SIRT1 nuclear positiveness (Fig 6 A). SIRT1 activity evaluated through a fluorimetric assay revealed that in rMC-1 cells exposed to the HG conditions have lower SIRT1 activity (P<0.0001). The presence of EOC-CM from both db/m and db/db mice prevented this abnormality, although db/db EOC-CM was more efficient (P=0.009 for db/m EOC-CM vs db/db EOC-CM) (Fig 6B). The combination of EOC-CM and siRNA or SIRT1 inhibitor EX-527 in cells exposed to HG completetly abolished the effect observed with both EOC-CM separately (Fig 6 B; Supplemental Fig 3A) (P<0.0001).

To further address the SIRT1 activity, we assessed one target of SIRT1 deacetylase, Lys310-RelA/p65 NFκB subunit. Cells exposed to HG conditions displayed an increase in total acetylated lysine protein with 2.8-fold

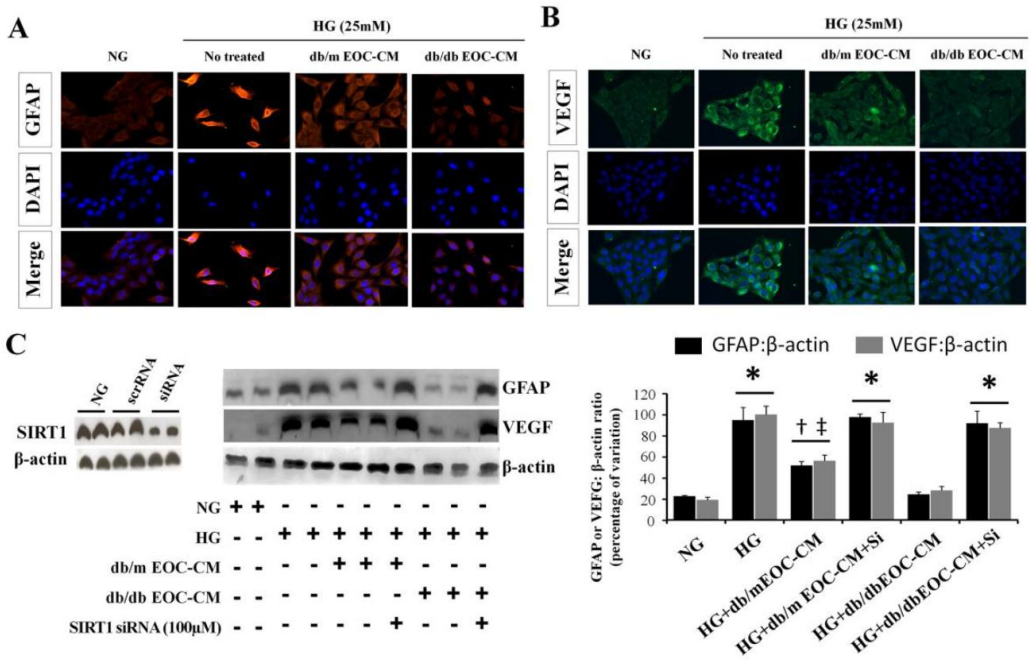

Figure 4. GFAP and VEGF were dowregulated by EOC-conditioned medium in rMC-1 via SIRT1 pathway. (A) A representative photomicrograph of GFAP immunofluoresecence in rMC-1. (B) A representative photomicrograph of VEGF immunofluoresecence in rMC-1. Magnification: X630. (C) Representative Western blots in total cell lysate against SIRT1. The efficiency of the Sirt1 small interfering RNA (100nM) was about 59%. Representative Western blots in total cell lysate against GFAP and VEGF antibodies from rMC-1. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. **P*<0.0001 vs NG and HG+db/db CM; †*P*<0.0001 vs HG, HG+db/m EOC-CM+Si and HG+db/m EOC-CM+Si; and ‡*P*<0.02 vs NG and HG+db/db EOC-CM. db/m EOC-CM: EOC-conditioned medium from db/m; db/db EOC-CM: EOC-conditioned medium from db/db; Scr: control; Si: SIRT1 small interfering RNA (100nM).

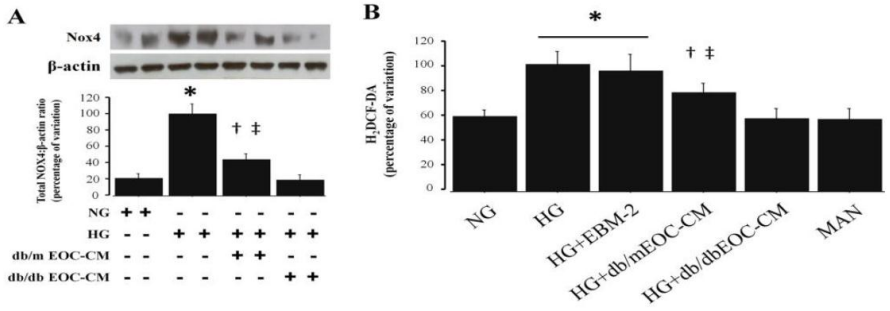

Figure 5. EOC-conditioned media reduce glucose-induced ROS production by dowregulating NOX4 expression in rMC-1. (A) Representative Western blots for NOX4 in rMC-1. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. (B) The quantification of total intracellular ROS levels in rMC-1 cultured for 24 hours by H_2DCF-DA. The values are expressed by mean ±SD and expressed as percentages of fluorescence units. Values were corrected by the number of cells at the end of each treatment. **P*<0.0001 vs NG, HG+db/db EOC-CM and MAN; †*P*<0.01 vs HG and HG+EBM-2; and ‡*P*<0.006 vs NG, HG+db/db EOC-CM and MAN. db/m EOC-CM, EOC-conditioned medium from db/m; db/db EOC-CM, EOC-conditioned medium from db/db, EBM-2, Endothelial Basal Medium-2 (10%); MAN: Manitol (25mM).

increment of Lys310-RelA/p65 acetylation. The treatment with db/m EOC-CM partially decreased this level (P=0.0002) and the db/db EOC-CM completely prevented this abnormality to normal levels (P<0.0001) (Fig 6 C). The presence of siRNA or SIRT1 inhibitor totally abolished this effect (Fig 6C and Supplemental Fig 3B, respectively). In agreement, these data suggest that EOC-CM either from db/m or db/db mice protects rMC under diabetic milieu conditions through reestablishment of SIRT1 activity which deacetylases Lys310-RelA/p65 NFκB subunit.

EOC-conditioned medium prevents SIRT1 post-translational modification in rMC-1

Finally, in order to further understand the mechanism by which EOC-CMs modulates SIRT1 activity, we assessed possible posttranslational modification of SIRT1 protein in HG condition. rMCs exposed to HG displayed a significant increase of SIRT1 carbonylation and nitrosylation (P<0.0001) (Fig 7). The treatment with db/m EOC-CMs partially decrease these modifications (P<0.0003) and db/db EOC-CMs totally restored (P<0.0001) to the levels of NG condition. This set of data indicates that EOC-CMs prevent carbonylation and nitration of SIRT1.

EOC-CM improves SIRT1 activity through Nox4 dowregulation

Finally, in order to further access the mechanism of EOC-CM on SIRT1 activity, the Nox4 small interfering RNA was approached. The presence of siRNA under normal glucose displayed about 25% of increment of SIRT1 activity (P=0.0002), suggesting that the Nox4 modulates Sirt1 at normal condition (Fig 8). Nox4 siRNA prevent the reduction of Sirt1 activity under HG; in the co-treatment with CM from heath donors (db/m), the presence of Nox4 siRNA increase even more the Sirt1 activity when compared with db/m EOC-CM alone, showing that db/m CM modulates Sirt1 through Nox4, but is not able to restore completely Sirt1 activity at normal levels. However, under HG condition, the combination of siRNA and CM from diabetic donors (db/db) does not show additive effect when compared with db/db EOC-CM alone, proving that the CM acts through this mechanism (Fig 8).

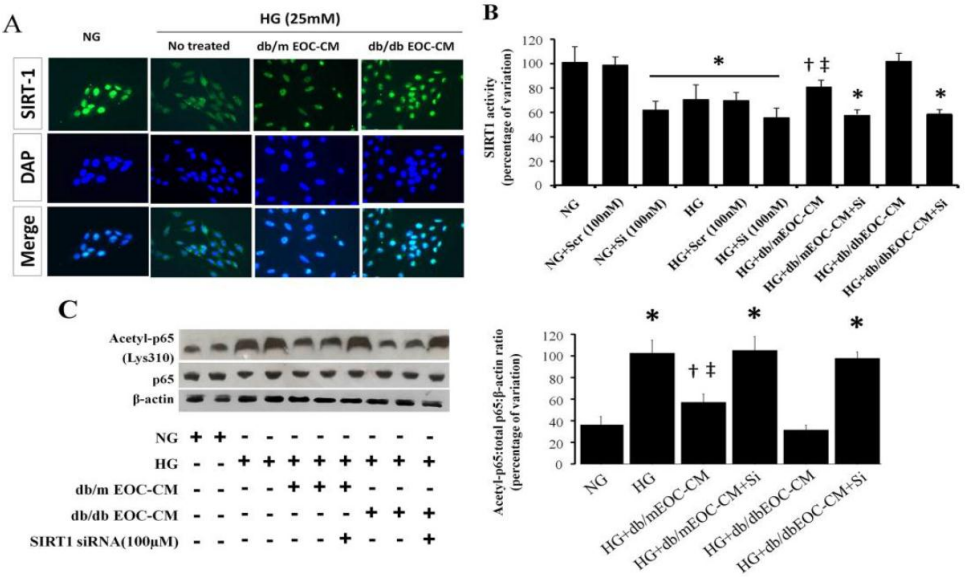

Figure 6. EOC-conditioned media improve SIRT1 deacetylation pathway in rMC-1 exposed to high glucose conditions. (A) Immunofluorescence for SIRT1 in rMC-1 cultured for 24 hours. (B) SIRT1 activity in rMC-1 cultured for 24 hours with different treatments by fluorescent method. (C) Representative Western blots for acetyl-Lys310-RelA/p65 in rMCs-1 lysates. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for total p65 subunit and for β-actin. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. *P<0.0001 vs NG and HG+db/db CM; †P<0.003 vs HG, HG+EBM-2, HG+db/m EOC-CM+Si and HG+db/db EOC-CM+Si; and ‡P<0.01 vs NG and HG+db/db EOC-CM. db/m EOC-CM: EOC-conditioned medium from db/m; db/db EOC-CM: EOC-conditioned medium from db/db; Scr: control of siRNA; Si: SIRT1 small interfering RNA (100nM).

DISCUSSION

This study provides evidence that EOCs intravenously is a potential cellular therapy for the treatment of diabetic retinopathy. A single intravenous injection of these cells in db/db mice was able to prevent the oxidative damage thus avoiding the early markers of DR namely GFAP and VEGF. The mechanistic pathways involved in these effects were studied in cultured retinal Muller cells and confirmed *in vivo*. rMCs exposed to HG and treated with EOCs-conditioned media had improvement of SIRT1 activity through modulation of Nox4-NADPH oxidase subunit, thus reducing ROS formation and preventing SIRT1 lost (carbonylation and nitration). This led to deacetylation of Lys310-p65, which in turn downregulates VEGF and GFAP expressions *in vitro* and *in vivo* model of diabetic retinopathy. These findings are in agreement with other researchers who reported that Nox4 plays an important role on vascular permeability present in pathogenesis of DR associated with VEGF upregulation in db/db/ mice (Li J et al, 2010). In db/db model and *in vitro* assays, the beneficial effects of using EOCs or even their conditioned media at least two mechanisms are operational: deacetylation of Lys310-p65 NFkB subunit and Nox-4 NADPH oxidase subunit inhibition. The high endocrine capacity of these cells in acting at distant sites may be one advantage since no direct manipulation of the eye is needed.

Since the landmark paper by Asahara in 1997 (Science), endothelial progenitor cells (EPCs) have been the center of regenerative medicine, involving mainly cardiovascular diseases and neovascularization (Dimmeler et al 2004; Kawamoto et al, 2002). Hur J and colleagues have differentiated the survival behaviors of the types of endothelial progenitor cells (EPCs) (Hur J et al 2004). The so called early EPCs showed peak growth at 2 to 3 weeks and died at 4 weeks and secreted angiogenic cytokines (vascular endothelial growth factor, interleukin 8) more than late EPC during culture in vitro. The EOCs are derived from CD14+ monocytic lineage display overlap between endothelial and monocyte and macrophage cell markers and functions as phagocytosis (Yoder et al, 2007; Krenning et al 2008) and produces vasoactive substances (Zhang et al, 2007) Presently, the EOCs used in this study display $CD34^+$ and VEGFR-2 surface markers therefore defined as endothelial progenitor cells (Yoder et al, 2007) and being for 7-10 days in culture accepted as the early outgrowth cells (EOCs) (Yoon et al, 2005).

The high glucose conditions driving changes in ratio of free $NADH/NAD^+$ is proposed as crucial mediator in the development of diabetic complication (Williamson et al, 1993). Excess in glucose flux activates a variety of metabolic pathways such as glycolysis, intracellular nonenzymatic glycation, PKC activation and oxidative stress (Giacco et al, 2010). Here we focused on possible protective effects of EOCs injection in diabetic retina and the role of oxidative sensitive pathway SIRT1. In current study, the EOCs administration significantly decreased both markers although GFAP expression in db/db retinal tissue from mice with 12 weeks of age is mild, suggesting the initial process of gliosis in the retina, articles shows that mice with 18 week of old the gliosis is already installed (Zhang et al, 2013). The same markers were evaluated in retinal Muller cell *in vitro* and the cells exposed to high glucose in presence of EOC-CM deeply reduced them. To address the oxidative markers, we estimated protein nitration through nitrotyrosine and DNA damage by 8-hydroxy-deoxyguanosine expressions both in tissue as *in vitro*. The EOCs injection in db/db mice and Muller cells in high glucose treated with EOC-CM exhibited an important decrease of both oxidative indicators. To further understand these effects, SIRT-1 activity was accessed. Its expression was restored in cells treated with EOC-CM and also in animals treated with a single intravenous injection of EOCs, as a consequence the p65-NFkB subunit acetylation is decreased leading to its inactivation. Since SIRT-1 is sensitive to redox balance, it was assessed ROS intracellular production and Nox-4 expression. The EOC-CM prevented Nox-4 expression and intracellular ROS production increments in Muller cells under diabetic milieu conditions. Collectively, these data indicate that EOC-CM is acting by anti-oxidant means. Recently, Gilbert and colleagues have demonstrated the complete proteome analyses of the conditioned medium from EOCs (Yuen DA, 2013). Among approximately three hundred proteins, there are several antioxidant proteins such as glutathione S transferase, peroxiredoxin, thioredoxin and $NADP^+$ that might contribute to antioxidant effects observed in EOC treated mice o EOC-CM treated cells.

In the current study, all the observed effects either from db/m EOCs or db/m conditioned media were milder than the effects from db/db EOCs or db/db conditioned media in a dose dependent manner. Finally, we addressed the modulation of Nox4 on Sirt1 activity. For this purpose, Nox4 was silenced in Muller cells and Sirt1 activity measured. Nox4 siRNA prevent the reduction of Sirt1 activity at HG and under normal condition increase even more; in combination of HG and CM from db/m or db/db donor animals there is no additive effect, proving that the CM acts through this mechanism.

In conclusion, compelling evidence is provided that EOCs are efficient in protecting the retinal tissue from the glucose related oxidative damage, mainly the EOCs from

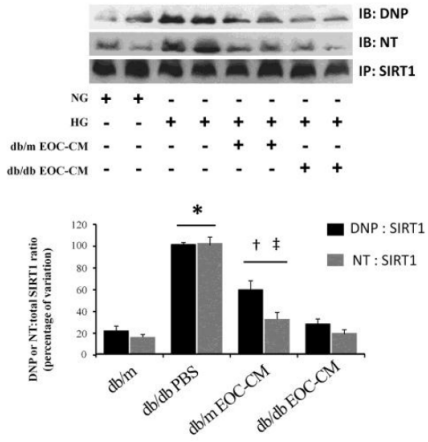

Figure 7. EOC-conditioned media prevent inactivation of SIRT1 through post-translational modification in rMC-1. SIRT1 immunoprecipitation (IP) in rMCs-1 lysates. Equal amount (100µg) of immunoprecipitated SIRT1 protein was used for immunoblotting (IB). Carbonylation was detected by first derivitizing the samples with DNPH followed by immunoblotting against anti-DNP antibody. Nitration was detected by immunoblotting against anti-nitrotyrosine (NT) antibody. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for total SIRT1. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. **P*<0.0001 vs NG and HG+db/db EOC-CM; †*P*<0.0003 vs HG; and ‡*P*<0.04 vs NG and HG+db/db EOC-CM. db/m EOC-CM, EOC-conditioned medium from db/m; db/db EOC-CM, EOC-conditioned medium from db/db; EX-527, SIRT1 blocker (10µM).

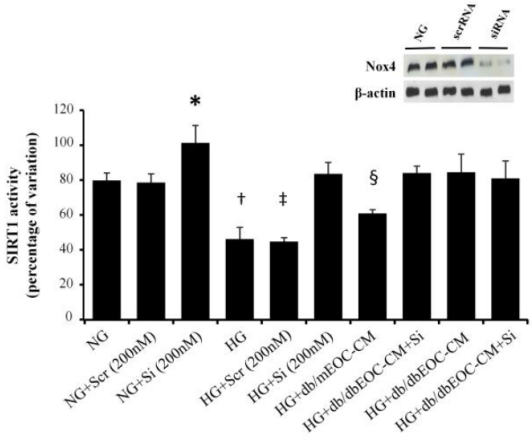

Figure 8. EOC-CM improves SIRT1 activity through Nox4 dowregulation. Finally, in order to further access the mechanism of EOC-CM on SIRT1 activity, the Nox4 small interfering RNA was approached. The presence of siRNA under normal glucose displayed about 25% of increment of SIRT1 activity (*P*=0.0002), suggesting that the Nox4 modulates Sirt1 at normal condition. Nox4 siRNA prevent the reduction of Sirt1 activity under HG; in the co-treatment with CM from heath donors (db/m), the presence of Nox4 siRNA increase even more the Sirt1 activity when compared with db/m EOC-CM alone, showing that db/m CM modulates Sirt1 through Nox4, but is not able to restore completely Sirt1 activity at normal levels. However, under HG condition, the combination of siRNA and CM from diabetic donors (db/db) does not show additive effect when compared with db/db EOC-CM alone, proving that the CM acts through this mechanism.

db/db mice and highlighted the possibility to further investigate the cellular therapy in patients with retinal diabetic complications. Since there was no EOC retained inside the eye, this may be consistent with the endocrine ability of these cells in balancing the oxidative status of the retinal tissue cells thus becomimg a non invasive eye therapy.

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D.A.D acquired the data, D.A.D, K.C.S contributed to technical assistance, A.P contributed to discussion and technical assistance, M.P.B.R and E.B.M.I.P. contributed to technical assistance, R.E.G. provided the retinal tissue, reviewed the data, and critically reviewed the manuscript, J.B.L.F. wrote the manuscript and reviewed the data, J.M.L.F designed the study, reviewed the data, and wrote, reviewed, and edited the manuscript. J.M.L.F. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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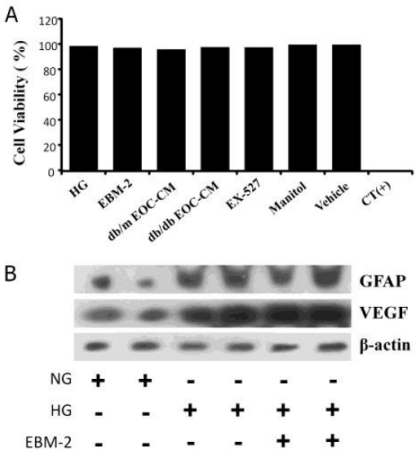
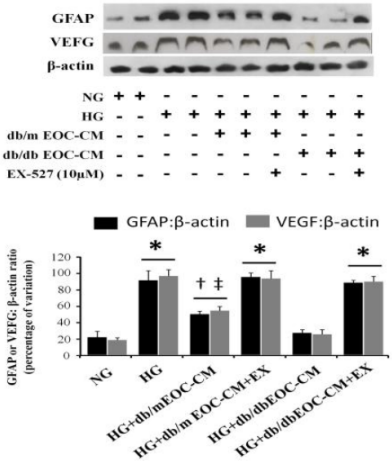
REFERENCES

1. Zheng, M. He, and N. Congdon, "The worldwide epidemic of diabetic retinopathy," Indian Journal of Ophthalmology, vol. 60, pp. 428–431, 2012.
2. M. Kitada, Z. Zhang, A. Mima, and G. L. King, "Molecular mechanisms of diabetic vascular complications," Journal of Diabetes Investigation, vol. 1, no. 3, pp. 77–89, 2010;
3. J. M. Santos, G. Mohammad, Q. Zhong, and R. A. Kowluru, "Diabetic retinopathy, superoxide damage and antioxidants," Current Pharmaceutical Biotechnology, vol. 12, no. 3, pp. 352–361, 2011;
4. R. N. Frank, "Diabetic Retinopathy," New England Journal of Medicine, vol. 350, no. 1, pp. 48–58, 2004
5. Buchholz DE, et al.*Derivation of functional retinal pigmented epithelium from induced pluripotent stem cells. Stem cells. 2009; 27: 2427–2434*
6. Schwartz SD, et al. *Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet. 2012; 379: 713–720.* Aftab U et al.*Growth kinetics and transplantation of human retinal progenitor cells. Exp Eye Res. 2009; 89: 301–310*
7. Inoue Y et al. *Subretinal transplantation of bone marrow mesenchymal stem cells delays retinal degeneration in the RCS rat model of retinal degeneration. Exp Eye Res. 2007; 85: 234–241;*
8. Siqueira RC et al. *Intravitreal injection of autologous bone marrow-derived mononuclear cells for hereditary retinal dystrophy: a phase I trial. Retina. 2011; 31: 1207–1214.)*
9. *Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischemia by autologous transplantation of bone marrow cells: a pilot study and a randomized control trial. Lancet.*2002; 360*:* 427*–435.*
10. Mund JA, Ingram DA, Yoder MC, Case J. Endothelial progenitor cells and cardiovascular cell-based therapies. Cytotherapy, 2009;11(2):103-13.
11. Hofmann M et al. Monitoring of bone marrow cell homing into the infarcted human myocardium. Circulation 2005;111:2198–2202.
12. Gnecchi M et al. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204–1219.
13. Hur J et al . Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004;24:288–293.
14. Yuen DA et al. Culture-modified bone marrow cells attenuate cardiac and renal injury in a chronic kidney disease rat model via a novel antifibrotic mechanism. PLoS ONE 2010;5:e9543.
15. Yang Z et al. Paracrine factors secreted by endothelial progenitor cells prevent oxidative stress-induced apoptosis of mature endothelial cells. Atherosclerosis 2010;211:103–109.
16. Zhang Y, Yuen DA, Advani A, Thai K, Advani SL, Kepecs D, Kabir MG, Connelly KA, Gilbert RE. Early-outgrowth bone marrow cells attenuate renal injury and dysfunction via an antioxidant effect in a mouse model of type 2 diabetes. Diabetes. 2012 Aug;61(8):2114-25.
17. Yuen DA, Connelly KA, Zhang Y, Advani SL, Thai K, Kabir G, Kepecs D, Spring C, Smith C, Batruch I, Kosanam H, Advani A, Diamandis E, Marsden PA, Gilbert RE. Early outgrowth cells release soluble endocrine antifibrotic factors that reduce progressive organ fibrosis. Stem Cells. 2013 Nov;31(11):2408-19
18. Chung S, Yao H, Caito S, Hwang JW, Arunachalam G, Rahman I. Regulation of SIRT1 in cellular functions: role of polyphenols. Arch.Biochem. Biophys. 501:79–90; 2010.
19. Rahman I, Kinnula VL, Gorbunova V, Yao H. SIRT1 as a therapeutic target in inflammaging of the pulmonary disease. Prev. Med. 54(Suppl): S20–28; 2012.
20. Yao H, Rahman I. Perspectives on translational and therapeutica spects of SIRT1 ininflammaging and senescence. Biochem. Pharmacol 84:1332–1339; 2012.
21. Yao H, Chung S, Hwang JW, Rajendrasozhan S, Sundar IK, Dean DA, McBurney MW, Guarente L, Gu W, Ronty M, Kinnula VL, Rahman I. SIRT1 protects against emphysema via FOXO3-mediated reduction of premature senescence in mice. J Clin Invest. 2012 Jun 1;122(6):2032-45.
22. Zhang, T, Kraus WL. SIRT1-dependent regulation of chromatin and transcription: Linking NAD+ metabolism and signaling to the control of cellular functions. Biochim. Biophys. Acta. 2009;10:022.
23. Yang H, Zhang W, Pan H, Feldser HG, Lainez E, Miller C, Leung S, Zhong Z, Zhao H, Sweitzer S, Considine T, Riera T, Suri V, White B, Ellis JL, Vlasuk GP, Loh C. SIRT1 activators suppress inflammatory responses through promotion of p65 deacetylation and inhibition of NF-κB activity. PLoS One. 2012;7(9):e46364.
24. Braidy N, Guillemin GJ, Mansour H, Chan-Ling T, Poljak A, Grant R. Age relatedchanges in NAD+ metabolism, oxidative stress and Sirt1 activity in Wistar rats. PLoS One 6:e19194;2011.
25. Furukawa A, Tada-Oikawa S, Kawanishi S, Oikawa S. H2O2 accelerates cellular senescence by accumulation of acetylated p53 via decrease in the function of SIRT1 by NAD+ depletion. Cell. Physiol.Biochem 20:45–54; 2007.
26. Kolthur-Seetharam U, Dantzer F, McBurney MW, de Murcia G, Sassone- Corsi P. Control of AIF-mediated cell death by the functional interplay of SIRT1 and PARP-1 in response to DNA damage. Cell Cycle 5:873–877; 2006.
27. Caito S, Rajendrasozhan S, Cook S, Chung S, Yao H, Friedman AE, Brookes PS, Rahman I. SIRT1 is a redox-sensitive deacetylase that is post translationally modified by oxidants and carbonyl stress. FASEBJ 24: 3145–3159;2010.
28. Ghezzi, P., and Bonetto, V. (2003) Redox proteomics: identification of oxidatively modified proteins. Proteomics **3,** 1145–1153.
29. Zhao YD, Courtman DW, Deng Y, Kugathasan L, Zhang Q, et al. (2005) Rescue of monocrotaline-induced pulmonary arterial hypertension using bone marrow-derived endothelial-like progenitor cells: efficacy of combined cell and eNOS gene therapy in established disease. Circ Res 96: 442–450
30. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983;**65**:55-63
31. Xu Y, Nie L, Yin YG, et al. Resveratrol protects against hyperglycemia-induced oxidative damage to mitochondria by activating SIRT-1 in rat mesangial cells. Toxicol. Appl. Pharmacol 2012;**259:** 395-401
32. Silva KC, Rosales MA, Hamassaki DE, et al. Green tea is neuroprotective in diabetic retinopathy. Invest Ophthalmol Vis Sci 2013;**54**:1325-36
33. Li J, Wang JJ, Yu Q, Chen K, Mahadev K, Zhang SX: Inhibition of reactive oxygen species by Lovastatin downregulates vascular endothelial growth factor expression and ameliorates blood-retinal barrier breakdown in db/db mice: Role of NADPH oxidase 4. Diabetes 59: 1528–1538, 2010
34. Dimmeler S, Zeiher AM. Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? J. Mol. Med., 82 (2004), pp. 671–677;
35. Kawamoto A, *et al.* Transplantation of endothelial progenitor cells for therapeutic neovascularization. Cardiovasc. Radiat. Med., 3 (2002), pp. 221–225.
36. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM, Park YB. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol. 2004 Feb;24(2):288-93. Epub 2003 Dec 29.
37. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood, 109 (2007), pp. 1801–1809)
38. Krenning G, Strate BWA, Schipper M, Gallego y van Seijen XJ, Fernandes BCA, Luyn MJA, Harmsen MC. $CD34^+$ cells augment endothelial cell differentiation of $CD14^+$ endothelial progenitor cells *in vitro*. J. Cell. Mol. Med. (2008)
39. J. Z. Shi, H. Zhang, M. Hou et al., "Is it possible to obtain "true endothelial progenitor cells" by in vitro culture of bone marrow mononuclear cells?" Stem Cells and Development, vol. 16, no. 4, pp. 683–690, 2007.
40. Yoder MC, Mead LE, Prater D, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 2007;109:1801–1809
41. Yoon CH, Hur J, Park KW, et al. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. Circulation 2005;112:1618–1627
42. Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, van den Enden M, Kilo C, Tilton RG. Hyperglycemic pseudohypoxia and diabetic complications. Diabetes, 1993 Jun;42(6):801-13.
43. Ferdinando Giacco and Michael Brownlee. Oxidative stress and diabetic complications. Circ Res. 2010 October 29; 107(9): 1058–1070.
44. Zhang SY, Li BY, Li XL, Cheng M, Cai Q, Yu F, Wang WD, Tan M, Yan G, Hu SL, Gao HQ. Effects of phlorizin on diabetic retinopathy according to isobaric tags for relative and absolute quantification-based proteomics in db/db mice. Mol Vis. 2013 Apr 5;19:812-21.

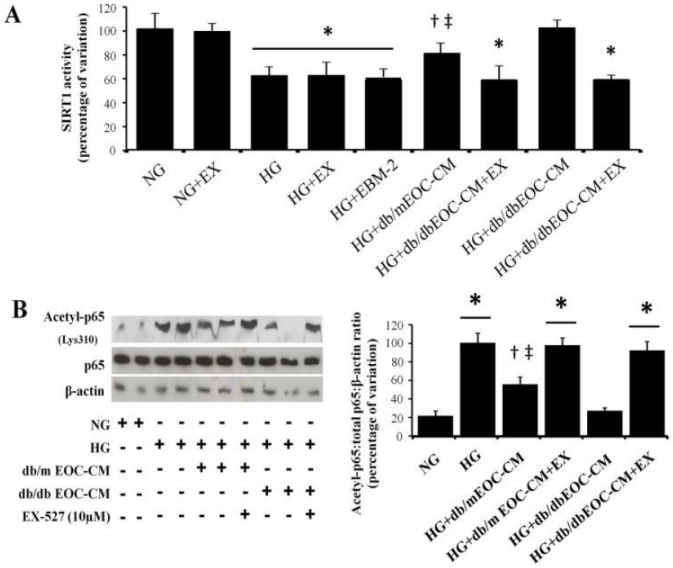
SUPPLEMENTARY FIGURES



Suppl. Figure 1. EBM-2 medium has no effect on GFAP or VEGF expression in rMCs exposed to HG. (A) The citotoxicity of the treatments and vehicle on rMC-1. The values are expressed as percentages of cell viability. (B) Representative Western blots for GFAP and VEGF in cell lysates. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. NG: normal D-glucose (5.5mM); HG: high D-glucose (25mM); EBM-2, Endothelial Basal Medium-2 (10%); db/m EOC-CM, EOC-conditioned medium from db/m; db/db EOC-CM, EOC-conditioned medium from db/db; EX-527, SIRT1 blocker (10μM); Manitol (25mM); Vehicle: DMSO (0.1%); CT(+): positive control for cell toxicity, TritomX100 (1μl/ml).



Suppl. Figure 2. SIRT1 blocker reverses EOC-conditioned media-induces GFAP and VEGF downregulation in rMC-1 exposed to high glucose conditions. Representative Western blots in total cell lysate against GFAP and VEGF antibodies from rMC-1.. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. **P*<0.0001 vs NG and HG+db/db CM; †*P*<0.0001 vs HG, HG+db/m EOC-CM+EX and HG+db/m EOC-CM+EX; and ‡*P*<0.01 vs NG and HG+db/db EOC-CM. db/m EOC-CM, EOC-conditioned medium from db/m; db/db EOC-CM, EOC-conditioned medium from db/db; EX-527, SIRT1 blocker (10μM).



Suppl. Figure 3. SIRT1 blocker abolished EOC-conditioned media improvement on SIRT1 deacetylation pathway in rMC-1 exposed to high glucose conditions. (A) SIRT1 activity in rMC-1 cultured for 24 hours with different treatments by fluorescent method. The mean ±SD expresses the percentage of fluorescence units. (B) Representative Western blots for acetyl-Lys310-RelA/p65 in rMCs-1 lysates exposed to HG and treated with EOC-CMs in presence or not of EX-527. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for total p65 subunit and for β-actin. The bars represent mean ± SD of band densities expressed as the percentage of variation from at least three independent experiments. **P*<0.0001 vs NG and HG+db/db CM; †*P*<0.002 vs HG, HG+EBM-2, HG+db/m EOC-CM+EX and HG+db/db EOC-CM+EX; and ‡*P*<0.03 vs NG and HG+db/db EOC-CM. db/m EOC-CM, EOC-conditioned medium from db/m; db/db EOC-CM, EOC-conditioned medium from db/db; EX-527, SIRT1 blocker (10μM).

5 – **SUMÁRIO DOS PRINCIPAIS RESULTADOS APRESENTADOS NOS ARTIGOS I E II**

- As rMC-1 expostas á alta glicose apresentaram aumento da expressão da subunidade Nox4-NADPH oxidase acompanhado do acréscimo na produção de ROS e redução da expressão e atividade da SIRT1.
- Mecanisticamente, mostramos que a inativação da SIRT1 nas rMC-1 em condição de alta glicose ocorreu por duas vias: 1) ativação da PARP-1 que levou ao consumo do NAD^+ intracelular; 2) modificação pós-tradução da SIRT1 por danos nitrosativos/oxidativos.
- A queda da atividade da SIRT1 levou à hiperacetilação da lisina 310 do complexo RelA/p65-NF-κB, ativando este fator de transcrição e consequente transcrição dos genes-alvo como VEGF e GFAP nas rMC-1 expostas á alta glicose.
- O bloqueio da PARP-1 ou a suplementação com NAD^+ restabeleceu a atividade da SIRT1 nas rMC-1 em condição de alta glicose. O mesmo foi verificado pelo silenciamento gênico da Nox4.
- O tratamento com cacau rico em polifenóis ou a epicatequina, composto mais abundante do cacau, reduziu a produção de ROS e a ativação da PARP-1, resultando na melhora dos níveis de NAD^+ e reestabelecimento da atividade da SIRT1 em rMC-1 exposta a alta glicose.
- O tratamento com o meio condicionado das células *early outgrowth* (provenientes de camundongos db/m e db/db, principalmente) reduziu a expressão de Nox4 resultando na queda da produção de ROS e consequente redução da nitração e carbonilação da SIRT1 e levando ao restabelecimento da atividade e expressão da sirtuínas em rMC-1 exposta a alta glicose.

- Ambos os tratamentos, pela ativação da SIRT1 preveniram o aumento do GFAP induzido pela alta glicose via redução da acetilação do p65. A inibição química ou silenciamento gênico da SIRT1 bloqueou tanto o efeito benéfico do cacau enriquecido com polifenóis quanto do tratamento celular com as células EOCs.

- Nos ratos SHR diabéticos, o tratamento com cacau rico em polifenóis reduziu o estresse tecidual e a ativação da PARP-1, resultando na melhora dos níveis de NAD^+ e reestabelecimento da atividade da SIRT1 no tecido retiniano.

- Nos camundongos diabéticos, o tratamento com a injeção única de células *early outgrowth* diminuiu a expressão de Nox4 resultando na redução da lesão oxidativa do DNA, preveniu a nitração e carbonilação da SIRT1 e levou ao restabelecimento da atividade e expressão da sirtuínas na retina diabética.

- Em ambos os modelos experimentais de diabetes, a ativação da SIRT1 preveniu o aumento do GFAP via desacetilação da lisina 310 do complexo RelA/p65-NF-κB.

6 - **CONCLUSÃO GERAL**

Em conclusão, a administração de cacau enriquecido com polifenóis melhorou o status redox e reduziu a ativação da PARP1, levando ao restabelecimento da razão NAD^+/NADH intracelular. Enquanto que o tratamento com células *early outgrowth*, por redução da expressão de Nox4 e consequente diminuição da produção de espécies reativas de oxigênio, preveniu danos nitrosativos/cabonilativos da SIRT1.

Ambos os tratamentos restabeleceram a atividade enzimática da SIRT1, o que culminou na desacetilação e inativação do complexo RelA/p65-NF-κB. Como consequência, os marcadores estruturais precoces da RD decorrente da neurodegeneração retina foram prevenidos tanto em modelo *in vivo* quanto *in vitro*.

É interessante ressaltar que este estudo mostrou pela primeira vez o mecanismo de regulação da expressão do GFAP via acetilação/desacetilação do complexo RelA/p65-NF-κB.

Em resumo, a presente tese fornece evidências que tanto a administração oral do cacau enriquecido com polifenóis quanto à terapia celular com células *early outgrowth*, conferem neuroproteção da retina aos insultos do diabetes. Portanto, intervenções que modulem a atividade das sirtuínas são promissoras ferramentas terapêuticas no tratamento farmacológico da retinopatia diabética.

7 - REFERÊNCIAS

Adamis AP. Is diabetic retinopathy an inflammatory disease? British Journal of Ophthalmology, vol. 86, no. 4, pp. 363-365, 2002.

Alaiti MA, Ishikawa M, Costa MA. Bone marrow and circulating stem/progenitor cells for regenerative cardiovascular therapy. Transl Res. 2010;156(3):112-29.

Alder VA, Su EN, Yu DY, Cringle S, Yu PY. Overview of studies on metabolic and vascular regulatory changes in early diabetic retinopathy. Aust NZJ Ophthalmol 1998; 26: 141-8.

Alder VA, Su EN, Yu DY, Cringle SJ, Yu PK. Diabetic retinopathy: early functional changes. Clin Exp Pharmacol Physiol. 1997; 24: 785-8.

Antonetti DA, Barber AJ, Khin S, et al. Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Diabetes 1998; 47: 1953-9.

Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221-228.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997;275(5302):964-7.

Baba S, Natsume M, Yasuda A, Nakamura Y. Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo- and hypercholesterolemic humans after intake of different levels of cocoa powder. J. Nutr. 2007;137:1436-1441.

Baeuerle PA, Baltimore D. NF-kB: Ten years after. Cell 1996;87:13-20.

Baldwin ASJ. The NF-kK and IkB proteins: new discoveries and insights. Annu Rev Immunol 1996; 14:649-83.

Balzer J, Rassaf T, Heiss C, Kleinbongard P. Sustained benefits in vascular function through flavanolcontaining cocoa in medicated diabetic patients a doublemasked, randomized, controlled trial. J. Am. Coll. Cardiol. 2008;51:2141-2149.

Barber AJ, Antonetti DA. Mapping the blood vessels with paracellular permeability in the retinas of diabetic rats. Investig. Ophthalmol Vis Sci 2003; 44: 5410-6.

Barber AJ. Comments on “A new view of diabetic Retinopathy”. Prog Neuropsychopharmacol Biol Psychiatry. 2004; 28: 747-748.

Barnes J, Anderson LA, Phillipson JD. St John's wort (Hypericum perforatum L.): a review of its chemistry, pharmacology and clinical properties. J Pharm Pharmacol 2001; 53(5):583-600.

Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. Large-scale characterization of HeLacell nuclear phosphoproteins. Proc. Natl. Acad. Sci. USA. 2004;101:12130-12135.

Benolken RM, Anderson RE, Wheeler TG. Membrane fatty acids associated with the electrical response in visual excitation. Science 1973; 182: 1253-4.

Berger F, Ramirez-Hernandez MH, Ziegler M. The new life of a centenarian: signaling functions of NAD(P). Trends Biochem. Sci. 2004; 29: 111–118.

Bi B, Schmitt R, Israilova M, Nishio H, Cantley LG. Stromal cells protect against acute tubular injury via an endocrine effect. J Am Soc Nephrol. 2007 Sep;18(9):2486-96.

Bordone L, Guarente L. Calorie restriction, SIRT1 and metabolism: understanding longevity. Nat Rev Mol Cell Biol. 2005 Apr;6(4):298-305.

Braidy N, Guillemin GJ, Mansour H, Chan-Ling T, Poljak A, Grant R. Age related changes in NAD^+ metabolism oxidative stress and Sirt1 activity in wistar rats. PLoS One. 2011 Apr 26;6(4):e19194.

Brankin B, Campbell M, Canning P. Endostatin modulates VEGF-mediated barrier dysfunction in the retinal microvascular endothelium. Exp Eye Res 2005; 81:22 31.

Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science. 2004 Mar 26;303(5666):2011-5.

Budd SL, Tenneti L, Lishnak T, Lipton SA. Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. Proc Natl Acad Sci U S A. 2000; 97: 6161-6166.

Buijsse B, Feskens EJ, Kok FJ, Kromhout D. Cocoa intake, blood pressure, and cardiovascular mortality: the Zutphen Elderly Study. Arch. Intern. Med. 2006;166:411-417.

Bulnes S, García-Blanco Á, Bengoetxea H, Ortuzar N, Argandoña EG, Lafuente JV. Glial stem cells and their relationship with tumour angiogenesis process. Rev Neurol. 2011 Jun 16;52(12):743-50.

Cai J, Boulton M. The pathogenesis of diabetic retinopathy: old concepts and new questions. Eye 2002; 16: 242-60.

Caito S, Rajendrasozhan S, Cook S, Chung S, Yao H, Friedman AE, Brookes PS, Rahman I. SIRT1 is a redox-sensitive deacetylase that is post-translationally modified by oxidants and carbonyl stress. FASEB J. 2010 Sep;24(9):3145-59.

Carnevale R, Loffredo L, Pignatelli P, Nocella C. Dark chocolate inhibits platelet isoprostanes via Nox2 down-regulation in smokers. J. Thromb. Haemost. 2012; 10:125-132.

Chen J, Zhou Y, Mueller-Steiner S, Chen LF, Kwon H, Yi S, Mucke L, Gan L. SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF-kappaB signaling. J Biol Chem. 2005 Dec 2;280(48):40364-74.

Chen LF, Williams SA, Mu Y, Nakano H, Duerr JM, Buckbinder L, Greene WC. NF-kappaB RelA phosphorylation regulates RelA acetylation. Mol Cell Biol. 2005 Sep;25(18):7966-75.

Chibber R, Ben-Mahmud BM, Chibber D, Kohner EM. Leukocytes in diabetic retinopathy. Current Diabetes Reviews, vol. 3, no. 1, pp. 3-14, 2007.

Cogan DG, Toussaint D, Kuwabara T. Retinal vascular patterns. IV. Diabetic retinopathy Arch. Ophthalmol 1961; 66: 366-78.

Control of AIF-mediated cell death by the functional interplay of SIRT1 and PARP-1 in response to DNA damage. Cell Cycle. 2006 Apr;5(8):873-7.

Cooper KA, Campos-Giménez E, Alvarez DJ, Rytz A, Nagy K, Williamson G. Predictive Relationship Between Polyphenols and Nonfat Cocoa Solids Content of Chocolate. Journal of Agricultural and Food Chemistry, 2008;56:260-265.

Copp SW, Inagaki T, White MJ, Hirai DM, Ferguson SK, Holdsworth CT, Sims GE, Poole DC, Musch TI. (-)-Epicatechin administration and exercising skeletal muscle vascular control and microvascular oxygenation in healthy rats. Am J Physiol Heart Circ Physiol. 2013 Jan 15;304(2):H206-14.

Cunha-Vaz JG. Blood–retinal barriers in health and disease. Trans. Ophthalmol 1980; 100: 337-40.

Dandona P, Thusu K, Cook S, Snyder B, Makowski J, Armstrong D, et al. Oxidative damage to DNA in diabetes mellitus. Lancet. 1996; 347: 444-445.

Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004; 116: 205-219.

Davison G, Callister R, Williamson G, Cooper KA. The effect of acute pre-exercise dark chocolate consumption on plasma antioxidant status, oxidative stress and immunoendocrine responses to prolonged exercise. Eur. J. Nutr. 2012;51:69-79.

Davison K, Coates AM, Buckley JD, Howe PR. Effect of cocoa flavanols and exercise on cardiometabolic risk factors in overweight and obese subjects. Int. J. Obes.(Lond) 2008;32:1289-1296.

DCCT Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin dependent diabetes Mellitus. N Engl J Med. 1993; 329: 977-986.

De Vries JH, Janseen PL, Hollman PC, Van Staveren WA, Katan MB. Consumption of quercetin and kaempferol in free living subjects eating a variety of diets, Cancer Lett 1997; 114:141-4.

Desch S, Schmidt J, Kobler D, Sonnabend M. Effect of cocoa products on blood pressure: systematic review and meta-analysis. Am. J. Hypertens. 2010;23:97-103.

Di Carlo G, Mascolo N, Izzo AA, Capasso F. Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sci 1999; 65(4):337-53.

Dornas WC, Oliveira TT, Rodrigues-das-Dores RG, Santos AF, Nagem TJ. Flavonóides: potencial terapêutico no estresse oxidativo. Rev. Ciênc. Farm. Básica Apl., v. 28, n.3, p. 241-249, 2007.

Dosso AA, Bonvin ER, Morel Y, et al. Risk factors associated with contrast sensitivity loss in diabetic patients. Graefes Arch Clin Exp Ophthalmol. 1996; 234: 300-5.

Drel VR, Xu W, Zhang J, Kador PF, Ali TK, Shin J, et al. Poly (ADP-ribose) polymerase inhibition counteracts cataract formation and early retinal changes in streptozotocin-diabetic rats. Invest Ophthalmol Vis Sci. 2009; 50: 1778-1790.

Duarte DA, Silva KC, Rosales MA, Lopes de Faria JB, Lopes de Faria JM. The concomitance of hypertension and diabetes exacerbating retinopathy: the role of inflammation and oxidative stress. Curr Clin Pharmacol. 2013 Nov;8(4):266-77.

Dusting GJ, Selemidis S, Jiang F. Mechanisms for suppressing NADPH oxidase in the vascular wall. Mem. Inst. Oswaldo Cruz. 2005, vol.100, suppl.1, pp. 97-103.

Early Treatment Diabetic Retinopathy Study Research Group (ETDRS). Photocoagulation of diabetic macular edema. Arch Ophthalmol 1985; 103: 1796-1809.

Elliott PJ, Jirousek M. Sirtuins: novel targets for metabolic disease. Curr Opin Investig Drugs. 2008 Apr;9(4):371-8.

Enayat S, Ceyhan MŞ, Başaran AA, Gürsel M, Banerjee S. Anticarcinogenic effects of the ethanolic extract of Salix aegyptiaca in colon cancer cells: involvement of Akt/PKB and MAPK pathways. Nutr Cancer. 2013;65(7):1045-58.

Estrutura molecular das catequinas [online]. [Acessado em 21 de setembro de 2014]. Disponível em: http://catequinas.weebly.com/

European Food Safety Authority (EFSA). Scientific opinion on the substantiation of a health claim related to cocoa flavanols and maintenance of normal endotheliumdependent vasodilation. Article 13(5) of Regulation (EC) No 1924/20061 EFSA. Panel on Dietetic Products, Nutrition and Allergies (NDA). EFSA J. 2012;10: 2809.

Faridi Z, Njike VY, Dutta S, Ali A. Acute dark chocolate and cocoa ingestion and endothelial function: a randomized controlled crossover trial. Am. J. Clin. Nutr. 2008; 88:58-63.

Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408(9):239-247.

Fong DS, Aiello LP, Ferris FL, Klein R. Diabetic retinopathy. Diabetes Care 2004; 27: 2540-2553.

Ford ES, Mokdad AH, Giles WH, et al. The metabolic syndrome and antioxidant concentrations: findings from the Third National Health and Nutrition Examination Survey. Diabetes 2003; 52: 2346-2352.

Ford J, Ahmed S, Allison S, Jiang M, Milner J. JNK2-dependent regulation of SIRT1 protein stability. Cell Cycle. 2008;7:3091-3097.

Fraga CG, Actis-Goretta L, Ottaviani J I, Carrasquedo F. Regular consumption of a flavanol-rich chocolate can improve oxidant stress in young soccer players. Clin. Dev. Immunol. 2005;12:1117.

Frank RN. Diabetic retinopathy. N Engl J Med. 2004; 350: 48-58.

Furukawa A, Tada-Oikawa S, Kawanishi S, Oikawa S. H2O2 accelerates cellular senescencebyaccumulationofacetylatedp53viadecreaseinthe function ofSIRT1byNAD+ depletion. Cell. Physiol.Biochem. 2007;20:45-54.

Galleano M, Bernatova I, Puzserova A, Balis P, Sestakova N, Pechanova O, Fraga CG. (-)-Epicatechin reduces blood pressure and improves vasorelaxation in spontaneously hypertensive rats by NO-mediated mechanism. IUBMB Life. 2013 Aug;65(8):710-5.

Garcia Soriano F, Virag L, Jagtap P, Szabó E, Mabley JG, Liaudet L, et al. Diabetic endothelial dysfunction: the role of poly (ADP-ribose) polymerase activation. Nat Med. 2001; 7: 108 –113.

Gardner TW, Lieth E, Khin SA, et al. Astrocytes increase barrier function and ZO-1 protein expression in cultured retinal capillary endothelial cells. Invest Ophthalmol Vis Sci 1997; 38:2423-7.

Ghezzi P, Bonetto V. Redox proteomics: identification of oxidatively modified proteins. Proteomics. 2003 Jul;3(7):1145-53.

Ghirlanda G, Di Leo MA, Caputo S, Falsini B, Porciatti V, Marietti G, et al. Detection of inner retina dysfunction by steady-state focal electroretinogram pattern and flicker in early IDDM. Diabetes 1991; 40: 1122-1127.

Gibbons TE, Pence BD, Petr G, Ossyra JM, Mach HC, Bhattacharya TK, Perez S, Martin SA, McCusker RH, Kelley KW, Rhodes JS, Johnson RW, Woods JA. Voluntary wheel running, but not a diet containing (-)-epigallocatechin-3-gallate and β-alanine, improves learning, memory and hippocampal neurogenesis in aged mice. Behav Brain Res. 2014 Oct 1;272:131-40.

Gilmore TD. Introduction to NF-κB: players, pathways, perspectives. Oncogene. 2006; 25 (51): 6680-4.

Glezera I, Marcourakisa T, Avellar MCW, Gorenstein C, Scavone C. The role of the transcription factor NF-kB in the molecular mechanisms of action of psychoactive drugs. Rev Bras Psiquiatr 2000; 22(1):26-30.

Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008 Nov 21;103(11):1204-19.

Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. Genes Dev 2000; 14: 1553–1577.

Gotti R, Furlanetto S, Pinzauti S, Cavrini V. Analysis of catechins in Theobroma cacao beans by cyclodextrin-modified micellar electrokinetic chromatography. J Chromatogr A. 2006 Apr 21;1112(1-2):345-52.

Grassi D, Desideri G, Necozione S, Lippi C. Blood pressure is reduced and insulin sensitivity increased in glucose-intolerant, hypertensive subjects after 15 days of consuming high-polyphenol dark chocolate. J. Nutr. 2008;138(9):1671-1676.

Grassi D, Desideri G, Necozione S, Ruggieri F. Protective effects of flavanol-rich dark chocolate on endothelial function and wave reflection during acute hyperglycemia. Hypertension 2012;60:827-832.

Grassi D, Lippi C, Necozione S, Desideri G. Shortterm administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons. Am. J. Clin. Nutr. 2005; 81: 611-614.

Grassi, D., Lippi, C., Necozione, S., Desideri, G. et al., Shortterm administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons. Am. J. Clin. Nutr. 2005;81(3):11–614.

Greene WC e Chen LF. Regulation of NF-kappaB action by reversible acetylation. Novartis Found Symp. 2004;259:208-17; discussion 218-25.

Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA. Oxidative stress and covalent modification of protein with bioactive aldehydes. J Biol Chem. 2008 Aug 8;283(32):21837-41.

Guarente L. Sir2 links chromatin silencing, metabolism, and aging. Genes Dev. 2000 May 1;14(9):1021-6.

Haimovich B, Lipfert L, Brugge JS, et al. Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. J Biol Chem 1993; 268: 15868–77

Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol. 2004; 142: 231-255.

Hanhineva K, Torronen R, Bondia-Pons I, Pekkinen J. Impact of dietary polyphenols on carbohydrate metabolism. Int. J. Mol. Sci. 2010;11:1365-1402.

Hannekens CH. Platelet Inhibitors and Antioxidant Vitamins in Cardiovascular Disease. American Heart Journal, 1994;128:1333-1336.

Hao LN, Wang M, Ma JL, Yang T. Puerarin decreases apoptosis of retinal pigment epithelial cells in diabetic rats by reducing peroxynitrite level and iNOS expression. Sheng Li Xue Bao. 2012 Apr 25;64(2):199-206.

Hassa PO, Buerki C, Lombardi C, Imhof R, Hottiger MO. Transcriptional coactivation of nuclear factor-κB-dependent gene expression by p300 is regulated by poly (ADP)-ribose polymerase-1. J. Biol. Chem. 2003; 278: 45145–45153.

He M, Pan H, Xiao C, Pu M. Roles for redox signaling by NADPH oxidase in hyperglycemia-induced heme oxygenase-1 expression in the diabetic retina. Invest Ophthalmol Vis Sci. 2013 Jun 12;54(6):4092-101.

Heiss C, Dejam A, Kleinbongard P, Schewe T. Vascular effects of cocoa rich in flavan-3-ols. JAMA 2003;290:1030-1031.

Heiss C, Jahn S, Taylor M, Real W.M. Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease. J. Am. Coll. Cardiol. 2010;56:218-224.

Heiss C, Kleinbongard P, Dejam A, Perre S. Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. J. Am. Coll. Cardiol. 2005;46:1276-1283.

Heiss C, Schroeter H, Balzer J, Kleinbongard P. Endothelial function, nitric oxide, and cocoa flavanols. J. Cardiovasc. Pharmacol. 2006:47(Suppl 2):S128-S135: discussion S172-S176.

Heptinstall S, May J, Fox S, Kwik-Uribe C. Cocoa flavanols and platelet and leukocyte function: recent in vitro and ex vivo studies in healthy adults. J. Cardiovasc. Pharmacol. 2006;47(Suppl 2): S197-S205; discussion S206-S209.

Herrmann J, Lerman LO, Lerman A. Ubiquitin and ubiquitin-like proteins in protein regulation. Circ. Res. 2007;100:1276–1291.

Hiroi M, Ohmori Y. The transcriptional coactivator CREB-binding protein cooperates with STAT1 and NF-kappa B for synergistic transcriptional activation of the CXC ligand 9/monokine induced by interferon-gamma gene. J Biol Chem. 2003 Jan 3;278(1):651-60. Epub 2002 Oct 25.

Hirschi KK, D'Amore PA. Control of angiogenesis by the pericyte: molecular mechanisms and significance. EXS 1997; 79: 419-428.

Hofman P, Blaauwgeers HG, Tolentino MJ, et al. VEGF-A induced hyperpermeability of blood–retinal barrier endothelium in vivo is predominantly associated with pinocytotic vesicular transport and not with formation of fenestrations. Vascular endothelial growth factor-A. Curr Eye Res 2000; 21: 637-45.

Hofmann M, Wollert KC, Meyer GP, Menke A, Arseniev L, Hertenstein B, Ganser A, Knapp WH, Drexler H. Monitoring of bone marrow cell homing into the infarcted human myocardium. Circulation. 2005;111(17):2198-202.

Hooper L, Kay C, Abdelhamid A, Kroon P A. Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. Am. J. Clin. Nutr. 2012; 95:740-751.

Hooper L, Kroon PA, Rimm EB, Cohn JS. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. Am. J. Clin. Nutr. 2008;88:38-50.

Hur J, Yoon CH, Kim HS, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol. 2004;24:288–293.

Hwang J, Yao H, Caito S, Sundar I, Rahman I. Redox regulation of SIRT1 ininflammation and cellular senescence. Free Radical Biology and Medicine. 2013;6:95-110.

Hwang JW, Chung S, Sundar IK, Yao H, Arunachalam G, McBurney MW, Rahman I. Cigarette smoke-induced autophagy is regulated by SIRT1–PARP1-dependent mechanism: implication in pathogenesis of COPD. Arch. Biochem. Biophys. 2010;500:203–209.

Hyvrinen L, Laurinen P, Rovano J. Contrast sensitivity in evaluation of visual impairment due to diabetes. Acta Ophthalmol. 1983; 61: 94-101.

IDF, International Diabetes Federation. EASD, 2011 [onlline]. [Acessado em 26 de setembro de 2014]. Disponível em: http://www.idf.org/types-diabetes

Imesch PD, Bindley CD, Wallow IHL. Clinicopathologic correlation of intraretinal microvascular abnormalities. Retina 1997;17:321-9.

Indacaterol provides 24-hour bronchodilation in COPD: a placebo-controlled blinded comparison with tiotropium. Respir Res. 2010 Oct 5;11:135. doi: 10.1186/1465-9921-11-135.

Innes AJ, Kennedy G, McLaren M, Bancroft AJ. Dark chocolate inhibits platelet aggregation in healthy volunteers. Platelets 2003;14:325-327.

International Diabetes Federation, 2013 [onlline]. [Acessado em 20 de setembro de 2014]. Disponível em Disponível em: http://www.idf.org/latestdiabetesfigurespaintgrim-global-picture

Ito K, Jazrawi E, Cosio B, Barnes PJ, Adcock IM. p65-activated histone acetyltransferase activity is repressed by glucocorticoids: mifepristone fails to recruit HDAC2 to the p65-HAT complex. J Biol Chem. 2001 Aug 10;276(32):30208-15. Epub 2001 Jun 6.

J.M. Morillas-Ruiz, J.M. Rubio-Perez, M.D. Albaladejo, P. Zafrilla, S. Parra, M.L. Vidal-Guevara. Effect of an antioxidant drink on homocysteine levels in Alzheimer's patients. J. Neurol. Sci., 299 (2010), pp. 175–178

Jacob Ra, Burri Bj. Oxidative Damage And Defense. American Journal Of Clinical Nutrition. 1996;63:985s-1996.

Jagtap P, Szabo C. Poly (ADP-ribose) polymerase and the therapeutic effects of its inhibitors. Nat Rev Drug Discov. 2005; 4: 421-440.

Jain SK. Superoxide dismutase overexpression and cellular oxidative damage in diabetes. A commentary on "Overexpression of mitochondrial superoxide dismutase in mice protects the retina from diabetes-induced oxidative stress". Free Radic Biol Med. 2006; 41: 1187-1190.

Jian B, Jones PL, Li Q, et al. Matrix metalloproteinase-2 is associated with tenascin-c in calcific aortic stenosis. Am J Pathol 2001; 159: 321-7.

Jonas JB, Witzens-Harig M, Arseniev L, Ho AD. Intravitreal autologous bone marrow-derived mononuclear cell transplantation: a feasibility report. Acta Ophthalmol. 2008;86(2):225-6.

Jones CP, Rankin SM. Bone Marrow-Derived Stem Cells and Respiratory Disease. Chest. 2011;140(1):205-211.

Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE, Adamis AP. Leukocytemediated endothelial cell injury and death in the diabetic retina. Am J Pathol. 2001; 158: 147-152.

Joussen AM, Poulaki V, Qin W, et al. Retinal vascular endothelial growth factor induces intercellular adhesion molecule-1 and endothelial nitric oxide synthase expression and initiates early diabetic retinal leukocyte adhesion in vivo. American Journal of Pathology, vol. 160, no. 2, pp. 501-509, 2002.

Jung KI, Kim JH, Park HY, Park CK. Neuroprotective effects of cilostazol on retinal ganglion cell damage in diabetic rats.J Pharmacol Exp Ther. 2013 Jun;345(3):457-63.

Kähkönen MP, Heinonem M. Antioxidant activity of anthocyanins and their aglycons. J Agric Food Chem 2003; 51(3):628-33.

Kang, H, Suh JY, Jung YS, Jung JW, Kim MK, Chung JH. Peptide switch is essential for Sirt1deacetylase activity. Mol. Cell. 2011;44:203–213.

Karrasch S, Holz O, Jörres RA. Aging and induced senescence as factors in the pathogenesis of lung emphysema. Respir Med. 2008 Sep;102(9):1215-30. doi: 10.1016/j.rmed.2008.04.013.

Kaštelan S, Tomić M, Gverović Antunica A, Salopek Rabatić J, Ljubić S. Inflammation and pharmacological treatment in diabetic retinopathy. Mediators Inflamm. 2013;2013:213130.

Kaur H, Chen S, Xin X, Chiu J, Khan ZA, Chakrabarti S. Diabetes-induced extracellular matrix protein expression is mediated by transcription coactivator p300. Diabetes 2006; 55: 3104–3111.

Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW, et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med. 2001;7(9):1035-40.

Kelly CJ. Effects of theobromine should be considered in future studies. Am. J. Clin. Nutr. 2005;82:486-487; author reply 487-488.

Kirimlioglu V, Ara C, Yilmaz M, Ozgor D, Isik B, Sogutlu G, Kirimlioglu H, Karabulut AB, Yilmaz S, Kayaalp C, Yologlu S. Resveratrol, a red wine constituent polyphenol, protects gastric tissue against the oxidative stress in cholestatic rats. Dig Dis Sci. 2006 Feb;51(2):298-302.

Klein R, Klein BE, Moss SE, Davis MD, DeMets DL. The Wisconsin Epidemiologic Study of Diabetic Retinopathy. III. Prevalence and risk of diabetic retinopathy when age at diagnosis is 30 or more years. Arch Ophthalmol. 1984; 102: 527-32.

Kolthur-Seetharam U, Dantzer F, McBurney MW, de Murcia G, Sassone-Corsi P.

Kong D, Melo LG, Gnecchi M, Zhang L, Mostoslavsky G, Liew CC, et al. Cytokine-induced mobilization of circulating endothelial progenitor cells enhances repair of injured arteries. Circulation. 2004;110(14):2039-46.

Kornberg MD, Sen N, Hara MR, Juluri KR, Nguyen JV, Snowman AM Law L, Hester LD, Snyder SH. GAPDH mediates nitrosylation of nuclear proteins. Nat. Cell Biol. 2010;12:1094-1100.

Kowluru RA, Chan PS. Oxidative stress and diabetic retinopathy. Exp Diabetes Res. 2007; 2007: 43603.

Kowluru RA, Santos JM, Zhong Q. Sirt1, a negative regulator of matrix metalloproteinase-9 in diabetic retinopathy. Invest Ophthalmol Vis Sci. 2014 Jun 3;55(9):5653-60

Kowluru RA. Diabetes-induced elevations in retinal oxidative stress, protein kinase C and nitric oxide are interrelated. Acta Diabetol. 2001; 38: 179-185.

Krenning G, van Luyn MJ, Harmsen MC. Endothelial progenitor cell-based neovascularization: implications for therapy. Trends Mol Med. 2009;15:180-189.

Kubota S, Ozawa Y, Kurihara T, Sasaki M, Yuki K, Miyake S, Noda K, Ishida S, Tsubota K. Roles of AMP-activated protein kinase in diabetes-induced retinal inflammation. Invest Ophthalmol Vis Sci. 2011 Nov 25;52(12):9142-8.

Kwik-Uribe C. Potential Health Benefits Of Cocoa Flavanols. The Manufacturing Confectioner. 2005; 85:43-49.

Lambooij AC, Kuijpers RW, Van Lichtenauer-Kaligis EG, et al. Somatostatin receptor 2A expression in choroidal neovascularization secondary to age-related macular degeneration. Invest Ophthalmol Vis Sci 2000; 41: 2329-35.

Lavu S, Boss O, Elliott PJ, Lambert PD. Sirtuins novel therapeutic targets to treat age-associated diseases. Nat Rev Drug Discov. 2008 Oct;7(10):841-53.

Lekstron JA, Bell WR. Aspirin in the Prevention of Thrombosis. Medicine, 1991;70:161-178.

Li Z, Ma L, Chen X, Li Y, Li S, Zhang J, Lu L. Glycogen synthase kinase-3: a key kinase in retinal neuron apoptosis in early diabetic retinopathy. Chin Med J (Engl). 2014 Oct;127(19):3464-70.

Lieth E, Barber AJ, Xu B, et al. Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy. Penn State Retina Research Group. Diabetes 1998; 47(5): 815-20.

Lin WJ, Kuang HY. Oxidative stress induces autophagy in response to multiple noxious stimuli in retinal ganglion cells. Autophagy. 2014 Aug 22;10(10).

Liu SH, Lu TH, Su CC, Lay IS, Lin HY, Fang KM, Ho TJ, Chen KL, Su YC, Chiang WC, Chen YW. Lotus leaf (Nelumbo nucifera) and its active constituents prevent inflammatory responses in macrophages via JNK/NF-κB signaling pathway. Am J Chin Med. 2014;42(4):869-89.

Lopes de Faria JM, Katsumi O, Cagliero E, Nathan D, Hirose T. Neurovisual abnormalities preceding the retinopathy in patients with long-term type 1 diabetes mellitus. Graefes Arch Clin Exp Ophthalmol. 2001; 239: 643-648.

Lopez-Revuelta A, Sanches-Gallego JI, Hernandez-Hernandez A, Sanchez-Yague J, Llanillo M. Membrane cholesterol contents influence the protective effects of quercetin and rutin in erythrocytes damaged by oxidative stress. Chem Biol Interact 2006; 161(1):79-91.

Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W. Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell. 2001 Oct 19;107(2):137-48.

Mabley, J. G. et al. Anti-inflammatory effects of a novel, potent inhibitor of poly (ADPribose) polymerase. Inflamm. Res. 2001; 50: 561–569.

MacGregor LC, Matschinsky FM. Experimental diabetes mellitus impairs the function of the retinal pigmented epithelium. Metabolism 1986; 35: 28-34.

Mann J. Secondary metabolism. Oxford: Clarendon Press; 1987. p.374.

Mao TK, Powell JW, Keen CL, Shimitz HH, Hammerstone JF, Gershwin ME. The Effect of Cocoa Procyanidins on the Transcription and Secretion of Interleukin 1β in Peripheral Blood Mononuclear Cells. Life Sciences. 2000;66:1377-1386.

Martin MA, Serrano AB, Ramos S, Pulido MI. Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stressinduced apoptosis in HepG2 cells. J. Nutr. Biochem. 2010;21:196-205.

McKinsey TA, Zhang CL, Olson EN. MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem Sci 2002; 27: 40–47.

Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. Biochem J. 2007 May 15;404(1):1-13.

Middleton EJr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000; 52:673-751.

Mitchell P, Foran S. Guidelines for the Management of Diabetic Retinopathy. The National Health and Medical Research Council (NHMRC).Australian Diabetes Society for the Department of Health and Ageing. 2008.

Monagas M, Khan N, Andres-Lacueva C, Casas R. Effect of cocoa powder on the modulation of inflammatory biomarkers in patients at high risk of cardiovascular disease. Am. J. Clin. Nutr. 2009;90:1144-1150.

Monahan KD, Feehan RP, Kunselman AR, Preston AG. Dose-dependent increases in flow-mediated dilation following acute cocoa ingestion in healthy olderadults. J. Appl. Physiol. 2011;111:1568-1574.

Moonen HJ, Geraets L, Vaarhorst A, Bast A, Wouters EF, Hageman GJ. Theophylline prevents NAD+ depletion viaPARP-1inhibitioninhuman pulmonary epithelialcells. Biochem. Biophys. Res. Commun. 2005;338:1805–1810.

Moura J, Børsheim E, Carvalho E. The Role of MicroRNAs in Diabetic Complications-Special Emphasis on Wound Healing. Genes (Basel). 2014 Sep 29;5(4):926-56.

Muniyappa R, Hall G, Kolodziej TL, Karne RJ. Cocoa consumption for 2 wk enhances insulin-mediated vasodilatation without improving blood pressure or insulin resistance in essential hypertension. Am. J. Clin.Nutr. 2008;88:1685-1696.

Nakanishi T, Mukai K, Hosokawa Y, Takegawa D, Matsuo T. Catechins inhibit vascular endothelial growth factor production and cyclooxygenase-2 expression in human dental pulp cells. Int Endod J. 2014 May 21.

Nanetti L, Raffaelli F, Tranquilli AL, Fiorini R. Effect of consumption of dark chocolate on oxidative stress in lipoproteins and platelets in women and in men. Appetite 2012;58:400-405.

North BJ, Verdin E. Sirtuins: Sir2-related NAD-dependent protein deacetylases. Genome Biol. 2004;5(5):224.

O'Neill LAJ, Kaltschmidt C. NF-kB: a crucial transcription factor for glial and neuronal cell function. TINS 1997;20:252-8.

O'Rourke MF, Hashimoto J. Mechanical factors in arterial aging: a clinical perspective. J. Am. Coll. Cardiol. 2007;50:1-13.

Obrosova IG, Drel VR, Kumagai AK, Sza´bo C, Pacher P, Stevens MJ. Obrosova IG, Drel VR, Pacher P, et al. Oxidative-nitrosative stress and poly (ADP-ribose) polymerase (PARP) activation in experimental diabetic neuropathy: the relation is revisited. Diabetes 2005; 54: 3435–3441.

Obrosova IG, Drel VR, Kumagai AK, Sza´bo C, Pacher P, Stevens MJ. Early diabetes induced biochemical changes in the retina: comparison of rat and mouse models. Diabetologia. 2006; 49: 2525–2533.

Obrosova IG, Xu W, Lyzogubov VV, et al. PARP inhibition or gene deficiency counteracts intraepidermal nerve fiber loss and neuropathic pain in advanced diabetic neuropathy. Free Radic Biol Med. 2008; 44: 972–981.

Ohara A. Radicais Livres Bons, Maus e Naturais. Oficina De Textos, 2006.

Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M. Global invivo and site-specific phosphorylation dynamic sinsignaling networks. Cell. 2006;127:635-648.

Osakabe N, Yamagishi M, Sanbongi C, Natsume M, Takizawa T, Osawa T. Antioxidative Substances in Cacao Liquor. Journal of Nutritional Science Vitaminology. 1998;44:313-21.

Pacher P, Liaudet L, Soriano FG, Mabley JG, Szabo E, Szabo C. The role of poly(ADPribose) polymerase activation in the development of myocardial and endothelial dysfunction in diabetes. Diabetes. 2002; 51: 514 –521.

Papadimitriou A, Peixoto EB, Silva KC, Lopes de Faria JM, Lopes de Faria JB. Increase in AMPK brought about by cocoa is renoprotective in experimental diabetes mellitus by reducing NOX4/TGFβ-1 signaling. J Nutr Biochem. 2014;25(7):773-84.

Pautler EL, Ennis SR. The effect of induced diabetes on the electroretinogram components of the pigmented rat. Invest Ophthalmol Vis Sci. 1980;19:702–705.

Pearson DA, Holt RR, Rein D, Paglieroni T. Flavanols and platelet reactivity. Clin. Dev. Immunol. 2005;12:1-9.

Pellegrini-Giampietro DE, Moroni ACF. Poly(ADP-ribose) Polymerase (PARP) and Excitotoxicity [onlline]. [Acessado em 29 de setembro de 2014]. Disponível em: http://www.landesbioscience.com/curie/chapter/2110/

Pietta G. Flavonoids as antioxidants. J Nat Prod 2000; 63:1035-42.

Pinto CC, Silva KC, Biswas SK, Martins N, De Faria JB, De Faria JM. Arterial hypertension exacerbates oxidative stress in early diabetic retinopathy. Free Radic Res 2007; 41: 1151-1158.

Pleschke JM., Kleczkowska HE, Strohm M, Althaus FR. Poly (ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. J. Biol. Chem. 2000; 275: 40974–40980.

Polak M, Newfield RS, Fioretto P, Czernichow P, Marchase R. Pathophysiology of diabetes complications. Diabetologia 1997;40:B65-7.

Portillo JA, Greene JA, Okenka G, Miao Y, Sheibani N, Kern TS, Subauste CS. CD40 promotes the development of early diabetic retinopathy in mice. Diabetologia. 2014 Jul 12.

Puvanendran K, Devathasan G, Wong PK.Visual evoked responses in diabetes. J Neurol Neurosurg Psychiatry.1983; 46: 643-647.

Qaum T, Xu Q, Joussen AM, et al. VEGF-initiated blood-retinal barrier breakdown in early diabetes. Invest Ophthalmol Vis Sci 2001, 42: 2408-13.

Rajendrasozhan S, Yang SR, Kinnula VL, Rahman I. SIRT1, an anti-inflammatory and anti-aging protein, is decreased in lungs of patients with chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 2008;177:861–870.

Rein D, Paglieroni TG, Pearson DA, Wun T. Cocoa and wine polyphenols modulate platelet activation and function. J. Nutr. 2000;130:2120S-2126S.

Rein D, Paglieroni TG, Wun T, Pearson DA, Schmitz HH, Gosselin R, Keen CL. Cocoa Inhibits Platelet Activation and Function. American Journal of Clinical Nutrition. 2000;72:30-35.

Richelle M, Tavazzi I, Enslen M, Offord EA. Plasma kinetics in man of epicatechin from black chocolate. Eur. J. Clin. Nutr. 1999;53:22-26.

Ried K, Sullivan T, Fakler P, Frank OR. Does chocolate reduce blood pressure? A meta-analysis. BMC Med. 2010;8:39.

Ried K, Sullivan TR, Fakler P, Frank OR. Effect of cocoa on blood pressure. Cochrane Database Syst. Rev. 2012;8:CD008893.

Riepe RE, Norenburg MD. Müller cell localization of glutamine synthetase in rat retina. Nature 1977; 268: 654-5.

Roberts CK, Sindhu KK. Oxidative stress and metabolic syndrome. Life Sci. 2009;84:705-712.

Romano C, Price MT, Olney JW. Delayed excitotoxic neurodegeneration induced by excitatory amino acid agonists in isolated retina. J Neurochem 1995; 65: 59-67.

Rongvaux A, Andris F, Van Gool F, Leo O. Reconstructing eukaryotic NAD metabolism. Bioessays 2003; 25: 683–690.

Rosales MA, Silva KC, Duarte DA, de Oliveira MG, de Souza GF, Catharino RR, Ferreira MS, Lopes de Faria JB, Lopes de Faria JM. S-nitrosoglutathione inhibits inducible nitric oxide synthase upregulation by redox posttranslational modification in experimental diabetic retinopathy. Invest Ophthalmol Vis Sci. 2014 May 2;55(5):2921-32.

Rosales MA, Silva KC, Duarte DA, Rossato FA, Lopes de Faria JB, Lopes de Faria JM. Endocytosis of Tight Junctions Caveolin Nitrosylation Dependent Is Improved by Cocoa Via Opioid Receptor on RPE Cells in Diabetic Conditions. Invest Ophthalmol Vis Sci. 2014;55(9):6090-100.

Rosales MA, Silva KC, Lopes de Faria JB, Lopes de Faria JM. Exogenous SOD mimetic tempol ameliorates the early retinal changes reestablishing the redox status in diabetic hypertensive rats. Invest Ophthalmol Vis Sci. 2010;51(8):4327-36.

Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr. 2002;22:19-34.

Runkle EA, Antonetti DA. The blood-retinal barrier: structure and functional significance. Methods Mol Biol 2011; 686: 133-48.

Salminen A, Kaarniranta K. SIRT1 regulates the ribosomal DNA locus: epigenetic candles twinkle longevity in the Christmas tree. Biochem Biophys Res Commun. 2009 Jan 2;378(1):6-9.

Sanbongi C, Osakabe N, Natsume M, Takizawa T, Gomi S, Osawa T. Antioxidative Polyphenols Isolated from Theobromacacao. Journal of Agricultural and Food Chemistry, 1998;46:454-457.

Sanbongi C, Suzuki N, Sakane T. Polyphenols in chocolate, which have antioxidant activity, modulate immune functions in humans in vitro. Cell. Immunol. 1997;177:129-136.

Sarria B, Mateos R, Sierra-Cinos JL, Goya L. Hypotensive, hypoglycaemic and antioxidant effects of consuming a cocoa product in moderately hypercholesterolemic humans. Food Funct. 2012;3:867-874.

Sasaki T, Maier B, Koclega KD, Chruszcz M, Gluba W, Stukenberg PT, Minor W, Scrable H. Phosphorylation regulates SIRT1 function. PLoS One. 2008;3: e4020.

Sauve AA, Wolberger C, Schramm VL, Boeke JD. The biochemistry of sirtuins. Annu. Rev.Biochem. 2006;75:435-465.

Scalbert A, Johnson IT, Saltmarsh M. Polyphenols: antioxidants and beyond. Am J Clin Nutr. 2005;81(1Supl.):S215-7.

Schnachenberg CG. Oxygen radicals in cardiovascular-renal disease. Curr Opin Pharmacol. 2002; 2: 121-125.

Schnorr O, Brossette T, Mommab, TY, Kleinbongard P, Keen CL, Schroeter H, Sies H. Cocoa Flavanols Lower Vascular Arginase Activity in Human Endothelial Cells in Vitro and in Erythrocytes in vivo. Archives of Biochemistry and Biophysics. 2008;476:211-215.

Schramm DD, Wang JF, Holt RR, Ensunsa JL. Chocolate procyanidins decrease the leukotrieneprostacyclin ratio in humans and human aortic endothelial cells. Am. J. Clin. Nutr. 2001;73:36-40.

Schreiber V, Dantzer F, Ame JC, de Murcia G. Poly (ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol. 2006; 7: 517-528

Sen R e Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 1986;46:705-16.

Shahani N, Sawa A. Nitricoxide signaling and nitrosative stress in neurons: role fors-nitrosylation. Antioxid. Redox Signaling. 2011;14:1493–1504.

Shrime MG, Bauer SR, McDonald AC, Chowdhury NH. Flavonoid-rich cocoa consumption affects multiple cardiovascular risk factors in a meta-analysis of short-term studies. J. Nutr. 2011;141:1982-1988.

Siebenlist U. NF-kB/IkB proteins. Their role in cell growth, differentiation and development. Biochim Biophys Acta 1997;1332:7-13.

Silva KC, Rosales MA, Biswas SK, Lopes de Faria JB, Lopes de Faria JM. Diabetic retinal neurodegeneration is associated with mitochondrial oxidative stress and is improved by an

angiotensin receptor blocker in a model combining hypertension and diabetes. Diabetes 2009; 58: 1382-1390.

Silva KC, Rosales MA, Hamassaki DE, Saito KC, Faria AM, Ribeiro PA, Faria JB, Faria JM. Green tea is neuroprotective in diabetic retinopathy. Invest Ophthalmol Vis Sci. 2013 Feb 15;54(2):1325-36.

Simó R, Villarroel M, Corraliza L, et al. The retinal pigment epithelium: something more than a constituent of the blood-retinal barrier–implications for the pathogenesis of diabetic retinopathy. J Biomed Biotechnol 2010; 2010:190724.

Singh SS, Sarma JA, Narasu L, Dayam R, Xu S, Neamati N. A Review on PARP1 Inhibitors: Pharmacophore Modeling, Virtual and Biological Screening Studies to Identify Novel PARP1 Inhibitors. Curr Top Med Chem. 2014 Sep 29.

Siqueira RC, Abad L, Benson G, Sami M. Behaviour of stem cells in eyes of rabbits with chorioretinal injuries caused by laser red diode 670N-M. In: Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), 2008, Fort Lauderdale. Investigative Ophthalmology & Visual Sciences. 2008;49:536-536.

Siqueira RC. Cell therapy in ophthalmology diseases. Rev. Bras. Hematol. Hemoter. 2009;31:120-127

Sokol S, Moskowitz A, Skarf B, et al. Contrast sensitivity in diabetics with and without background retinopathy. Arch Ophthalmol 1985; 103: 51-5

Stitt AW, Gardiner TA, Archer DB. Histological and ultrastructural investigation of retinal microaneurysm development in diabetic patients. Br J Ophthalmol 1995; 79: 362–7.

Sugawara R, Hikichi T, Kitaya N, Mori F, Nagaoka T, Yoshida A, Szabo C. Peroxynitrite decomposition catalyst, FP15, and poly (ADP-ribose) polymerase inhibitor, PJ34, inhibit leukocyte entrapment in the retinal microcirculation of diabetic rats. Curr Eye Res. 2004; 29:11–16.

Sullivan PG, Geiger JD, Mattson MP, Scheff SW. Dietary supplement creatine protects against traumatic brain injury. Ann Neurol. 2000; 48: 723-729.

Szabo C, Biser A, Benko R, Bottinger E, Susztak K. Poly (ADP-ribose) polymerase inhibitors ameliorate nephropathy of type 2 diabetic Leprdb/db mice. Diabetes 2006; 55: 3004–3012.

Tamada Y, Fukiage C, Nakamura Y, Azuma M, Kim YH, Shearer TR. Evidence for apoptosis in the selenite rat model of cataract. Biochem Biophys Res Commun. 2000; 275: 300–306.

Taubert D, Roesen R, Lehmann C, Jung N. Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: a randomized controlled trial. JAMA 2007;298:49-60.

The Longevity Gene SIRT1 – CR, Fasting and Aging Diseases [onlline]. [Acessado em 21 de setembro de 2014]. Disponível em: http://physiologyonline.physiology.org/content/21/6/404/F4.medium.gif

Tokede OA, Gaziano JM, Djousse L. Effects of cocoa products/dark chocolate on serum lipids: a meta-analysis. Eur. J. Clin. Nutr. 2011;65:879-886.

Toledano MB, Leonard WJ. Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction in vitro. Proc Natl Acad Sci U S A. 1991 May 15;88(10):4328-32.

Tretiach M, Madigan MC, Wen L, et al. Effect of Müller cell coculture on in vitro permeability of bovine retinal vascular endothelium in normoxic and hypoxic conditions. Neurosci Lett 2005; 378: 160-5.

Tuder RM. Aging and cigarette smoke: fueling the fire. Am J Respir Crit Care Med. 2006 Sep 1;174(5):490-1.

United Kingdom Prospective Diabetes Study Group. Intensive blood glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 1998; 352: 837-853.

Vinores SA, Gadegbeku C, Campochiaro PA, et al. Immunohistochemical localization of blood-retinal barrier breakdown in human diabetics. Am J Pathol 1989; 134: 231-5.

Vinores SA, Van Niel E, Swerdloff JL, et al. Electron microscopic immunocytochemical demonstration of blood–retinal barrier breakdown in human diabetics and its association with aldose reductase in retinal vascular endothelium and retinal pigment epithelium. Histochem J 1993;

Vogelmeier C, Ramos-Barbon D, Jack D, Piggott S, Owen R, Higgins M, Kramer B; INTIME study investigators (INdacaterol & TIotropium: Measuring Efficacy).

Wang X, Tang X, Gong X, Albanis E, Friedman SL, Mao Z. Regulation of hepatic stellate cell activation and growth by transcription factor myocyte enhancer factor 2. Gastroenterology 2004; 127: 1174-1188.

WESDR. The Wisconsin Epidemiologic Study of Diabetic Retinopathy XXIII. Ophthalmology. 2009; 116(3):497-503.

Weseler AR, Geraets L, Moonen HJ, Manders RJ, vanLoon LJ, Pennings HJ, Wouters EF, Bast A, Hageman GJ. Poly(ADP-ribose) polymerase 1-inhibiting flavonoids attenuate cytokine release in blood from male patients with chronic obstructive pulmonary disease or type 2 diabetes. J. Nutr. 2009;139:952–957.

Westphal CH, Dipp MA, Guarente L. A therapeutic role for sirtuins in diseases of aging? Trends Biochem Sci. 2007 Dec;32(12):555-60. Epub 2007 Nov 5.

Williamson G, Manach C. Bioavailability and Bioefficacy of Polyphenols in Humans. Ii. Review of 93 Intervention Studies. American Journal of Clinical Nutrition.2005;8: 243s-255s.

Wiswedel I, Hirsch D, Kropf S, Gruening M. Flavanol-rich cocoa drink lowers plasma F(2)-isoprostane concentrations in humans. Free Radic. Biol. Med. 2004;37:411-421.

Wollgast J, Anklam E. Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Research International, Essex, v. 33, n. 6, p. 423-447, 2000b.

Xu B, Chiu J, Feng B, Chen S, Chakrabarti S. PARP activation and the alteration of vasoactive factors and extracellular matrix protein in retina and kidney in diabetes. Diabetes Metab Res Rev. 2008; 24: 404-412.

Xu HZ, Le YZ. Significance of outer blood-retina barrier breakdown in diabetes and ischemia. Invest Ophthalmol Vis Sci 2011; 52(5): 2160-4.

Yang SR, Wright J, Bauter M, Seweryniak K, Kode A, Rahman I. Sirtuin regulates cigarette smoke-induced pro inflammatory mediator release via RelA/p65NF-kappaB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. Am. J. Physiol. Lung Cell. Mol. Physiol. 2007;292:L567–576.

Yang Y, Fu W, Chen J, Olashaw N, Zhang X. Nicosia SV. Bhalla K, Bai W. SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. Nat. Cell Biol. 2007;9:1253-1262.

Yang Z, von Ballmoos MW, Faessler D, et al. Paracrine factors secreted by endothelial progenitor cells prevent oxidative stress-induced apoptosis of mature endothelial cells. Atherosclerosis 2010;211:103-109.

Yao H, Rahman I. Perspectives on translational and therapeutic aspects of SIRT1 in inflammaging and senescence. [Acessado em 21 de setembro de 2014]. Disponível em: http://physiologyonline.physiology.org/content/21/6/404/F4.medium.gif.

Yao H, Chung S, Hwang JW, Rajendrasozhan S, Sundar IK, Dean DA, McBurney MW, Guarente L, Gu W, Ronty M, Kinnula VL, Rahman I. SIRT1 protects against emphysema via FOXO3-mediated reduction of pre-mature senescence in mice. J. Clin. Invest. 2012;122:2032–2045.

Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. 2004 Jun 16;23(12):2369-80.

Yoder MC, Mead LE, Prater D, Et Al. Redefining Endothelial Progenitor Cells Via Clonal Analysis And Hematopoietic Stem/Progenitor Cell Principals. Blood. 2007;109:1801-1809.

Yoon CH, Hur J, Park KW, et al. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. Circulation 2005;112:1618-1627.

Yu, SW. et al. Mediation of poly (ADP-ribose) polymerase-1-dependent cell death by apoptosis inducing factor. Science 2002; 297: 259-263.

Yuen DA, Connelly KA, Advani A, et al. Culture-modified bone marrow cells attenuate cardiac and renal injury in a chronic kidney disease rat model via a novel antifibrotic mechanism. PLoS ONE 2010;5:e9543.

Yuen DA, Connelly KA, Zhang Y, Advani SL, Thai K, Kabir G, Kepecs D, Spring C, Smith C, Batruch I, Kosanam H, Advani A, Diamandis E, Marsden PA, Gilbert RE. Early outgrowth cells release soluble endocrine antifibrotic factors that reduce progressive organ fibrosis. Stem Cells. 2013;31(11):2408-19.

Zee RS, Yoo CB, Pimentel DR, Perlman DH, Burgoyne JR, Hou X, McComb, ME, Costello CE, Cohen RA, Bachschmid MM. Redox regulation of sirtuin-1 by S-glutathiolation. Antioxid. RedoxSignaling. 2010. 13:1023-1032.

Zhang T, Kraus WL. SIRT1-dependent regulation of chromatin and transcription: linking NAD(+) metabolism and signaling to the control of cellular functions. Biochim Biophys Acta. 2010 Aug;1804(8):1666-75.

Zhang W, Liu H, Rojas M, Caldwell RW, Caldwell RB. Anti-inflammatory therapy for diabetic retinopathy. Immunotherapy, vol. 3, no. 5, pp. 609-628, 2011.

Zhang Y, Yuen DA, Advani A, Thai K, Advani SL, Kepecs D, Kabir MG, Connelly KA, Gilbert RE. Early-outgrowth bone marrow cells attenuate renal injury and dysfunction via an antioxidant effect in a mouse model of type 2 diabetes. Diabetes. 2012;61(8):2114-25.

Zheng L, Gong B, Hatala DA, Kern TS. Retinal ischemia and reperfusion causes capillary degeneration: similarities to diabetes. Invest Ophthalmol Vis Sci. 2007; 48: 361-367.

Zheng L, Szabo C, Kern TS. Poly(ADP-ribose) polymerase is involved in the development of diabetic retinopathy via regulation of nuclear factor-κB. Diabetes 2004; 53: 2960–2967.

Zheng Z, Chen H, Li J, Li T, Zheng B, Zheng Y, Jin H, He Y, Gu Q, Xu X. Sirtuin 1-mediated cellular metabolic memory of high glucose via the LKB1/AMPK/ROS pathway and therapeutic effects of metformin. Diabetes. 2012 Jan;61(1):217-28.

Zhu D, Wang L, Yan S, Zhou Q, Li Z, Sheng J, Zhang W. (+) Catechin ameliorates diabetic nephropathy by trapping methylglyoxal in type 2 diabetic mice. Mol Nutr Food Res. 2014 Sep 22.

8 – APÊNDICE

EDITORIAL

Future Therapeutic Targets for the Treatment of Diabetic Retinopathy—What Comes Next?

Diabetic retinopathy (DR) is still the leading cause of blindness among productive-age individuals in developed countries. Several mechanisms have been postulated to explain the pathogenesis of DR, and many of them have focused on particular abnormalities at the molecular level. In this special issue, the authors conducted comprehensive mini-reviews addressing different systems and pathways involved in the development and progression of DR, such as rennin angiotensin aldosterone system, mitochondria dysfunction, and oxidative stress. Nowadays, besides the improvement of surgical techniques, innovative pharmacological treatments are needed in order to treat this devastating complication of diabetes that affects approximately 5%–10% of patients with diabetes around the world. In proposing new target molecules to treat DR, a better understanding of the underlying mechanisms that are acting in the retinal tissue under diabetic milieu conditions is needed.

Classically, DR is understood as ischemic retinopathy leading to microvasculopathy. Although the ischemic stimulus is pivotal to the progression of the retinopathy to more severe forms, retinal neurodegeneration is present in the early phases of the disease. The retina displays a sophisticated organization and the intrinsic cross-talk between vascular elements and the neuro-glial complex should be considered. Valuable animal models have been used to elucidate which retinal mechanisms are altered in the presence of diabetes. Rodent experimental models such as the pharmacological induction of diabetes, the galactose diet, selective inbreeding, or genetic modification are commonly used. Among these, inherited hypertension and/or obesity are of particular interest. However, there is still no ideal animal model that fully reproduces the retinal alterations observed in patients with diabetes, either in the neuro-glial complex and/or in vascular sites of the retina.

Observations from pre-clinical and clinical studies indicate that both the renin-angiotensin system (RAS) and advanced glycation end products (AGEs) influence DR and also the cross-talk between both systems. In this issue, Miller and colleagues described the implications of the identification of all RAS components in the retina, and the interactions of these pathways might influence new developments of therapeutic targets. Blockade of the RAS and inhibition of AGEs improve experimental DR. The authors suggest that the crosstalk between the RAS and AGE pathways involves the glyoxalase 1 enzyme and that it is crucial to detoxify methylglyoxal-derived AGEs. A possible treatment through the combination of an RAS blockade and the augmentation of GLO1 would provide further protection against the cellular dysfunction that occurs in DR.

Mitochondria are the cellular energy-producing organelles and are determinants of cell lifespan. The function of mitochondria has been intensely studied in metabolic disorders, including DR. Their aberrant changes, described as fission and fusion machineries undergo morphological changes. Mitochondrial fission and fusion play important role in diabetes establishment and progression of diabetes as well as in DR. Elucidating the role of mitochondrial morphology dynamics in the pathogenesis of DR may provide important evidence in identifying new therapeutic targets. Revising this important topic, Roy and collaborators recognize mitochondria as a key organelle responsible for high glucose-mediate endoplasmatic reticulum stress in retinal tissue, and these events are important in producing apoptosis, a hallmark in the pathogenesis of DR.

Convincing evidence demonstrates that inflammation and oxidative stress are pivotal in the early stages of the pathogenesis of DR. From large, randomized clinical studies as UKPDS and DIRECT, tight blood pressure control reduces the progression of DR in patients with type 2 diabetes, indicating that the operating mechanisms in hypertension and diabetes are interacting in the retinal tissue, exacerbating the retinopathy. These observations motivated experimental studies aimed at addressing the possible mechanisms that worsen the retinopathy. In the mini-review about the mechanisms by which hypertension intensifies DR, findings from experimental studies indicate that oxidative stress and inflammation are up regulated in hypertensive diabetic animals when compared with normotensive ones and that the blockage of RAS protects the retina from the toxicity of the hyperglycemia by its antioxidant effect.

Future directions in providing novel, non-invasive ocular treatment modalities depend on a better understanding of the pathogenesis of DR. In this special issue, some important aspects are discussed.

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The Concomitance of Hypertension and Diabetes Exacerbating Retinopathy: The Role of Inflammation and Oxidative Stress

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Abstract: Diabetes and hypertension frequently coexist and constitute the most notorious combination for the pathogenesis of DR. Large clinical trials have clearly demonstrated that tight control of glycaemia and/or blood pressure significantly reduces the incidence and progression of DR. The mechanism by which hypertension interacts with diabetes to exacerbate the retinal disease is not completely understood. From experimental studies, increasing evidence demonstrates that chronic inflammation and oxidative stress are involved. In the present review, we summarize data obtained from our research along with those from other groups to better understand the role of hypertension in the pathogenesis of DR. It is suggested that oxidative stress and inflammation may be common denominators of retinal damage in the presence of hypertension in diabetic patients.

Keywords: Diabetes, DR, hypertension, renin-angiotensin system, inflammation, oxidative stress.

SCOPE OF THIS ARTICLE

According to the World Health Organization, 346 million people worldwide have diabetes and it is becoming apparent at increasingly younger ages in both developed and developing nations [1]. Each year, another seven million people develop diabetes, and the disease is expected to affect some 428 million people worldwide by 2030. After 15 years of diabetes, approximately 2% of people become blind (approximately 7 million people), and about 10% develop severe visual impairment (approximately 35 million people) [2]. Thus, the increased prevalence of diabetes represents a major growing health burden worldwide.

Hypertension is frequently observed in association with diabetes, with up to 20–60% of diabetic patients having hypertension [3]. The coexistence of hypertension and diabetes substantially increases the risk of developing organ damage, such as kidney and retina leading to a greater incidence of cardiovascular mortality [4, 5]. Thus, the control of modifiable risk factors such as diabetes and hypertension is critical to reducing the burden of cardiovascular events.

Diabetes is associated with several micro- and macrovascular sequelae that result from hyperglycemia, dyslipidemia, and other metabolic disturbances. The control of metabolic factors, particularly tight glycemic control, and reducing hypertension to optimal levels is fundamental to reducing cardiovascular risk in patients with type 2 diabetes [6]. The United Kingdom Prospective Diabetes Study (UKPDS) Group found that each 10-mmHg decrease in mean systolic blood pressure (SBP) was associated with a 12% reduction in risk for any complication related to diabetes, a 15% decrease for a death related to diabetes, an 11% reduction for myocardium infarction, and a 37% decrease for DR [7].

Diabetes causes a number of metabolic and physiologic abnormalities in different organs, specifically in the eye. DR is a major cause of blindness in the working-age population in developed countries [8]. It manifests as a multifactorial progressive disease of the retina, the pathogenesis of which is extremely complex and involves a cascade of events affecting different cells and molecules [9, 10] Fig. (**1**). Furthermore, numerous factors have been found in early DR induced by hyperglycemia, characterized by activation of retinal cell markers before the damage [11-13]. In this article, we explore the possibility that exaggerated oxidative stress and inflammation may underlie the mechanism of retinal diseases in the concomitance of diabetes and hypertension. This review will focus on clinical and experimental evidences showing that the presence of hypertension aggravates retinopathy in diabetic patients.

DIABETES AND ASSOCIATED FACTORS IN THE WORLD

Diabetes induces disordered levels of metabolites such as lipids, amino acids, hormones, and nutrients and is characterized by hyperglycemia. Epidemiologic studies have shown the effects of hyperglycemia, hypertension, and dyslipidemia, insulin resistance, a high body mass and low level of physical activity on the incidence and progression of DR and clinically significant macular edema [13-16].

Hyperglycemia induces the progression of retinopathy, but this excess in plasma glucose cannot account solely for

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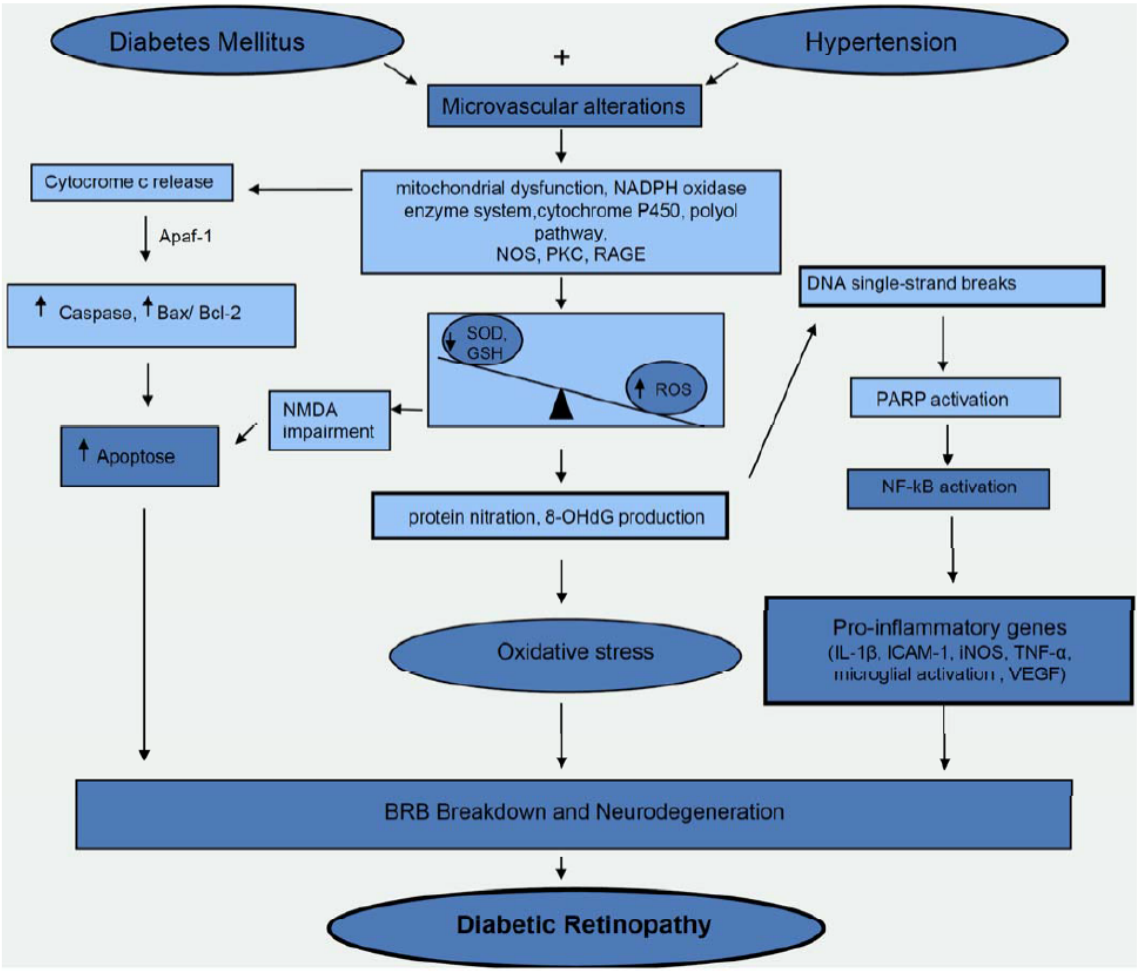

Fig. (1). Both diabetes and hypertension affect microvessels on the same target organs such as the retina, thus exacerbating the long-term damage. Among the biochemical pathways are mitochondrial dysfunction, NADPH oxidase system, polyol pathway and nitric oxide syntases. Mitochondrial oxidative stress releases the cytochrom *c*, which will complex with Apaf-1, activating the caspase cascade leading to apoptosis of vascular and neural cells. Also, the GSH depletion will impair the glutamate/glutamine cycle and this is the main mechanism of excitotoxicity and neurodegeneration. On the other hand, the oxidative stress will produce nitration of protein and DNA damage with consequent PARP activation, NFkB nuclear translocation, and transcription of several pro-inflammatory genes. As a consequence, multiple structural alterations are detected in retinal tissue, namely acellular capillaries, pericyte loss, and BRB leakage.
SOD: Superoxide dismutase; GSH: reduced glutathione; ROS: reactive oxygen species; NO: nitric oxide; PARP: poly-(ADP-ribose) polymerase; NF-kB: nuclear factor kappa B; IL-1β: interleukin-1B; ICAM-1: intercellular adhesion molecule 1; iNOS: inducible nitric oxide synthase; TNF-α: tumor necrosis factor- α; BRB: blood retinal barrier.

the range of cellular and functional damages [17-19]. In addition, a number of studies suggest that even with intensive therapy to tightly control blood sugar, long-term effects on the risk of kidney disease (50%), nerve disease (60%), and eye disease (76%) are still observed [20, 21]. Studies have shown that the control of blood glucose, blood pressure, and blood lipid levels helps prevent complications in people with type 1 or type 2 diabetes [22-26].

Clinically, although the glycated-hemoglobin (HbA1c) level is the strongest risk factor for predicting the development and progression of DR, HbA1c accounted for only 11% of the risk of retinopathy [27]. Similarly, blood pressure and total serum cholesterol together accounted for only 9 to 10% of the risk of retinopathy as described in the Wisconsin Epidemiologic Study of DR [28]. Data from clinical studies have shown that hypertension increases large artery dilation by as much as 15% [29] and retinal artery dilation by as much as 35% [30]. At the cellular and molecular levels, mechanical stretching can initiate intracellular signaling and alter the secretion of numerous factors, including nitric oxide

[31], endothelin-1 [32], platelet-derived growth factor [33], fibroblast growth factor [34, 35], and angiotensin II (ANG-II) [36].

THE EFFECTS OF HYPERTENSION ON RETINAL VASCULOPATHY

In the past 30 years, few papers addressed the effects of hypertension solely on the vascular bed of the retina. It has been shown that retinal capillaries are especially sensitive to sustained high blood pressure. In severe forms of arterial hypertension, so-called malignant hypertension, it is observed areas of retinal hemorrhages exudation are observed with ischemic manifestations of retinal edema and cotton-wool spots. From the classical studies from Garner, Ashton and Tripathi in the earlier 1975, the clinical and pathological changes of the retina from monkeys experimentally induced hypertension were described. Hypertensive retinopathy (HR) is characterized by tortuosity and dilation of the arteries and the light reflex decreased in those animals that became hypertensive. The earliest abnormality was a development of many points of fluorescein leakage on terminal arterioles or small arteries. Many precapillary arteries were constricted and some were virtually occluded, progressing to plasma insudation into the vessel wall with muscle necrosis results in secondary occlusion and the typical picture of advanced fibrinoid necrosis (For more detailed description, please refer to Ashton N, Peltier S, Garner A: Experimental hypertensive retinopathy in the monkey. Trans Ophthalmol Soc UK 1969; 88: 167– 186) [37]. By using mophometric techniques, Flückiger and collaborators [38] in 1984 described that in SHR rats the retinal capillary basement membrane is thickened. Morphometric studies on retinal microangiopathy and myocardiopathy in hypertensive rats (SHR) with induced diabetes). In a more exuberant animal model of hypertension, the stroke-prone SHR, the retinal hypertensive lesions are observed earlier than in SHR rats, and include neural damages in the ganglion cell layer, exhibiting some ganglion cells realized cytoid bodies corresponding to a lysed cell and with most of the axons destroyed, the inner and outer plexiform layers, have most of the contacts were lost because of fibrinous deposits and numerous dense bodies in the inner rod segment and the vesiculation of the rod outer segment were observed. The capillaries showed markedly hypertensive lesions with thickening of basement membrane [39]. In another study from Gin and colleagues [40], aimed to investigate the contributions of hypertension and diabetes to microvascular dysfunction in the kidney and eye, the authors observed that the concomitance of hypertension and diabetes accentuated the retinal and anterior segment vascular dysfunctions estimated by I-albumin leakage and that antihypertensive therapies as converting enzyme inhibitor (perindopril), a calcium-channel blocker (lacidipine), or triple therapy (hydrochlorothiazide, reserpine, and hydralazine) prevented these changes, although more readily by calcium-channel blockade than by converting-enzyme inhibition. These collective data suggest that hypertension and diabetes have the vascular bed as their target organs and the pathology of these alterations is very similar in both diseases involving not only vascular cells but also neuro elements of the retina.

More recently, an elegant study decribed the early ultrastructural changes in retinal capillaries under hypertension and diabetes conditions [41]. In Wistar rats, the investigators induced hypertension by unilateral nephrectomy and administered subcutaneous mineralocorticoid and 0.9% oral saline weekly thus inducing experimental diabetes. A significant and sustained increase in systolic blood pressure occurred in nephrectomized rats. There was a significant increase in the number of caveolae in both pericytes and endothelial cells in animals with hypertension and diabetes together compared with all other groups and in pericytes in animals with diabetes alone. The number of direct contacts between pericytes and endothelial cells was reduced in diabetic and hypertensive diabetic animals. Based on these observations, the authors concluded that hypertension and diabetes had an interactive effect in producing retinal capillary basement membrane thickening and blood retinal barrier breakdown by increasing the number of caveolae in both endothelial cells and pericytes. Confirming these data, in a more recent study, the ACE inhibitor cilazapril was effective in preventing endothelial alterations and basement membrane thickness in diabetic hypertensive conditions, and thus may account for the well-known improvement of the blood-retinal barrier observed during antihypertensive treatment [42].

Then, clinical trials, such as EUCLID were conducted in order to better elucidate the participation of hypertension of the development of diabetic retinopathy. In this particular study, the role of rennin-angiotensin system (RAAS) gained more importance. From these observations, Wilkinson-Berka, Miller A and collaborators not only described the presence of all components of RAAS in the retina but also that these components are up regulated in presence of diabetes. This topic is fully convered in a comprehensive mini review included in this special issue.

THE RETINA: VASCULAR NEURO-GLIAL UNIT IN DIABETIC RETINOPATHY

Diabetic retinopathy is characterized by early neuro-glial changes followed by extensive vascular changes, namely acellular capillaries, pericyte loss, microaneurysm formation, basement membrane thickening, and blood-retinal barrier (BRB) breakdown [43].

There is increasing evidence that functional impairment precedes the earliest clinical manifestations of diabetic retinal vasculopathy. Sensitive psychophysical examinations, such as contrast sensitivity [44-46] and color vision [47, 48] and also electrophysiological methods including electro-retinography (ERG) [49-51] and visual evoked potentials (VEP) [52-55] clearly show early abnormalities to be a potential primary consequence of hyperglycaemia before the onset of retinopathy. These alterations characterize DR as a neurodegenerative disease in which visual dysfunction is initiated in early diabetes.

The influence of diabetes on neural retinal cells has also been analyzed at the molecular level. Under diabetic conditions, the increase in superoxide anions in the neural retina leads to a decreased bioavailability of nitric oxide, which is originally induced in the retina for tissue protection;

the decrease in nitric oxide action increases the formation of peroxynitrite, which inhibits the downstream signaling of a neuroprotective factor, nerve growth factor (NGF), and neuronal survival [56].

The pathological mechanisms that lead to neuronal deficits are unknown. However, it seems that retinal glial cells play an essential role in maintaining the normal function and integrity of the retina, including Müller cells, the principal glial cells in the retina [57].

Several observations indicate that the degenerative processes begin in the neural retina shortly after the onset of diabetes and precede the characteristic vascular changes in DR. Glial cells play a central role in the homeostatic regulation of the retina [58], interacting with most neurons in a symbiotic relationship [59]. Normal neuronal and vascular function depends on interactions with glial cells. Glial cells are implicated in the maintenance of the BRB at the endothelial lining of retinal microvessels [60] and also the extracellular ionic environment is optimized by glial cells for proper electrophysiological function of neurons. Also, glial cells maintain low synaptic levels of neurotransmitters. The major excitatory neurotransmitter in the retina is glutamate, an amino acid that is toxic to retinal neurons when present in high amounts [61]. Thus, the regulation of retinal glutamate by glial cells [62] is essential for normal vision. A previous study in the streptozotocin rat model of short-term DR demonstrated that glial function and glutamate metabolism are altered in the retina within 3 months after the onset of diabetes in rats. This observation is consistent with the possibility that similar alterations occur in humans within the first years after onset of diabetes but remain subclinical until degeneration proceeds to the point where irreparable vision impairment is incurred. Furthermore, increased retinal glutamate may reduce the efficacy of neurotransmission in the inner retina [63].

The BRB restricts the permeability between the blood elements and retinal tissue. This barrier has particular permeability characteristics and appears to play a role of major importance in the pathophysiology and therapeutics of retinal disease [64]. A well-established early marker of DR is the inner BRB breakdown, which is formed by tight junctions between retinal capillary endothelial cells [65] and in the outer BRB, constituted by tight junctions between retinal pigment epithelial (RPE) cells [66]. Tight junctions (TJs) in both cells are essential for the strict control of ion, protein, and water flux into and out of the retina, as well as to prevent the entrance of toxic molecules and plasma components into the retina. The BRB is essential to maintaining the eye as a privileged site and is essential for normal visual function [64]. The vascular components of the BRB maintain the appropriated environment of the neural retina. The inner BRB acts as a selective barrier providing immune privilege and regulating osmotic balance, ionic concentration, and the transport of nutrients (sugars, lipids, and amino acids), thereby helping to control the specialized environment of the retina. In addition, pericytes, which are modified smooth muscle cells, share a common basal lamina and directly contact vascular endothelial cells. Formation of the inner BRB requires specialized differentiation of the vascular endothelial cells induced by astrocytes, Muller cells, and pericytes [64]. As demonstrated in a preclinical study, there is an early and diffuse retinal vascular permeability, which may represent an important mechanism accelerating the retinal changes caused by DM [67]. Early vascular changes include leukostasis [68], aggregation of platelets [69], altered blood flow [70], degeneration of pericytes [71], and basement membrane thickening [72]. The vascular permeability may be affected through the paracellular and transcellular pathways; the paracellular involves the endothelial tight junctions, and the transcellular pathway is mediated by endocytotic vesicles, known as caveolae [73-78].

Retinal Pigmented Epithelium (RPE) is a specialized epithelium lying in the interface between the neural retina and the choriocapillaris that plays an essential role in the proper functioning and maintenance of the neural retina by allowing the diffusion of oxygen from the choroidal circulation to the highly metabolic rods and cones, controlling the flow of solutes and fluids, thereby preventing the accumulation of extracellular fluid in the subretinal space of the retina and removing metabolic wastes by the transport of Cl^- and K^+ [79]. Thus, the outer BRB plays a crucial role in the transport and recycling of fatty acids, which is a major component of the photoreceptors and is important to retinal function [80]. Since the retina is the most active tissue metabolically, the transport of glucose and lactose as major energy sources through glucose and monocarboxylate transporters in the RPE is important to visual function [81]. The transport of retinoids from the blood circulation to the retina through the RPE is essential to a normal visual cycle [82]. These active transports are performed by the Na^+-K^+-ATPases, located in the apical side of the RPE [79]. The diabetes-induced outer BRB dysfunction has been clearly suggested in a study by Vinores and collaborators [83]. In this study, the authors localized inner and outer extravascular albumin sites from diabetic patients with and without various stages of retinopathy. The presence of extravascular albumin was earlier detected in inner layers of the retina; by contrast, the outer sites of extravascular albumin were only observed in the more advanced stages of DR [83]. By electrophysiology, the morphologic changes in the RPE can be readily detected in diabetic animals, which are reflected by an alteration in the *c*-wave of electroretinography [84, 85]. Despite these observations, the contribution of outer BRB dysfunction in DR deserves further studies. The development of innovative therapies that allow the transport of specific compounds across these barriers depends upon the understanding of the mechanisms of the functioning of BRB in a diabetic milieu.

EVIDENCE SHOWING THAT HYPERTENSION IS A CENTRAL RISK FACTOR ASSOCIATED WITH DIABETIC RETINOPATHY

In recent years, the concomitance of diabetes and hypertension has been frequently studied in patients and experimental models. In a survey conducted in the United States by Third National Health and Nutrition Examination between 1988 and 1994, 71% of adults with diabetes were found to have elevated blood pressure (mean blood pressure $\geq$ 130/85 mmHg or using antihypertensive medication) [86]. In individuals with type 1 diabetes, blood pressure levels are

usually normal at diagnosis, whereas, in individuals with type 2 diabetes, approximately 33% of the patients have elevated blood pressure when diabetes is diagnosed [87]. The concomitant presence of hypertension and diabetes significantly magnifies the risk of diabetic microvascular complications, namely DR (DR) [25, 26, 88].

A growing literature suggests that hypertension is an important risk factor associated with the development and progression of DR Fig. (**1**). In this setting of the micro-vascular complications, the United Kingdom Prospective Diabetes Study (UKPDS) has demonstrated that blood pressure and glycemia intensive controls are effective in substantially reducing the incidence and progression of DR [88, 89]. The same group has also suggested that blood pressure control supersedes glycemic control in the primary prevention of complications of diabetes [23].

Earlier studies have focused on improving glycemic control for the prevention and treatment of DR [82, 83]. Therefore, data from EURODIAB EUCLID (Controlled Trial of Lisinopril in Insulin-Dependent Diabetes) [90] and U.K. Prospective Diabetes Study results [86], on the controlling of blood pressure and, specifically, interference in the renin-angiotensin system have emerged as important strategies for treating DR. More recently, DIRECT (Diabetic Retinopathy Candesartan Trial), a randomized double-blind placebo-controlled study with type 1 or type 2 diabetic patients into daily placebo or 32 mg candesartan groups, an angiotensin II receptor blocker [25, 26], showed that in patients with type 2 diabetes, treatment with candesartan decreased the progression of retinopathy by 34% in participants with early retinopathy. These data showed that the potential benefits of the angiotensin II type 1 (AT_1) receptor blocker (ARB) candesartan might be seen in the early stages of diabetic retinopathy.

In a large population-based study, the Wisconsin Epidemiology Study of Diabetic Retinopathy (WESDR), the presence of hypertension in type 1 diabetic patients was associated with a 73% increase in the risk of incident proliferative DR (PDR) [91]. From other reports, the presence of arterial hypertension and poorer glycemia control increases the risk of visual impairment by 72% [92]. However, the mechanisms by which diabetes and hypertension interact to exacerbate DR are poorly established.

An important study demonstrated that lowering blood pressure in normotensive type 2 diabetic patients offers beneficial results on vascular complications [24]. In this study, the achieved blood pressure (BP) in the intensive group was 128/75 mm Hg versus 137/81 mm Hg in the moderate group. The intensive BP control group demonstrated a lesser progression of DR and a lower incidence of strokes and slowed the progression to incipient and overt diabetic nephropathy [24]. Specifically in individuals with type 2 DM, the risk of DR was independently and additively attributable to hypertension and hyperglycemia [93].

A multicenter controlled trial involving 285 normotensive type-1 diabetic patients has shown that losartan (angiotensin receptor blocker) slowed the progression of retinopathy [94], demonstrating the beneficial effects of primary intervention on this system. This finding is in line with another important multicentric clinical trial, DIRECT-1 (Prevent) [25]. A previous study has suggested that elevated blood pressure levels even in normotensive diabetic individuals are associated with microvascular complications, including DR. Rodrigues *et al*. demonstrated that in type 1 diabetic subjects, each increment of 5 mmHg in night-time systolic and diastolic blood pressure leads to an increase of about 40% in the risk of DR [94]. In type 2 diabetic patients, the risk for development diffuse macular edema is 3.2 times greater in patients with high blood pressure (HBP) (95% confidence interval [CI], 1.5 to 6.9) than in those with normal blood pressure [95].

From Animal Studies

We explore the influence of hypertension on the progression of diabetic retinal complication, suggesting the possibility that exaggerated oxidative stress and inflammation may underlie the mechanism of retinal diseases in diabetes and hypertension. It has been shown in spontaneously hypertensive rats (SHR) that the frequency of acellular capillaries in the presence of diabetes was nearly twice that in normotensive diabetic Wistar Kyoto (WKY) rats [96]. Supporting this finding, previous work also demonstrated that the control of blood pressure ameliorates retinal disease in diabetic SHR [97]. These studies support the concept that elevated blood pressure contributes to DR in SHR rats.

To better understand the molecular interaction between hypertension and diabetes-exacerbating DR, our group and other investigators have addressed this question in experimental models of diabetes and hypertension. Increased expression of fibronectin associated with elevation in retinal capillary permeability, higher expression of retinal vascular endothelial growth factor (VEGF), and decrease in retinal cell replication were observed after as little as 15 days of induction of diabetes by streptozotocin in hypertensive animals compared with normotensive control WKY rats [98]. In the same study, we assessed the total number of retinal cell replication evaluated by BrdU-positive retinal cells; it was found to be significantly higher in hypertensive SHR than in normotensive WKY rats. BrdU-positive-stained cells were rare but clearly identifiable in retinal sections of adult rats. The proliferating retinal cells of adult rats were characterized by the use of specific cell markers, including GFAP (marker of glial cell), the tyrosine-kinase receptor Flk1 (a surface receptor protein characteristic of endothelial cells), the intermediate filament protein nestin (an intermediate filament structural protein expressed in primitive neural tissue), and PKC-α (cellular maturation involved in a bipolar–amacrine neural cell). The findings indicate that adult rat retina contains different populations of replicating cells with characteristics of glial or endothelial cells. The detection of cells that coexpressed BrdU and nestin or PKC-α suggests that subpopulations of replicating cells may differentiate into neural cell subtypes [98]. We have also assessed the cell cycle regulators, cyclins $p27^{kip-1}$ and $p21^{Cip-1}$ in retina from diabetic SHR rats. The concomitancy of diabetes and hypertension attenuated the proliferating retinal cells and it was associated with an increase in $p27^{Kip1}$ expression, fibronectin accumulation, and BRB breakdown compared with normotensive WKY rats [98]. Findings from diabetic kidney disease demonstrate that the presence of

hypertension in diabetic rats modulates the expression of cyclin $p27^{Kip1}$, a negative cell cycle regulator, leading to a lesser cellular proliferation rate and enhanced cell hypertrophy with extra cellular accumulation [99]. Additional studies are required to clarify the mechanisms by which these cell cycle changes contribute to the structural abnormalities present in the early pathogenesis of DR.

From in vitro Studies

In the presence of hypertension, there will be transmission of systemic hypertension to the retinal capillary bed due to loss of the autoregulation of arterial vessels, thus contributing to the mechanism of the exacerbation of DR by hypertension [100]. Rassam *et al*. showed *in vitro* that there is a positive correlation between loss of vascular autoregulation and levels of glucose in the human retina. Numerous conditions, including atherosclerosis, aging, cigarette smoking, and diabetes, are associated with a decline in the production and/or biological activity of endothelium-derived NO• [101], thus interfering with vascular autoregulation. NO exerts numerous beneficial antiatherogenic endothelial functions by the elevation of cGMP and vasorelaxation *via* activation of soluble guanylate cyclase (sGC) and inhibition of platelet aggregation and inflammatory response. The reaction between $O_2^{\bullet-}$ and NO results not only in loss of NO bioactivity but also in the formation of $ONOO^-$, a potent oxidant, which may contribute to lipid peroxidation and membrane damage. $ONOO^-$ has multiple effects including oxidation of the endothelial NOS (eNOS) cofactor BH_4 [102]. This effect can produce eNOS "uncoupling," a condition in which the normal flow of electrons within the enzyme is diverted such that eNOS produces $O_2^{\bullet-}$ rather than NO. As an essential cofactor, BH4 is necessary for optimal eNOS activity [103]. It facilitates NADPH-derived electron transference from the eNOS reductase to the oxygenase domain to convert l-arginine to NO and l-citrulline. When BH4 levels are inadequate, eNOS becomes unstable and uncoupled, subsequently leading to less NO production and more superoxide generation. Moreover, the interaction between NO and superoxide leads to the formation of peroxynitrite, a potent oxidant, which further oxidizes BH4.

Evidence indicates that endothelial dysfunction could play an initial and ultimately crucial role in the development of macrovascular and microvascular complications caused by diabetes mellitus (DM) in human and animal models of DM [104]. Insulin is an essential hormone of metabolic homeostasis that has vasodilator action *via* PI3K/Akt pathway-dependent eNOS activation [105]. In addition to the PI3K/Akt pathway, insulin can modulate eNOS activity by increasing BH4 synthesis *via* the activation of GTPCH. Shinozaki *et al*. [106] have demonstrated that the GTPCH activity is significantly decreased in a rat model of insulin resistance, which leads to the attenuation of endothelial BH4 levels and substantially increased BH2 levels, resulting in impairment of endothelium-dependent vasodilatation. These sets of evidence demonstrate another mechanism by which hypertension contributes to diabetic microvascular complication in the retina. The alterations caused by diabetes in the retina also have been investigated using retinal cells subjected to mechanical stretching. VEGF has an important role in increasing vascular retinal permeability and in the development of intraocular neovascularization in DR [107]. Seko and collaborators demonstrated for the first time, the fact that retinal pigment epithelial cells respond to stretching by increasing the expression of VEGF [108]. This finding was confirmed by other studies showing that bovine retinal endothelial and pericyte cells exposed to cyclic stretching increase protein and mRNA expression of VEGF and its receptor [109]. Later, it was suggested by Suzuma *et al*. that stretching-induced retinal VEGF expression was mediated by phosphatidylinositol (PI) 3-kinase and protein kinase C (PKC) [110]. In addition, it has been demonstrated that stretching induces apoptosis in porcine retinal pericytes, which may be mediated by oxidative stress [111]. Pericyte loss is a hallmark of DR. Recently, it has been shown that stretching in bovine retinal pericytes induces a reduction in cell proliferation, increased apoptosis, and profound morphological changes in the actin cytoskeleton assembly [112]. When the pericytes were exposed to high levels of glucose, these alterations induced by stretching were magnified [112].

Using an experimental model of hypertension in spontaneously hypertensive rats (SHR), Suzuma *et al*. showed that VEGF-R2 (kinase insert domain-containing receptor [KDR]) is increased in retinal tissue compared with normotensive WKY control animals [110]. In recent years, our group has been evaluating retinal disease in hypertensive animals with experimentally induced diabetes. In these efforts, we have demonstrated that within as soon as 20 days after induction of diabetes, the redox status reflected an unbalance: in diabetic rats, there was a higher superoxide production. The superoxide production was higher in diabetic WKY rats than in control WKY rats and SHR rats showed elevated superoxide production compared with WKY groups accompanied by an impaired glutathione defense system. The presence of diabetes induced an additional DNA damage in SHR rats [113].

In conclusion, hypertension aggravates oxidative-induced cytotoxicity in diabetic retina due to the increase of superoxide production and impairment of the antioxidative system [113] Fig. (**1**). Another study with SHR, with 4-week-old (underdeveloping hypertensive rats) and 12-week-old (fully hypertensive rats), demonstrated that ED1-positive cells, ICAM-1, and VEGF levels, markers of inflammation, were significantly higher in diabetic SHR in both prehypertensive and hypertensive ages compared with the respective control groups. NF-kappaB p65 levels were higher in prehypertensive SHR and in hypertensive diabetic SHR rats ($p < 0.05$) [114]. Aggravation of the inflammatory process may be involved in the mechanism by which essential hypertension exacerbates retinopathy in the presence of diabetes. The abnormalities present in diabetic normotensive SHR rats (before the full development of hypertension) suggest that the genetics of hypertension might display a predisposition to a more severe diabetic retinal disease by exacerbation of inflammation/oxidative stress in retinal tissue.

Common mechanisms in hypertension and diabetes exacerbating the retinopathy Fig. (**1**).

In addition to strict control of blood glucose levels, tight blood pressure control with rennin angiotensin system (RAS) inhibition has been shown to prevent the progression of DR in the UK Prospective Diabetes Study (UKPDS) [115]. Recent studies indicate that glycemic insult compounds hemodynamic injury in microvascular complications of diabetes by activating the sometimes common pathways that lead to organ damage. Components of the DM include high glucose, hypertension-induced mechanical stress, and activation of the reninangiotensin system (RAS) [116]. Both hypertension and diabetes mellitus affect the same major target organs and the common denominator of hypertensive/ diabetic target organ-disease is the vascular tree [117].

Among diabetic patients, a large incidence and prevalence of hypertension in various populations has been documented; overall, hypertension is approximately twice as common in patients with diabetes mellitus than in non-diabetic people [118, 119], and increases in prevalence as kidney complications of diabetes worsen. In addition, patients with both diabetes and hypertension not only have a higher incidence of DR, but also a greater prevalence of cardiovascular risk factors, including dyslipidemia and hyperuricemia, a thrombotic tendency, and left ventricular hypertrophy [120].

Both hypertension and diabetes induce oxidative stress and inflammation, which, in turn, contribute to DR. The interaction between hypertension and diabetes-inducing inflammation and oxidative stress in DR has been assessed in SHR and WKY rat strains with streptozotocin-induced DM. Oxidative stress and inflammation are closely related events, because the generation of reactive species by the inflammatory cells can induce oxidative stress. On the other hand, oxidative stress may induce inflammation through NF-κB-mediated pro-inflammatory gene expression [121]. Numerous studies have demonstrated this intrinsic relationship between inflammation and oxidative stress in an inseparable manner in different organs, particularly in the retina [122, 123]. The mitochondrial respiratory chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme system, Nox4 (homologue of gp91phox subunit of NADPH oxidase), and AGE-receptor for AGE (RAGE) interaction have been found to induce oxidative stress in high-glucose conditions [124, 125]. Oxidative stress is conventionally defined as an imbalance between pro-oxidant stress and antioxidant defense. However, recent evidence indicates a new definition of oxidative stress has been proposed as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" [126].

Our group demonstrated an increase in the levels of ED1/microglial positive cells in the retina of SHR rats, mainly in diabetic SHR rats when compared with normotensive control WKY rats associated with an increase in ICAM-1 and the NF-κB p65 transcription factor [127]. We have demonstrated that the concomitance of systemic diseases, diabetes and hypertension, leads to earlier and more pronounced retinal inflammatory alterations. As already mentioned, the diabetic SHR rats demonstrate increased levels of ICAM-1, VEGF, and NF-κB p65 in the retina when compared with normotensive diabetic WKY rats [127].

Oxidative stress may lead to cell death [128] *via* apoptotic means, and it is widely known that apoptosis of retinal cells is a consummated phenomenon in DR. Retinal capillary cells undergo accelerated apoptosis, which precedes the detection of any of the histopathological changes characteristic of DR. Beside vascular cells, neuro and glial cells are also affected by the concomitance of hypertension and diabetes. The presence of all components of RAS along all retinal tissues precludes the suggestion that RAS might play a role in the surveillance of neuro/glial cells [129]. In our study, it was observed that the apoptotic rate was higher in the retina of diabetic SHRs compared with control WKY rats, and the cells exhibited neural and glial characteristics, as demonstrated by specific antigens. The oxidative imbalance was higher in diabetic rats and accentuated in diabetic SHRs in the presence of mitochondrial involvement, as demonstrated by decreased expression of Bcl-2 and UCP-2 mitochondrial proteins. The treatment with losartan, an angiotensin receptor blocker (AT-1), led to amelioration of the apoptotic rate in neural and glial retinal cells, reestablishment of redox status by decreasing superoxide production, improving the antioxidative enzymatic system GSH, and restoration of mitochondrial protein expression levels [130]. These findings indicate that the blockage of RAS exerts a variety of effects, including antioxidant, anti-apoptotic, and anti-inflammatory properties [131, 132].

The retina has a high content of polyunsaturated fatty acids and, relative to any other tissue, has the highest oxygen uptake and glucose oxidation. This phenomenon renders the retina more susceptible to oxidative stress [133]. The principal source of ROS in the retina is the mitochondria [134-136]. A number of studies have shown that there is an increase in oxidative markers after the induction of DM [125, 137, 138]. We previously demonstrated that the concomitance of diabetes and hypertension led to oxidative retinal damage in tissue from short-term STZ-induced diabetes in SHRs by an increase in nitrotyrosine staining throughout the retinal layers, mainly in the plexiform layers and heterogeneous 8-OHdG positivity in the nuclei of retinal tissue, presenting in all cellular layers of diabetic SHRs [113, 130].

Superoxide dismutase (SOD) catalyzes the conversion of superoxide (O_2^-) to hydrogen peroxide (H_2O_2), which can then be turned into water by catalase or the glutathione (GSH) peroxidase system. Although SOD is the first line of physiological defense against oxidative stress, the reaction of O_2^- with nitric oxide (NO) is about three times faster than its reaction with SOD [139]. The effects of free radicals on DNA are the most damaging to cell function. Nitrosative stress induces DNA single-strand breaks and leads to overactivation of the DNA repair enzyme poly-(ADP-ribose) polymerase (PARP). PARP is the enzyme that cleaves nicotinamide adenine dinucleotide (NAD+) to form nicotinamide and a PAR polymer. Generally, the activation of PARP contributes to energy failure, transcriptional gene regulation, and the induction of apoptosis/necrosis [140]. It has been demonstrated that nitroxides such as tempol exert a cytoprotective action against diverse oxidative insults [141] and catalyze the dismutation of superoxide to H_2O_2 plus O_2 (SOD-like activity). Previous studies have demonstrated the beneficial effects of tempol in diabetic retinas for reducing

leukostasis [142] and protecting the retina's neural cells [143].

These oxidative markers are produced by oxidative retinal damage, which is a consequence of the increase in superoxide production and depletion of the gluthatione-reduced antioxidant system [113, 132]. Concomitant with these findings, we also observed increased expression of a DNA repair enzyme poly-(ADP-ribose) polymerase (PARP) in SHR rats compared with normotensive WKY rats at 4 weeks of age, and the presence of hyperglicemia by only 20 days further increased these levels in DM SHR rats [144]. In our study, the administration of a superoxide dismutase mimetic, tempol, to diabetic SHRs re-established the redox status and PARP activation and improved the early molecular markers of DR [144]. The ribosylation of PARP can cause NF-κB activation [145]. By using PARP inhibitors or knocking out PARP genes, both NF-κB activation and transcription of NF-κB-dependent genes such as inducible nitric oxide synthase (iNOS) or ICAM-1 can be reduced [146]. PARP activation also contributes to the formation of VEGF [147] and angiogenesis [148]. These evidences suggest that ribosylation of PARP is one possible mechanism linking oxidative/nitrosative stress with inflammation in DR and this pathway is also enhanced in diabetic hypertensive rats.

CONCLUDING REMARKS AND PERSPECTIVES

About a fifth of the world's adult population are thought to have metabolic syndrome, a cluster of factors associated with an increased risk of type 2 diabetes, hypertension, and cardiovascular disease [149, 150].

DR is a major complication of uncontrolled diabetes mellitus, and increases the necessity to put an emphasis on the preventive aspect of visual loss through the development of new diagnostic tools for earlier detection of retinal changes and also to provide novel non-invasive ocular treatment modalities. In this context, besides an optimal blood glucose control, normalization of arterial pressure and the use of drugs that interfere in the RAAS could be efficient approaches for the prevention and treatment of DR. Supported by experimental and clinical studies, maneuvers that reduce superoxide generation and/or increase anti-oxidant defenses are of special interest [132, 133, 151, 152]. In the present review, we have provided evidence that local generation of oxidative stress and inflammation can be a common mechanism of retinal lesion in the presence of diabetes and hypertension. We have suggested a multifactorial approach, in which diet as nutritional therapy could be an alternative treatment. Studies have shown that the consumption of dark chocolate is associated with reductions in both blood pressure and oxidative stress and an increase in nitric oxide bioavailability [153], with a concomitant substantial reduction in the risk of cardiometabolic disorders [154]. Also, green tea has been widely studied for its alleged beneficial properties in the treatment or prevention of human diseases. Green tea is a rich source of polyphenols, particularly flavonoids, which are reported to delay or prevent cardiovascular disease [155] and also display a strong antioxidant activity [156, 157, 158]. Finally, to more conclusively prove the efficacy of anti-oxidant diets, further studies are required, especially experimental studies to demonstrate causation.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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REFERENCES

[1] International Diabetes Federation (2010) Diabetes atlas, 4th edn. Available from: http://www.diabetesatlas.org/

[2] World Health Organization [homepage on the Internet]. Available from: http://www.who.int/diabetes/en/

[3] American Diabetes Association (ADA). Treatment of hypertension in adults with diabetes. Diabetes Care 2003; 26(Suppl 1): S80-2.

[4] Stamler J, Vaccaro O, Neaton JD, *et al.* Diabetes, other risk factors and 12-years cardiovascular mortality for men screened in the multiple risk factor intervention trial. Diabetes Care 1993; 16: 434-44.

[5] Cea-Calvo L, Conthe P, Gómez-Fernández P, *et al.* Target organ damage and cardiovascular complications in patients with hypertension and type 2 diabetes in Spain: a cross-sectional study. Cardiovasc Diabetol 2006; 3: 5-23.

[6] American Diabetes Association (ADA). Standards of medical care in diabetes 2010. Diabetes Care 2010; 33(Suppl 1): S11-S61.

[7] United Kingdom Prospective Diabetes Study Group. Association of systolic blood pressure with macrovascular and microvascular complications of type 2 diabetes. BMJ 2000; 321: 412-9.

[8] Wild S, Roglic G, Green A, *et al.* Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27: 1047-1053.

[9] Coral K, Angayarkanni N, Gomathy N, *et al.* Homocysteine levels in the vitreous of proliferative diabetic retinopathy and rhegmatogenous retinal detachment: its modulating role on lysyl oxidase. Investigative Ophthalmology & Visual Science 2009; 50: 3607-12.

[10] Ganapathy PS, Roon P, Moister TK, *et al.* Diabetes accelerates retinal neuronal cell death in a mouse model of endogenous hyperhomocysteinemia. Ophthalmology Eye Diseases 2009; 1: 3-11.

[11] Garcia-Valenzuela E, Shareef S, Walsh J, *et al.* Programmed cell death of retinal ganglion cells during experimental glaucoma. Experimental Eye Research 1995; 61(1): 33-44.

[12] Jing G, Wang JJ, Zhang SX. ER Stress and Apoptosis: A NewMechanism for Retinal Cell Death. Exp Diabetes Res 2012; 2012: 589589.

[13] Sasaki M, Ozawa Y, Kurihara T, *et al.* Neurodegenerative influence of oxidative stress in the retina of a murine model of diabetes. Diabetologia 2010; 53: 971-9.

[14] Klein R, Klein BE, Moss SE, *et al.* The Wisconsin epidemiologic study of diabetic retinopathy. IV. Diabetic macular edema. Ophthalmology 1984; 91(12): 1464-74.

[15] Klein R, Klein BE, Moss SE, *et al.* The Wisconsin epidemiologic study of diabetic retinopathy. III. Prevalence and risk of diabetic retinopathy when age at diagnosis is 30 or more years. Arch Ophthalmol 1984; 102(4): 527-32.

[16] Klein R, Knudtson MD, Lee KE, *et al.* The Wisconsin Epidemiologic Study of Diabetic Retinopathy XXIII: the twenty-five-year incidence of macular edema in persons with type 1 diabetes. Ophthalmology 2009; 116(3): 497-503.

[17] Barnes AJ, Kohner EM, Johnston DG, *et al.* Severe retinopathy and mild carbohydrate intolerance: possible role of insulin deficiency and elevated circulating growth hormone. Lancet 1985; 1: 1465-8.

[18] Harrower AD, Clarke BF. Diabetic retinopathy with normal glucose tolerance. The British Journal of Ophthalmology 1976; 60: 459-63.

[19] Warboys CM, Fraser PA. Hyperglycemia attenuates acute permeability response to advanced glycation end products in retinal microvasculature. Microvascular Research 2010; 80: 174-6.

[20] Ismail-Beigi F, Craven T, Banerji MA *et al.* Effect of intensive treatment of hyperglycaemia on microvascular outcomes in type 2 diabetes: an analysis of the ACCORD randomised trial. Lancet 2010; 376: 419-30.
[21] Patel A, MacMahon S, Chalmers J, *et al.* Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. The New England Journal of Medicine 2008; 358: 2560-72.
[22] United Kingdom Prospective Diabetes Study Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes. Lancet 1998; 352: 837-53.
[23] Prospective Diabetes Study Group. Relationship between ethnicity and glycemic control, lipid profiles, and blood pressure during the first 9 years of type 2 diabetes: U.K. Diabetes Care 2001; 24(7): 1167-74.
[24] Schrier RW, Estacio RO, Esler A, *et al.* Effects of aggressive blood pressure control in normotensive type 2 diabetic patients on albuminuria, retinopathy and strokes. Kidney International 2002; 61: 1086–97.
[25] Chaturvedi N, Porta M, Klein R, *et al.* Effect of candesartan on prevention (DIRECT-Prevent 1) and progression (DIRECT-Protect 1) of retinopathy in type 1 diabetes: randomised, placebo-controlled trials. Lancet 2008; 372: 1394-402.
[26] Sjølie AK, Klein R, Porta M, *et al.* DIRECT Programme Study Group: Effect of candesartan on progression and regression of retinopathy in type 2 diabetes (DIRECT-Protect 2): a randomized placebo-controlled trial. Lancet 2008; 372: 1385-93.
[27] Hirsch IB, Brownlee M. Beyond hemoglobin A1c - need for additional markers of risk for diabetic microvascular complications. JAMA 2010; 303: 2291-2.
[28] Klein R. The epidemiology of diabetic retinopathy. In: Duh E, ed. Diabetic retinopathy. Totowa: Humana 2008; pp. 67-107.
[29] Safar ME, Peronneau PA, Levenson JA, *et al.* Pulsed Doppler: diameter, blood flow velocity and volumic flow of the brachial artery in sustained essential hypertension. Circulation 1981; 63: 393-400.
[30] Houben AJ, Canoy MC, Paling HA, *et al.* Quantitative analysis of retinal vascular changes in essential and renovascular hypertension. J Hypertens 1995; 13: 1729-33.
[31] Ziegler T, Silacci P, Harrison VJ, *et al.* Nitric oxide synthase expression in endothelial cells exposed to mechanical forces. Hypertension 1998; 32: 351-5.
[32] Yamazaki T, Komuro I, Kudoh S, *et al.* Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. J Biol Chem 1996; 271: 3221-8.
[33] Hu Y, Bock G, Wick G, *et al.* Activation of PDGF receptor alpha in vascular smooth muscle cells by mechanical stress. FASEB J 1998; 12: 1135-42.
[34] Park JM, Borer JG, Freeman MR, *et al.* Stretch activates heparin-binding EGF-like growth factor expression in bladder smooth muscle cells. Am J Physiol 1998; 275: 1247-54.
[35] Marrero MB, Schieffer B, Paxton WG, *et al.* Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. Nature 1995; 375: 247-50.
[36] Tamura K, Umemura S, Nyui N, *et al.* Activation of angiotensinogen gene in cardiac myocytes by angiotensin II and mechanical stretch. Am J Physiol 1998; 275: 1-9.
[37] Ashton N, Peltier S, Gamer A: Experimental hypertensive retinopathy in the monkey. Trans Ophthalmol Soc UK 1969; 88: 167-186.
[38] Flückiger W, Perrin IV, Rossi GL. Virchows Arch B Cell Pathol Incl Mol Pathol 1984; 47(1): 79-94.
[39] Ruchoux MM, Droy-Lefaix MT, Bakri F, Guillemain J, Ruchoux P, Autret A. Cicletanine and hypertensive retinopathy. Ophthalmic Res 1990; 22(5): 318-29.
[40] Gin T, Joon TL, Panagiotopoulos S, Cooper M, Taylor H, Jerums G. Organ specificity of antihypertensive therapy on ocular albumin vascular clearance and albuminuria in the hypertensive diabetic rat. Invest Ophthalmol Vis Sci 1996; 37(2): 281-9.
[41] Hillman N, Cox S, Noble AR, Gallagher PJ. Increased numbers of caveolae in retinal endothelium and pericytes in hypertensive diabetic rats. Eye (Lond) 2001;15: 319-25.
[42] Dosso AA, Rungger-Brändle E, Leuenberger PM. Ultrastructural alterations in capillaries of the diabetic hypertensive rat retina: protective effects of ACE inhibition. Diabetologia 2004; 47(7): 1196-201.
[43] Cogan DG, Toussaint D, Kuwabara T. Retinal vascular patterns. IV. Diabetic retinopathy Arch. Ophthalmol 1961; 66: 366-78.
[44] Hyvrinen L, Laurinen P, Rovano J. Contrast sensitivity in evaluation of visual impairment due to diabetes. Acta Ophthalmol 1983; 61: 94-101.
[45] Dosso AA, Bonvin ER, Morel Y, *et al.* Risk factors associated with contrast sensitivity loss in diabetic patients. Graefes Arch Clin Exp Ophthalmol 1996; 234: 300-5.
[46] Sokol S, Moskowitz A, Skarf B, *et al.* Contrast sensitivity in diabetics with and without background retinopathy. Arch Ophthalmol 1985; 103: 51-5
[47] Hardy KJ, Lipton J, Foster DH, *et al.* Detection of color vision abnormalities in uncomplicated type 1 diabetic patients with angiographically normal retinas. Br J Ophthalmol 1992; 76: 461-4.
[48] Greenstein VC, Shapiro A, Zaidi Q, *et al.* Psychophysical evidence for post-receptoral sensitivity loss in diabetics. Invest Ophthalmol Vis Sci 1992; 33: 2781-90.
[49] Bresnick GH, Korth K, Groo A, *et al.* Electroretinographic oscillatory potentials predict progression of diabetic retinopathy. Arch Ophthalmol 1984; 102: 1307-11.
[50] Ghirlanda G, Di Leo MA, Caputo S, *et al.* Detection of inner retina dysfunction by steady-state focal electroretinogram pattern and flicker in early IDDM. Diabetes 1991; 40: 1122-7.
[51] Prager TC, Garcia CA, Mincher CA, *et al.* The pattern electro-retinogram in diabetes. Am J Ophthalmol 1990; 109: 279-84.
[52] Puvanendran K, Devathasan G, Wong PK. Visual evoked responses in diabetes. J Neurosurg Psychiatry 1983; 46: 643-7.
[53] Moreo G, Mariani E, Pizzamiglio G, *et al.* Visual evoked potentials in NIDDM: a longitudinal study. Diabetologia 1995; 38: 573-6.
[54] Uccioli L, Parisi V Monticone G, *et al.* Electrophysiological assessment of visual function in newly diagnosed IDDM patients. Diabetologia 1995; 38: 804-8.
[55] Lopes de Faria JM, Katsumi O, Cagliero E, *et al.* Neurovisual abnormalities preceding the retinopathy in patients with long-term type 1 diabetes mellitus. Graefes Arch Clin Exp Ophthalmol 1939; 9: 643-8.
[56] Lambooij AC, Kuijpers RW, Van Lichtenauer-Kaligis EG, *et al.* Somatostatin receptor 2A expression in choroidal neovascularization secondary to age-related macular degeneration. Invest Ophthalmol Vis Sci 2000; 41: 2329-35.
[57] Tretiach M, Madigan MC, Wen L, *et al.* Effect of Müller cell co-culture on *in vitro* permeability of bovine retinal vascular endothelium in normoxic and hypoxic conditions. Neurosci Lett 2005; 378: 160-5.
[58] Newman E, Reichenbach A. The Müller cell: a functional element of the retina. TINS 1996; 19:307-11.
[59] Bringmann A, Pannicke T, Grosche J, *et al.* Müller cells in the healthy and diseased retina. Prog Retin Eye Res 2006; 25: 397-424.
[60] Gardner TW, Lieth E, Khin SA, *et al.* Astrocytes increase barrier function and ZO-1 protein expression in cultured retinal capillary endothelial cells. Invest Ophthalmol Vis Sci 1997; 38:2423-7.
[61] Romano C, Price MT, Olney JW. Delayed excitotoxic neuro-degeneration induced by excitatory amino acid agonists in isolated retina. J Neurochem 1995; 65: 59-67.
[62] Riepe RE, Norenburg MD: Müller cell localization of glutamine synthetase in rat retina. Nature 1977; 268: 654-5.
[63] Lieth E, Barber AJ, Xu B, *et al.* Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy. Penn State Retina Research Group. Diabetes 1998; 47(5): 815-20.
[64] Runkle EA, Antonetti DA. The blood-retinal barrier: structure and functional significance. Methods Mol Biol 2011; 686: 133-48.
[65] Qaum T, Xu Q, Joussen AM, *et al.* VEGF-initiated blood-retinal barrier breakdown in early diabetes. Invest Ophthalmol Vis Sci 2001, 42: 2408-13.
[66] Xu HZ, Le YZ. Significance of outer blood-retina barrier breakdown in diabetes and ischemia. Invest Ophthalmol Vis Sci 2011; 52(5): 2160-4.
[67] Frank RN. Diabetic retinopathy. N Engl J Med 2004; 350: 48-58.
[68] Haimovich B, Lipfert L, Brugge JS, *et al.* Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. J Biol Chem 1993; 268: 15868–77

[69] Jian B, Jones PL, Li Q, et al. Matrix metalloproteinase-2 is associated with tenascin-c in calcific aortic stenosis. Am J Pathol 2001; 159: 321-7.
[70] Alder VA, Su EN, Yu DY, Cringle S, Yu PY. Overview of studies on metabolic and vascular regulatory changes in early diabetic retinopathy. Aust NZJ Ophthalmol 1998; 26: 141-8.
[71] Cai J, Boulton M. The pathogenesis of diabetic retinopathy: old concepts and new questions. Eye 2002; 16: 242-60.
[72] 76 Stitt AW, Gardiner TA, Archer DB. Histological and ultrastructural investigation of retinal microaneurysm development in diabetic patients. Br J Ophthalmol 1995; 79: 362–7.
[73] Antonetti DA, Barber AJ, Khin S, *et al*. Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Diabetes 1998; 47: 1953-9.
[74] Barber AJ, Antonetti DA. Mapping the blood vessels with paracellular permeability in the retinas of diabetic rats. Investig. Ophthalmol Vis Sci 2003; 44: 5410-6.
[75] Brankin B, Campbell M, Canning P, *et al*. Endostatin modulates VEGF-mediated barrier dysfunction in the retinal microvascular endothelium. Exp Eye Res 2005; 81: 22-31.
[76] Cunha-Vaz JG. Blood–retinal barriers in health and disease. Trans. Ophthalmol 1980; 100: 337-40.
[77] Hofman P, Blaauwgeers HG, Tolentino MJ, *et al*. VEGF-A induced hyperpermeability of blood–retinal barrier endothelium *in vivo* is predominantly associated with pinocytotic vesicular transport and not with formation of fenestrations. Vascular endothelial growth factor-A. Curr Eye Res 2000; 21: 637-45.
[78] Vinores SA, Van Niel E, Swerdloff JL, *et al*. Electron microscopic immunocytochemical demonstration of blood–retinal barrier breakdown in human diabetics and its association with aldose reductase in retinal vascular endothelium and retinal pigment epithelium. Histochem J 1993;
[79] Simo R, Villarroel M, Corraliza L, *et al*. The retinal pigment epithelium: something more than a constituent of the blood-retinal barrier–implications for the pathogenesis of diabetic retinopathy. J Biomed Biotechnol 2010; 2010:190724.
[80] Benolken RM, Anderson RE, Wheeler TG. Membrane fatty acids associated with the electrical response in visual excitation. Science 1973; 182: 1253-4.
[81] Philp NJ, Ochrietor JD, Rudoy C, *et al*. Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse. Invest Ophthalmol Vis Sci 2003; 44: 1305-11.
[82] Saari JC. Biochemistry of visual pigment regeneration: the Friedenwald lecture. Invest Ophthalmol Vis Sci 2000; 41: 337-48.
[83] Vinores SA, Gadegbeku C, Campochiaro PA, *et al*. Immunohistochemical localization of blood-retinal barrier breakdown in human diabetics. Am J Pathol 1989; 134: 231-5.
[84] Pautler EL, Ennis SR. The effect of induced diabetes on the electroretinogram components of the pigmented rat. Invest Ophthalmol Vis Sci. 1980;19:702–705.
[85] MacGregor LC, Matschinsky FM. Experimental diabetes mellitus impairs the function of the retinal pigmented epithelium. Metabolism 1986; 35: 28-34.
[86] Geiss LS, Rolka DB, Engelgau MM. Elevated blood pressure among U.S. adults with diabetes, 1988-1994. Am J Prev Med 2002; 22: 42-8.
[87] Parving HH. Controlling hypertension in diabetes. Acta Diabetol 2002; 39: 35-40.
[88] UK Prospective Diabetes Study Group. Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38. Br Med J 1998; 317: 703-13.
[89] Klein R, Klein BE, Moss SE, *et al*. The Wisconsin Epidemiologic Study of Diabetic Retinopathy. XVII. The 14-year incidence and progression of diabetic retinopathy and associated risk factors in type 1 diabetes. Ophthalmology 1998; 105: 1801-15.
[90] Chaturvedi N, Sjolie AK, Stephenson JM, *et al*. Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes. The EUCLID Study Group. EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus. Lancet 1998; 351: 28-31.
[91] Klein R, Knudtson MD, Lee KE, *et al*. The Wisconsin Epidemiologic Study of Diabetic Retinopathy: XXII the twenty-five-year progression of retinopathy in persons with type 1 diabetes. Ophthalmology 2008; 115: 1859-68.
[92] Klein R, Lee KE, Gangnon RE, *et al*. The 25-year incidence of visual impairment in type 1 diabetes mellitus the wisconsin epidemiologic study of diabetic retinopathy. Ophthalmology 2010; 117: 63-70.
[93] Stratton IM, Cull CA, Adler AI, *et al*. Additive effects of glycaemia and blood pressure exposure on risk of complications in type 2 diabetes: a prospective observational study (UKPDS 75). Diabetologia 2006; 49: 1761-9.
[94] Rodrigues TC, Canani LH, Viatroski RS, *et al*. Masked hypertension, nocturnal blood pressure and retinopathy in normotensive patients with type 1 diabetes. Diabetes Res Clin Pract 2010; 87:240-5.
[95] Lopes de Faria JM, Jalkh AE, Trempe CL, *et al*. Diabetic macular edema: risk factors and concomitants. Acta Ophthalmol Scand 1999; 77(2): 170-5.
[96] Hammes HP, Brownlee M, Edelstein D, *et al*. Aminoguanidine inhibits the development of accelerated diabetic retinopathy in the spontaneous hypertensive rat. Diabetologia 1994; 37: 32-5.
[97] Gin T, Joon TL, Panagiotopoulos S, *et al*. Organ specificity of antihypertensive therapy on ocular albumin vascular clearance and albuminuria in the hypertensive diabetic rat. Invest Ophthalmol Vis Sci 1996; 37: 281-9.
[98] Lopes de Faria JM, Silva KC, Boer PA, *et al*. A decrease in retinal progenitor cells is associated with early features of diabetic retinopathy in a model that combines diabetes and hypertension. Mol Vis 2008; 14: 1680-91.
[99] Silveira LA, Bacchi CE, Pinto GA, *et al*. The genetics of hypertension modifies the renal cell replication response induced by experimental diabetes. Diabetes 2002; 51(5): 1529-34.
[100] Rassam SM, Patel V, Kohner EM. The effect of experimental hypertension on retinal vascular autoregulation in humans: a mechanism for the progression of diabetic retinopathy. Exp Physiol 1995; 80: 53-68.
[101] Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc Res 1999; 43: 562-71.
[102] Laursen JB, Somers M, Kurz S, *et al*. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. Circulation 2001; 103: 1282-8.
[103] Alp NJ, Channon KM. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. Arterioscler Thromb Vasc Biol 2004; 24: 413-20.
[104] Vanhoutte PM, Shimokawa H, Tang EHC, *et al*. Endothelial dysfunction and vascular disease. Acta Physiologica 2009; 196: 193-222.
[105] Montagnani M, Chen H, Barr VA, *et al*. Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser1179. J Biol Chem 2001; 276: 30392-8.
[106] Shinozaki K, Kashiwagi A, Nishio Y, *et al*. Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O2- imbalance in insulin-resistant rat aorta. Diabetes 1999; 48: 2437-45.
[107] Aiello LP, Avery RL, Arrigg PG, *et al*. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 1994; 331: 1480-7.
[108] Seko Y, Seko Y, Fujikura H, *et al*. Induction of vascular endothelial growth factor after application of mechanical stress to retinal pigment epithelium of the rat *in vitro*. Invest Ophthalmol Vis Sci 1999; 40: 3287-91.
[109] Suzuma I, Hata Y, Clermont A, *et al*. Cyclic stretch and hypertension induce retinal expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2: potential mechanisms for exacerbation of diabetic retinopathy by hypertension. Diabetes 2001; 50: 444-54.
[110] Suzuma I, Suzuma K, Ueki K, *et al*. Stretch-induced retinal vascular endothelial growth factor expression is mediated by phosphatidylinositol 3-kinase and protein kinase C (PKC)-zeta but not by stretch-induced ERK1/2, Akt, Ras, or classical/novel PKC pathways. J Biol Chem 2002; 277: 1047-57.

[111] Suzuma I, Murakami T, Suzuma K, *et al.* Cyclic stretch-induced reactive oxygen species generation enhances apoptosis in retinal pericytes through c-jun NH2-terminal kinase activation. Hypertension 2007; 49: 347-54.
[112] Beltramo E, Berrone E, Giunti S, *et al.* Effects of mechanical stress and high glucose on pericyte proliferation, apoptosis and contractile phenotype. Exp Eye Res 2006; 83: 989-94.
[113] Pinto CC, Silva KC, Biswas SK. Arterial hypertension exacerbates oxidative stress in early diabetic retinopathy. Free Radic Res 2007; 41(10): 1151-8.
[114] UK Prospective Diabetes Study Group. Efficacy of atenolol and captopril in reducing risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 39. BMJ 1998; 317: 713-20.
[115] Mark E. Williams. The Goal of Blood Pressure Control for Prevention of Early Diabetic Microvascular Complications. Curr Diab Rep 2011: 11: 323-9.
[116] Satofuka S, Ichihara A, Nagai N. (Pro)renin receptor-mediated signal transduction and tissue renin-angiotensin system contribute to diabetes-induced retinal inflammation. Diabetes 2009; 58(7): 1625-33.
[117] No authors listed. National High Blood Pressure Education Program Working Group report on hypertension in diabetes. Hypertension 1994; 23: 145-58.
[118] Ganne S, Arora SK, Hypertension in people with diabetes and the metabolic syndrome: Pathophysiologic insights and therapeutic update. Current Diabetes Reports 2007; 7(3): 208-17.
[119] Alderman MH, Cohen H, Madhavan S. Diabetes and Cardiovascular Events in Hypertensive Patients. Hypertension 1999; 33: 1130-4.
[120] Calcutt NA, Cooper ME, Kern TS, *et al.* Therapies for hyperglycaemia-induced diabetic complications: from animal models to clinical trials. Nat Rev Drug Discov 2009; 8: 417-29.
[121] Kern TS. Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Exp Diabetes Res 2007; 2007: 95103.
[122] Vaziri ND, Rodriguez-Iturbe B. Mechanisms of disease: oxidative stress and inflammation in the pathogenesis of hypertension. Nat Clin Pract Nephrol 2006; 2: 582-93.
[123] Gorin Y, Block K, Hernandez J, *et al.* Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. J Biol Chem 2005; 280: 39616-26.
[124] Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005; 54: 1615-25.
[125] Jones DP. Redefining oxidative stress. Antioxid Redox Signal 2006; 8: 1865-79.
[126] Silva KC, Pinto CC, Biswas SK, *et al.* Prevention of hypertension abrogates early inflammatory events in the retina of diabetic hypertensive rats. Exp Eye Res 2007; 85: 123-9.
[127] Silva KC, Pinto CC, Biswas SK, *et al.* Hypertension increases retinal inflammation in experimental diabetes: a possible mechanism for aggravation of diabetic retinopathy by hypertension. Curr Eye Res 2007; 32: 533-41.
[128] Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. J Clin Invest 1996; 97: 2883-90.
[129] Fletcher EL, Phipps JA, Ward MM, *et al.* The renin-angiotensin system in retinal health and disease: Its influence on neurons, glia and the vasculature. Prog Retin Eye Res. 2010 Jul; 29(4): 284-311.
[130] Silva KC, Rosales MA, Biswas SK, *et al.* Diabetic retinal neurodegeneration is associated with mitochondrial oxidative stress and is improved by an angiotensin receptor blocker in a model combining hypertension and diabetes. Diabetes. 2009; 58(6): 1382-90.
[131] Silva KC, Rosales MA, de Faria JB, *et al.*. Reduction of inducible nitric oxide synthase *via* angiotensin receptor blocker prevents the oxidative retinal damage in diabetic hypertensive rats. Curr Eye Res 2010; 35: 519-28.
[132] Jung KH, Chu K, Lee ST, *et al.* Blockade of AT1 receptor reduces apoptosis, inflammation, and oxidative stress in normotensive rats with intracerebral hemorrhage. J Pharmacol Exp Ther 2007; 322: 1051-8.
[133] Naash MI, Anderson RE. Characterization of glutathione peroxidase in frog retina. Curr Eye Res 1984; 3(11): 1299-304.
[134] Kowluru RA, Abbas SN. Diabetes-induced mitochondrial dysfunction in the retina. Invest Ophthalmol Vis Sci 2003; 44(12): 5327-34.
[135] Liang FQ, Godley BF. Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration. Exp Eye Res 2003; 76(4): 397-403.
[136] Kowluru RA. Diabetic retinopathy: mitochondrial dysfunction and retinal capillary cell death. Antioxid Redox Signal. 2005; 7(11-12): 1581-87.
[137] Madsen-Bouterse SA, Kowluru RA. Oxidative stress and diabetic retinopathy: pathophysiological mechanisms and treatment perspectives. Rev Endocr Metab Disord 2008; 9: 315-27.
[138] Du Y, Smith MA, Miller CM, *et al.* Diabetes-induced nitrative stress in the retina, and correction by aminoguanidine. J Neurochem 2002; 80: 771-9.
[139] Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am J Physiol Cell Physiol. 1996; 40: 1424-37.
[140] Schreiber V, Dantzer F, Ame JC, *et al.* Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol 2006; 7: 517-28.
[141] Krishna MC, Russo A, Mitchell JB, *et al.* Do nitroxide antioxidants act as scavengers of O2− or as SOD mimics? J Biol Chem 1996; 271: 26026–31.
[142] Chen P, Guo AM, Edwards PA, *et al.* Role of NADPH oxidase and ANG II in diabetes-induced retinal leukostasis. Am J Physiol Regul Integr Comp Physiol 2007; 293: 1619-29.
[143] El-Remessy AB, Khalil IE, Matragoon S, *et al.* Neuroprotective effect of (−) Delta9-tetrahydrocannabinol and cannabidiol in N-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite. Am J Pathol 2003; 163: 1997-2008.
[144] Rosales MA, Silva KC, Lopes de Faria JB, *et al.* Exogenous SOD mimetic tempol ameliorates the early retinal changes restablishing the redox status in diabetic hypertensive rats. Invest Ophthalmol Vis Sci 2010; 51: 4327-36.
[145] Zheng L, Szabó C, Kern TS. Poly(ADP-ribose) polymerase is involved in the development of diabetic retinopathy *via* regulation of nuclear factor-kappaB. Diabetes 2004; 53(11): 2960-7.
[146] Chiarugi A, Moskowitz MA. Poly(ADP-ribose) polymerase-1 activity promotes NF-kappaB-driven transcription and microglial activation: implication for neurodegenerative disorders. J Neurochem 2003; 85(2): 306-17.
[147] Obrosova IG, Minchenko AG, Frank RN, *et al.* Poly(ADP-ribose) polymerase inhibitors counteract diabetes- and hypoxia-induced retinal vascular endothelial growth factor overexpression. Int J Mol Med 2004; 14(1): 55-64.
[148] Tentori L, Lacal PM, Muzi A, *et al.* Poly(ADP-ribose) polymerase (PARP) inhibition or PARP-1 gene deletion reduces angiogenesis. Eur J Cancer 2007; 43(14): 2124-33.
[149] Ogbera AO. Prevalence and gender distribution of the metabolic syndrome. Diabetol Metab Syndr 2010; 2:1.
[150] World Health Organization [homepage on the Internet]. 2008-2013 action plan for the global strategy for the prevention and control of non-communicable diseases. 2000. Available from: http://whqlibdoc.who.int/publications/ 2009/9789241597418_eng.pdf
[151] Tang Z, Shou I, Wang LN, *et al.* Effects of antihypertensive drugs or glycemic control on antioxidant enzyme activities in spontaneously hypertensive rats with diabetes. Nephron 1997; 76: 323-330.
[152] Ogawa S, Kobori H, Ohashi N, *et al.* Angiotensin II Type 1 Receptor Blockers Reduce Urinary Angiotensinogen Excretion and the Levels of Urinary Markers of Oxidative Stress and Inflammation in Patients with Type 2 Diabetic Nephropathy. Biomark Insights 2009; 4: 97-102.
[153] Taubert D, Roesen R, Lehmann C, *et al.* Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: a randomized controlled trial. JAMA 2007; 298: 49-60.
[154] Buitrago-Lopez A, Sanderson J, Johnson L. Chocolate consumption and cardiometabolic disorders: systematic review and meta-analysis. BMJ 2011; 343: 4488.
[155] Mulvihill EE, Huff MW. Antiatherogenic properties of flavonoids: implications for cardiovascular health. Can J Cardiol 2010; 26: 17-21.
[156] Higdon JV, Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Crit Rev Food Sci Nutr 2003; 43: 89-143.

[157] Rice-Evans CA, Miller NJ, Bolwell PG, *et al*. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res 1995; 22: 375-83.

[158] Mustata GT, Rosca M, Biemel KM, *et al*. Paradoxical effects of green tea (Camellia sinensis) and antioxidant vitamins in diabetic rats: improved retinopathy and renal mitochondrial defects but deterioration of collagen matrix glycoxidation and cross-linking. Diabetes 2005; 54: 517-26.

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Reliability and validity of digital assessment of perifoveal capillary network measurement using high-resolution imaging

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ABSTRACT

Background Assessment of the perifoveal capillary network (PCN) might indicate macular function and could reflect the systemic microcirculation. The quantification and reliability of this measurement is currently unknown. The aim of this study was to validate quantification of the PCN by a non-invasive technique from high-resolution retinal images.

Methods Ten healthy volunteers were included in this validation study. At least 320 high-resolution retinal images were used for assessment of inter- and intra-observer reliability. Non-invasive capillary perfusion mapping was performed using a retinal function imager. After the images were enhanced and segmented, the reproducibility was verified by comparing the values of two independent examiners and of a single examiner at two different time points.

Results The inter-observer concordance coefficients were highly significant for PCN (intraclass correlation coefficient (ICC)=0.901, 95% CI 0.655 to 0.975, p<0.001) and normalised PCN (ICC=0.727, 95% CI 0.262 to 0.923, p=0.004). The intra-observer measurements at two different time points were also highly concordant for PCN (ICC=0.879, 95% CI 0.598 to 0.968, p<0.001) and for normalised PCN (ICC=0.960, 95% CI 0.851 to 0.990, p<0.001).

Conclusions The reliability of PCN measurement is reproducible and could be used as a new tool to quantify the capillary perfusion network of the macular area.

INTRODUCTION

Imaging of the retinal vasculature is an important tool for the early diagnosis, targeted treatment and follow-up of ocular diseases and also to assess the status of the systemic microcirculation. The retinal arteries and venules are easily seen by direct visualisation and share similarities with the microcirculation of the brain, heart and kidneys.[1–6] An important multicentre clinical trial (ACCORD) recently showed that the retina may provide an anatomical index of the effect of metabolic and haemodynamic factors on future cardiovascular outcomes.[7]

Optical imaging techniques use visible, ultraviolet and infrared wavelengths to probe an imaged tissue target for its optical properties, which include fluorescence, absorption and scattering. Small changes in reflectance due to the intrinsic optical properties of the blood have been used to explore haemodynamic activity.[8] The Retinal Function Imager (RFI; Optical Imaging, Rehovot, Israel) was developed to capture these changes in reflectance as a function of time under stroboscopic illumination. This instrument incorporates a non-contact and non-invasive method of imaging and mapping the capillaries using the intrinsic contrast chromophore in the haemoglobin. Fast acquisition of images at a wavelength strongly absorbed by haemoglobin enables the motion of the red blood cells to be seen and, by tracing the paths of this perfusing motion, the capillaries can be visualised in detail, as previously described.[9 10]

The aim of the present study was to validate a new method of digitally quantifying the perifoveal capillary network (PCN) using RFI.

METHODS

Study population

Staff members from the University Hospital of the State University of Campinas were asked to participate in this study. The criteria for inclusion were volunteers less than 35 years of age who were free of any disease. Patients were excluded from the analysis if the quality of the images was deemed unsatisfactory. Ten normal volunteers participated in the study.

Image acquisition

The acquisition of the retinal images was as described elsewhere.[9 10] Retinal images were acquired by RFI using fundus illumination at green wavelengths (red-free 548 bandpass, 8.5 nm) at which red blood cells are strongly absorbed. Using fast stroboscopic lighting and a charge coupled device camera with an exposure time of 700 µs at a resolution of 1024×1024 pixels, we obtained eight images in 122.5 ms (figure 1A). For high-resolution images of the foveal avascular zone (FAZ), the images were acquired at 20° centred on the fovea resulting in 4.27 micron/pixel resolution. After image registration, 'differential images' were created by dividing each single retinal image by the average of the entire series to extract the motion signal from the stationary retinal background. In this work, a 4–5 capillary mapping perfusion series was used.

Localisation and measurement of the FAZ area

After the retinal images were obtained, two independent specialists selected the perifoveal region. Using images from the capillary perfusion map, the FAZ was manually drawn as an ellipse (figure 2A) and the radius (r) was then determined, with r_1 as the major radius ellipse and r_2 as the minor radius

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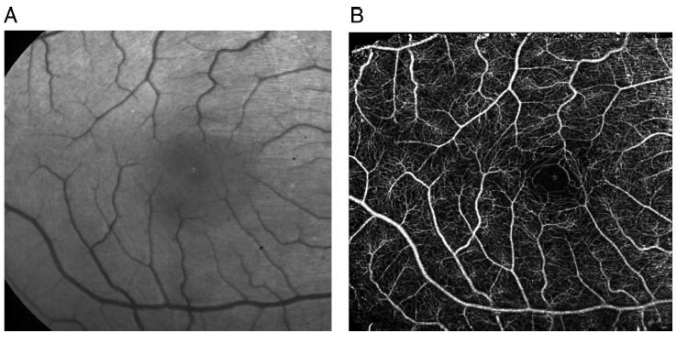

Figure 1 Normal fundus image from a normal participant. The macular area is located in the centre of the image. (A) Retinal functional imager (RFI) raw data red-free image with fovea 20°, 1024×1024 pixels; (B) The capillary perfusion map acquired with RFI software tools. Due to the high resolution, it is possible to visualise the smallest capillary net around the foveal avascular area.

ellipse. The selected zone was cropped with radius 3r (ie, annular width 3r of FAZ; figure 2). The annular band area with 3r of FAZ was chosen for the measurements in this present study because, by using this methodology, the width of the annular band corresponds to approximately 1500 μm diameter (ie, it is within the macular area considered for clinical estimation of clinically significant macular oedema according to the Early Treatment Diabetic Retinopathy Study protocol). The Contrast-Limited Adaptive Histogram Equalisation technique was used to enhance the images.[11]

PCN measurement

The image was submitted to a binary operation based on Otsu's method (figure 3). The vascular and non-vascular elements were easily identified and quantified in pixels with a pixel value equal to 1 (sum of the binary image). To allow comparisons between two different examinations, the PCN obtained in pixels was normalised by the total annular band area as follows: nPCN=PCN (k)/annular area (k). This ratio represented the index of perifoveal perfusion.

Inter- and intra-observer reliability of PCN measurements

The PCN measurement images were taken on a single occasion for each volunteer. For inter-observer reliability testing, two observers drew the area of FAZ in one processed image for each participant and were blinded to each other's measurements.

For the intra-observer component, one assessor determined the area of PCN measurement twice with the same images at two different time points to assure consistency of FAZ diameter measurement.

Statistical analyses

SPSS for Windows was used. To compare measurements between two independent observers and at two different time points for a single observer, the Wilcoxon signed rank test for related samples and the intraclass correlation coefficient (ICC) were applied, respectively. Statistical differences were considered significant at $p < 0.05$. For sample size calculation, the Estimation of Mean Value formula was applied with a significance level at 5% and a sample error of 37% of SD.

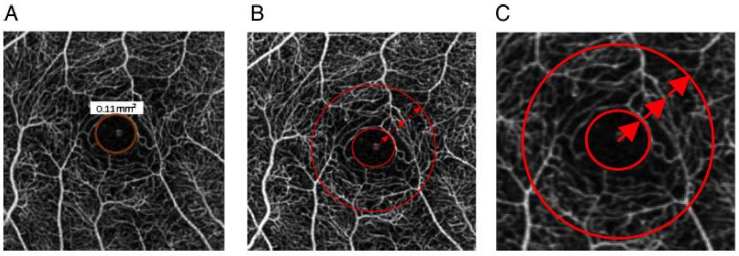

Figure 2 (A) Manual drawing of the foveal avascular zone (FAZ) done by a specialist. (B) The radius (r) is determined from the centre of the FAZ, and the area of the perifoveal capillary network corresponds to an annular band with 3r of FAZ. (C) Centred zoom 200%.

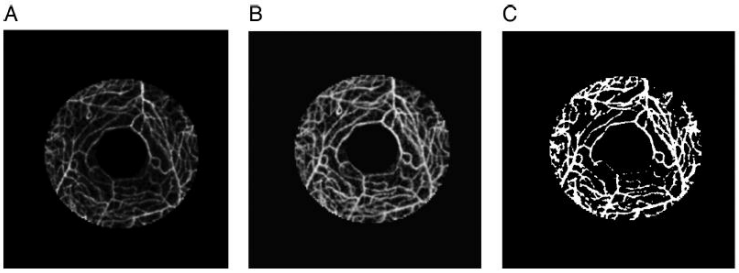

Figure 3 (A) The perifoveal capillary network (PCN) was cropped from the high-resolution retinal image to obtain an annular band (B) and enhanced by contrast-limited adaptive histogram equalisation. (C) The binary operation based on Otsu's method was applied to the enhanced PCN image, discriminating the vascular and non-vascular elements; the pixel value is 1.

Table 1 Wilcoxon signed rank test for related samples for inter-observer reliability assessment

Variables	Sample (n)	ICC	95% CI	p Value
Perifoveal annular band	10	0.975	0.904 to 0.994	<0.001
PCN	10	0.901	0.655 to 0.975	<0.001
Normalised PCN	10	0.727	0.262 to 0.923	0.004

ICC, intraclass correlation coefficient; PCN, perifoveal capillary network.

Table 2 Intraclass correlation coefficient (ICC) for intra-observer reliability assessment

Variables	Sample (n)	ICC	95% CI	p Value
Perifoveal annular band	10	0.968	0.883 to 0.992	<0.001
PCN	10	0.879	0.598 to 0.968	<0.001
Normalised PCN	10	0.960	0.851 to 0.990	<0.001

Concordance of measurements taken from one observer at two different time points.
ICC, intraclass correlation coefficient; PCN, perifoveal capillary network.

RESULTS

Images were obtained from 10 healthy participants (5 men and 5 women) with a mean age of 30 years (range 25–35).

Inter- and intra-observer reliability of PCN and normalised PCN

For inter-observer reliability testing, 320 images were taken and the two observers drew the FAZ on one good quality image while blinded to each other's results (table 1). The FAZ drawings from the same participant were highly reproducible in repeated measurements. For observer 1 the mean±SD FAZ measurement was 218±50 μm^2 (range 120–300 μm^2) and, for observer 2, the mean ± SD FAZ measurement was 218±60 μm^2 (range 110–310 μm^2, p>0.05). The mean±SD annular area was highly reproducible and concordant, ranging from 54 to 139 pixels for observer 1 and from 52 to 142 pixels for observer 2 (mean ± SD 100.5±26.9 and 101.2±29.1 pixels for observers 1 and 2, respectively, p=0.6). The PCN normalised for the annular band area ranged from 0.20 to 0.36 for observer 1 and from 0.20 to 0.35 for observer 2 (p=0.3). These indices were shown to be concordant between the two independent observers both for PCN (ICC=0.901, 95% CI 0.655 to 0.975, p<0.001) and also for normalised PCN (ICC=0.727, 95% CI 0.262 to 0.923, p=0.004; table 1, figure 4A).

For the intra-observer component, the observer determined the area of FAZ from the same images at two different time points and then the normalised PCN was obtained. The mean ±SD perifoveal annular area was 103.9±32.10 pixels (range 51–149) and 101.2±29.17 pixels (range 52–142) at time points 1 and 2, respectively (p=0.4). The normalised PCN was 0.27 ±0.05 (range 0.19–0.31) and 0.27±0.05 (range 0.20–0.35) at time points 1 and 2, respectively (p=0.6). The concordance between the two normalised PCN measurements for the same observer at different time points was highly significant (ICC 0.960, 95% CI 0.851 to 0.990, p<0.001; table 2 and figure 4B).

DISCUSSION

In this validation study we propose a new method to assess PCN perfusion using digital high-resolution images of the retina. This non-invasive technology allows us to search the motion path of red blood cells to directly visualise the microvasculature and quantify the perfusion rate of the annular perifoveal band, which represents the more distal capillary bed. Previous observations by Sakata *et al* have shown that the reduction in perifoveal capillary blood flow may occur before the increase in retinal thickness at the central fovea in patients with diabetes. This parameter could be used as a prognostic factor for the development/progression of macular oedema.[12] Previous studies have attempted to improve the visualisation of the retinal microcirculation using a scanning laser ophthalmoscope. In these studies, intravenous or oral dye with or without

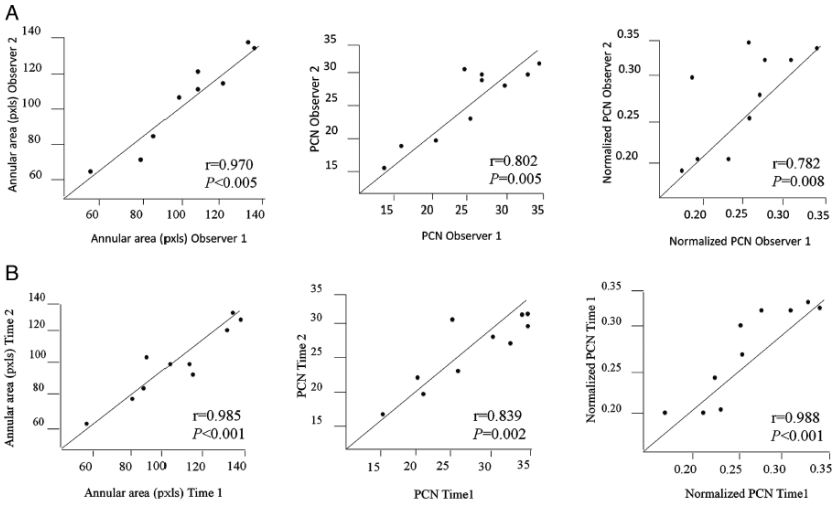

Figure 4 Scatter plots for concordance between (A) two independent observers (inter-observer concordance) and (B) for one observer at two different time points (intra-observer concordance). PCN, perifoveal capillary network.

confocal technology was used.[13–15] In the present study, with the use of RFI technology and post-processing images, it was possible to visualise the most distal capillary bed in the retinal circulation without dye and also to quantify it.

The normalised PCN indices among normal volunteers varied from 0.198 to 0.357, which indicated that the normal PCN ranged from 19% to 35% perfusion. To our knowledge, this is the first attempt to quantify the microcirculation of the retina, and it is important because the retinal microcirculation is accepted as an in vivo index of the haemodynamic status for future cardiovascular outcomes.

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Contributors DAD, AMA and RFLC acquired the data. RA, RFLC and YI reviewed and analysed the data. JMLF designed the study, reviewed the data and wrote, reviewed and edited the manuscript. JMLF is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Competing interests RFLC received a scholarship from FAPESP.

Patient consent Written informed consent was obtained from participants prior to conducting the ophthalmic examination.

Ethics approval The research was carried out in conformity with the Declaration of Helsinki and was approved by the local ethics committee (approval number 745/2011).

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REFERENCES

1 Blonde L. Benefits and risks for intensive glycemic control in patients with diabetes mellitus. *Am J Med Sci* 2012;343:17–20.
2 Klein R, Klein BE, Moss SE, *et al*. Retinal vascular caliber in persons with type 2 diabetes: the Wisconsin Epidemiological Study of Diabetic Retinopathy: XX. *Ophthalmology* 2006;113:1488–98.
3 Taylor B, Rochtchina E, Wang JJ, *et al*. Body mass index and its effects on retinal vessel diameter in 6-year-old children. *Int J Obes* 2007;31:1527–33.
4 McGeechan K, Liew G, Macaskill P, *et al*. Prediction of incident stroke events based on retinal vessel caliber: a systematic review and individual-participant meta-analysis. *Am J Epidemiol* 2009;170:1323–32.
5 Sun C, Wang JJ, Mackey DA, *et al*. Retinal vascular caliber: systemic, environmental, and genetic associations. *Surv Ophthalmol* 2009;54:74–95.
6 Flammer J, Konieczka K, Bruno RM, *et al*. The eye and the heart. *Eur Heart J*. 2013;34:1270–8.
7 Gerstein HC, Ambrosius WT, Danis R, *et al*. Diabetic retinopathy, its progression, and incident cardiovascular events in the ACCORD trial. *Diabetes Care* 2013;36:1266–71.
8 Grinvald A, Lieke E, Frostig RD, *et al*. Functional architecture of cortex revealed by optical imaging of intrinsic signals. *Nature* 1966;324:361–4.
9 Nelson DA, Burgansky-Eliash Z, Barash H, *et al*. High-resolution wide-field imaging of perfused capillaries without the use of contrast agent. *Clin Ophthalmol* 2011;5:1095–106.
10 Izhaky D, Nelson D, Burgansky-Eliash Z, *et al*. Functional imaging using the retinal function imager: direct imaging of blood velocity, achieving fluorescein angiography-like images without any contrast agent, qualitative oximetry, and functional metabolic signals. *Jpn J Ophthalmol* 2009;53:345–51.
11 Zuiderveld K. Contrast limited adaptive histogram equalization. In: Heckbert PS. eds. *Graphics gems IV*. Academic Press Professional, 1994:474–85.
12 Sakata K, Funatsu H, Harino S, *et al*. Relationship between macular microcirculation and progression of diabetic macular edema. *Ophthalmology* 2006;113:1385–91.
13 Garcia CR, Rivero ME, Bartsch DU, *et al*. Oral fluorescein angiography with the confocal scanning laser ophthalmoscope. *Ophthalmology* 1999;106:1114–18.
14 Khoobehi B, Peyman GA. Fluorescent labeling of blood cells for evaluation of retinal and choroidal circulation. *Ophthalmic Surg Lasers* 1999;30:140–5.
15 Squirrell D, Dinakaran S, Dhingra S, *et al*. Oral fluorescein angiography with the scanning laser ophthalmoscope in diabetic retinopathy: a case controlled comparison with intravenous fluorescein angiography. *Eye* 2005;19:411–17.

S-Nitrosoglutathione Inhibits Inducible Nitric Oxide Synthase Upregulation by Redox Posttranslational Modification in Experimental Diabetic Retinopathy

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Purpose. Diabetic retinopathy (DR) is associated with nitrosative stress. The purpose of this study was to evaluate the beneficial effects of S-nitrosoglutathione (GSNO) eye drop treatment on an experimental model of DR.

Methods. Diabetes (DM) was induced in spontaneously hypertensive rats (SHR). Treated animals received GSNO eye drop (900 nM or 10 μM) twice daily in both eyes for 20 days. The mechanisms of GSNO effects were evaluated in human RPE cell line (ARPE-19).

Results. In animals with DM, GSNO decreased inducible nitric oxide synthase (iNOS) expression and prevented tyrosine nitration formation, ameliorating glial dysfunction measured with glial fibrillary acidic protein, resulting in improved retinal function. In contrast, in nondiabetic animals, GSNO induced oxidative/nitrosative stress in tissue resulting in impaired retinal function. Nitrosative stress was present markedly in the RPE layer accompanied by *c*-wave dysfunction. In vitro study showed that treatment with GSNO under high glucose condition counteracted nitrosative stress due to iNOS downregulation by S-glutathionylation, and not by prevention of decreased GSNO and reduced glutathione levels. This posttranslational modification probably was promoted by the release of oxidized glutathione through GSNO denitrosylation via GSNO-R. In contrast, in the normal glucose condition, GSNO treatment promoted nitrosative stress by NO formation.

Conclusions. In this study, a new therapeutic modality (GSNO eye drop) targeting nitrosative stress by redox posttranslational modification of iNOS was efficient against early damage in the retina due to experimental DR. The present work showed the potential clinical implications of balancing the S-nitrosoglutathione/glutathione system in treating DR.

Keywords: diabetic retinopathy, nitrosative stress, S-nitrosoglutathione, retinal pigment epithelium, S-glutathionylation

Diabetic retinopathy (DR) is the leading cause of blindness and visual disability in working-age adults.[1] The pathogenesis of DR is complex and multifactorial, and includes molecular alterations to reactive oxygen species (ROS) and reactive nitrogen species (RNS), elevated nitric oxide (NO) and superoxide production, expression of different isoforms of nitric oxide synthase (NOS), nitrate and polyADP-ribosylate proteins (PARP), and downregulation of antioxidant enzymes. Therefore, better understanding of these mechanisms is a valuable tool for the pharmacologic treatment of DR.[2]

The NO formed by constitutive endothelial NOS (eNOS) and neuronal NOS (nNOS) has an important role in regulating physiologic functions from the cardiovascular system to the central and peripheral nervous systems. However, NO produced by inducible NOS (iNOS) in excessive amounts for long periods of time promotes nitrosative stress and results in cytotoxicities, such as apoptosis, inhibition of mitochondrial respiration, regulation of oxidative phosphorylation, neurodegeneration, and circulatory failure.[3–6] This can be achieved through reaction with superoxide anions to yield peroxynitrite, which can produce toxic hydroxyl radicals and promote oxidative injury via the formation of peroxynitrous acid, a reactive nitrogen-containing species. Endogenous NO is unstable, and some of its main biological actions are mediated through S-nitrosylation,[7] that is, the covalent incorporation of nitric oxide moiety into thiol groups (C-SH or R-SH) to form S-nitrosothiol (SNO). The S-nitrosylation promotes posttranslational modification of certain proteins and affects their activities, such as transcription factors, enzymes, and structural proteins. Thus, S-nitrosylation demonstrates action in vasodilation, inflammation, and neurodegeneration.[8,9]

In the diabetic setting, the increase in NO production as a result of iNOS induction is associated with inflammatory responses and oxidative stress, in retinas of experimental

models.[10] Studies by our group showed an increase in iNOS protein expression in the retinas of animals with short duration of experimental diabetes.[11,12] Retinas from donors with diabetes (DM) and nonproliferative DR (NPDR) showed higher iNOS immunoreactivity localized on the inner nuclear layer, probably on Müller glial cells, compared to subjects without DM and without ocular disease.[13] In addition, NO stable end product concentrations (nitrites and nitrates) in the vitreous were significantly elevated in patients with proliferative DR (PDR) compared to the control group.[14] These data suggested that high concentrations of NO mainly produced by iNOS might contribute to the pathogenesis of DR.

The existence of more stable transport forms of endogenous NO has been postulated in view of the increased half-life of NO in vivo.[15] Low-molecular-weight thiols, such as cysteine, reduced glutathione (GSH), and penicillamine, are prime candidates for such carrier molecules, and they can form SNO on reaction with nitrogen oxides.[16] S-nitrosoglutathione (GSNO) is formed by the S-nitrosylation reaction of NO with GSH in the extracellular setting. Also, GSNO is the most abundant endogenous SNO and the most important form of nitric oxide in vivo, due to its ability to modulate cellular signaling through posttranslational modifications of redox-sensitive proteins by S-nitrosylation and/or S-glutathionylation. The intracellular stability of GSNO is regulated by chemically-driven degradation reactions, thiol, and metal-mediated decomposition and enzymatic reactions.[17–19] The main enzymatic-dependent degradation described is the reduction of GSNO to oxidized glutathione (GSSG) and ammonia (NH_3) by glutathione-dependent formaldehyde dehydrogenase (or alcohol dehydrogenase III); also called GSNO reductase (GSNO-R). This enzyme uses the reducing power of NADH to convert GSNO to glutathione S-hydroxysulfenamide (GSNHOH), which, in turn, is converted into GSSG. The GSNO-R turnover significantly influences the whole-cell level of S-nitrosation.[20,21] Its relative redox activities depend on substrate concentrations of nicotinamide adenine dinucleotide and its reduced form (NAD^+/NADH) ratio.

Previous studies showed that GSNO administration provided protection in an experimental model of cerebral ischemia by downregulating the expression of iNOS and nuclear factor κB (NF-κB).[22] The therapeutic effects of GSNO have been demonstrated in experimental autoimmune uveitis,[23] in which the oral administration of GSNO significantly suppressed the levels of inflammatory mediators associated with maintaining normal retinal histology and function. The RPE cells constitutes a site of immunosuppressive/inflammatory factor secretion inside the eye,[24] such as iNOS and TNF-α.[25,26] For this reason, human RPE cell line (ARPE-19) cells constitute an adequate model for assessing nitrosative stress in vitro.

To our knowledge, no study has addressed the possible effects of GSNO in the development or progression of DR. Based on these observations, we hypothesized that diabetes increases iNOS protein expression and NO production, and that treatment with a GSNO compound could modulate iNOS expression/activity in the diabetic retina, and further determine the importance of the S-nitrosoglutathione/glutathione system in DR pathogenesis. The hypothesis was tested with an in vivo model of diabetes and through in vitro exposure of ARPE-19 cells to a high glucose (HG) condition.

Materials and Methods

Animals Study

The animal study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, in accordance with the local Committee for Ethics in Animal Research (1834-1/CEEA/IB/UNICAMP). Spontaneously hypertensive rats (SHR) 4 weeks old were provided by Taconic (Germantown, NY, USA) and bred in our animal facility. We have chosen to use SHR rats, because these animals display earlier and extensive retinal changes after streptozotocin (STZ, 50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) induction when compared to normotensive counterparts.[27,28] Enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany) was used to measure plasma glucose levels 48 hours after the STZ or citrate buffer injection to confirm the induction; rats with plasma glucose values of ≥15 mM were considered diabetic for the present study. After the confirmation, the rats were randomized to be treated with either eye drop vehicle only, low-dose eye drop GSNO (900 nM), or high-dose eye drop GSNO (10 μM) twice daily in both eyes for 20 days. The preparation of GSNO eye drops was performed at the Chemistry Institute, State University of Campinas (UNICAMP). The GSNO was synthesized by the equimolar reaction between glutathione (GSH) and sodium nitrite, in the dark, and remained dormant for 40 minutes to allow complete nitrosation of thiol. After this period, it was precipitated with acetone, filtered, and lyophilized in the dark. To prepare the eye drops, the GSNO was dissolved in a phosphate buffer and added to a solution vehicle hydroxypropyl methylcellulose (HPMC) at a final concentration of 10 μm or 900 nm of GSNO and 2% (wt/wt) of HPMC. The basal and final systolic blood pressures (SBP) were obtained by noninvasive tail cuff blood pressure amplifier with a built-in automatic cuff pump (Model 229; ITC, Inc., Life Science, Woodland Hills, CA, USA). At the end of the experiment (20 days of treatment), the rats were submitted to full-flash electroretinography, blood collected for measurement of glycated hemoglobin ($GHbA_{1c}$) levels with colorimetric kit (Helena Glyco-Tek Affinity Column Method; Helena Laboratories, Beaumont, TX, USA), and then euthanized. Levels of NO estimated by Nitric Oxide Analyzer (NOA) method in aqueous humor and vitreous from control treated animals was performed to demonstrate the ability of GSNO in penetrating ocular tissues (see Supplementary Method and Fig. S1).

ARPE-19 Cell Line Culture. The ARPE-19 was obtained from the Federal University of Rio de Janeiro (RJCB Collection). Cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12 (DMEM:F12) supplemented with 10% FBS and 1% penicillin/streptomycin. The ARPE-19 cell cultures were serum starved and then treated with normal glucose (NG), HG, with or without the following treatments. The cytotoxicity of the treatments with GSNO (from 10 μM to 10 nM) and NOS inhibitors (from 2 mM to 2 μM) after 24 hours in ARPE-19 cells was obtained by MTT cell viability assay.[29] We considered no cytotoxicity if cell death was below 10% (data not shown). For NOS inhibitors, the cells were pretreated for one hour with a nonselective L-NAME (Sigma-Aldrich), and specific for iNOS, aminoguanidine (AG; Sigma-Aldrich) and N6-(1-iminoethyl)-lysine, hydrochloride (L-NIL; Cayman Chemical, Ann Arbor, MI, USA).

Full-Flash Electroretinogram (ERG) Recording. Retinal function was measured in SHR animals as described previously, with some modification.[30] For retinal function analysis, we used −10 dB light stimulus for recordings of *a*- and *b*-waves, and 0 dB for *c*-wave in which better signal responses are evoked.

Immunohistochemistry for Glial Fibrillary Acidic Protein (GFAP), Nitrotyrosine (NT), and Inducible Nitric Oxide Synthase (iNOS) in Retinal Tissues and Immunofluorescence of Nitrotyrosine in ARPE-19 Cells. Immunohistochemistry was performed as described previously by our group.[31] The retinal sections were incubated with goat polyclonal anti-GFAP (Santa Cruz Biotechnologies, Santa Cruz,

TABLE. Physiological Parameters of the Animals

Groups	Initial Body Weight, g	Final Body Weight, g	SBP, mm Hg	% HbA_{1c}
CT, $n = 5$	170 ± 16	215 ± 18	191 ± 18	7.31 ± 0.44
CT-low dose, $n = 6$	178 ± 13	245 ± 10	184 ± 5	7.59 ± 0.30
CT-high dose, $n = 5$	163 ± 14	240 ± 8	182 ± 4	7.69 ± 0.29
DM, $n = 6$	166 ± 22	127 ± 13*	185 ± 8	11.67 ± 0.77*
DM-low dose, $n = 5$	169 ± 9	123 ± 14*	188 ± 11	11.30 ± 1.69*
DM-high dose, $n = 6$	173 ± 16	136 ± 14*	182 ± 6	10.45 ± 0.73*

The DM groups had lower final body weight and higher glycated hemoglobin levels compared to the CT animals (*$P < 0.01$). The treatment did not change any parameters. CT, nondiabetic rats treated with vehicle of eye drop; CT-low dose, nondiabetic rats treated with GSNO 900 nM; CT-high dose, nondiabetic rats treated with GSNO 10 μM; DM, diabetic rats treated with vehicle of eye drop; DM-low dose, diabetic rats treated with GSNO 900 nM; DM-high dose, diabetic rats treated with GSNO 10 μM; SBP, systolic blood pressure; % HbA_{1c}, percentage of hemoglobin A_{1c} glycated.

CA, USA) or rabbit polyclonal anti-NT (Upstate Cell Signaling Solutions, Lake Placid, NY, USA) or rabbit polyclonal anti-iNOS (Santa Cruz Biotechnologies) overnight at 4°C.

The immunofluorescence for ARPE-19 cells was performed as published previously.[32] Rabbit anti-NT (1:20) for overnight incubation at 4°C and secondary antibody Alexa 488 goat anti-rabbit (Invitrogen, San Diego, CA, USA) at 1:200 for 1 hour at room temperature were applied.

Western Blotting Analysis for INOS or NF-κB in Whole Retinal Tissue and in ARPE-19 Cells. The Western blotting was performed as described previously.[31] Membranes were incubated with rabbit polyclonal iNOS antibody (Cell Signaling

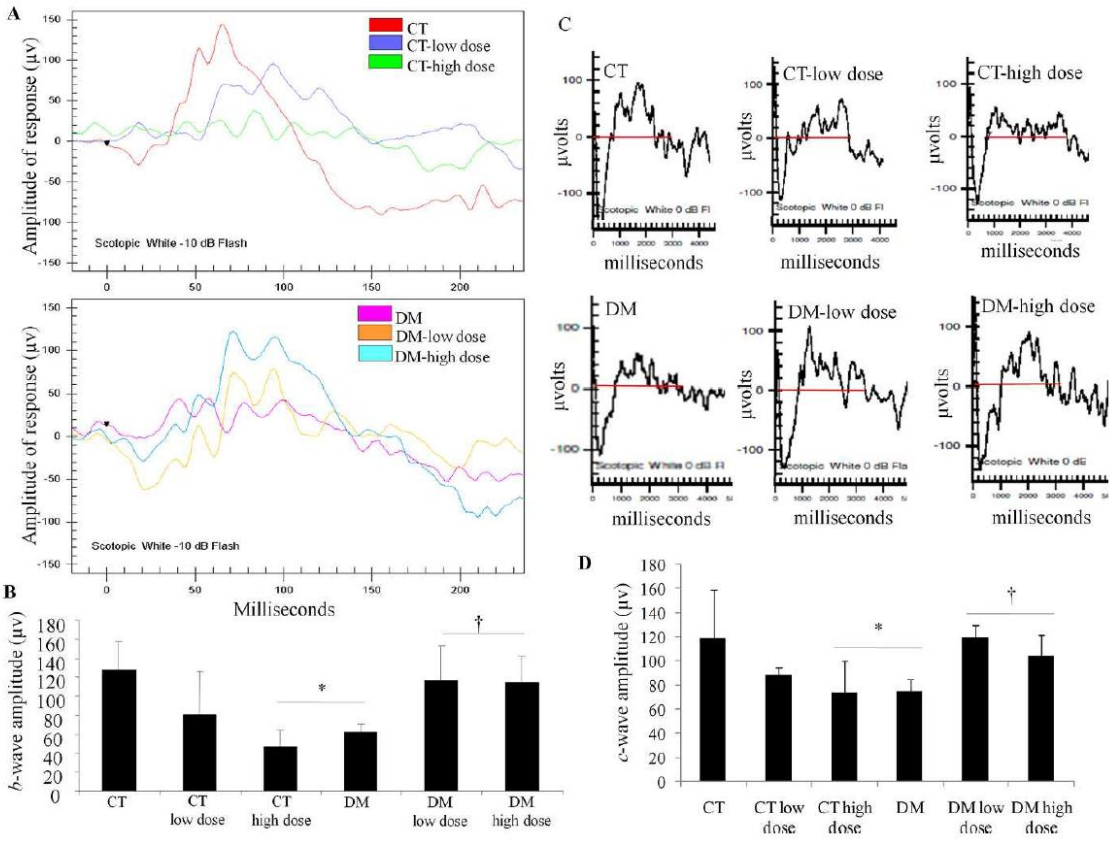

FIGURE 1. Retinal function evaluated by electroretinography. (**A**) Representative waveforms of the *a*- and *b*-waves in the CT and DM groups, which corresponds to the photoreceptor and inner retinal cell responses, respectively, in response to light stimulus intensity at −10 dB. (**B**) The *error bars* represent the mean ± SD of the *b*-wave amplitude in μvolts. *$P \leq 0.02$ versus CT group; †$P \leq 0.05$ versus DM and CT high-dose group. (**C**) Waveforms of *c*-waves in normal and diabetic groups in response to light stimulus intensity at 0 dB. (**D**) The *error bars* represent the mean ± SD of the *c*-wave amplitude in μvolts. *$P \leq 0.02$ versus CT group; †$P \geq 0.01$ versus DM and CT high-dose group. There was no significant difference in *a*-waves and implicit *b*-wave time between the studied groups (data not shown).

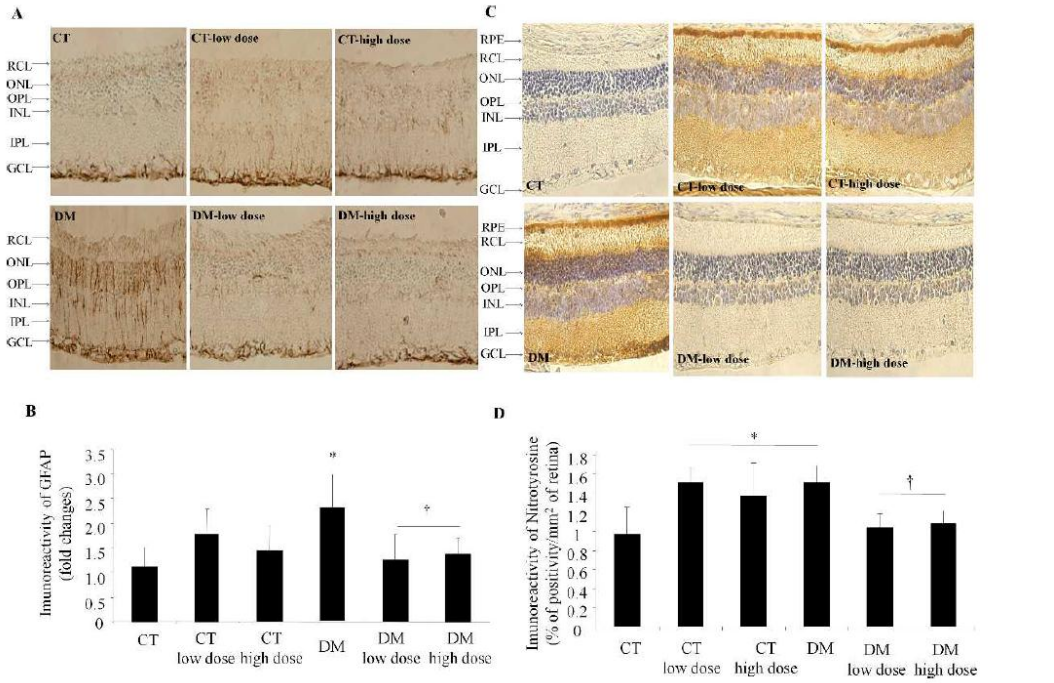

Figure 2. Early marker of diabetic retinopathy and nitrosative stress of the studied groups. (**A**) Photomicrograph representing immunolocalization of glial fibrillary acidic protein (GFAP) on retinal tissue. The GFAP in retinal tissue sections (5 µm) is shown in *brown* color (magnification ×400). (**B**) *Error bars* represent the mean ± SD of GFAP positivity analyses. The percentage of positivity per retinal field (mm²) was transformed to changes/fold in relation to the media of control in each experiment to compare independent experiments.*$P = 0.02$ versus CT group; †$P \leq 0.05$versus DM group. The treatment in the CT low- and high-dose groups did not alter GFAP immunoreactivity compared to the CT group ($P = 0.2$). (**C**) Representative photomicrograph of NT immunoreactivity. The presence of NT is shown in *brown* on retinal tissue sections (5 µm) (magnification ×400). The positivity was widely expressed among all retinal layers, and especially in RPE. (**D**) The *error bars* represent the mean ± SD of the percentage of positivity per retinal field (mm²). *$P \leq 0.03$ versus CT group; †$P \leq 0.03$ versus DM group. At least 3 independent experiments were performed for each assay. RCL, rods and cones layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Technology, Beverly, MA, USA) or rabbit polyclonal NFκB p65 (Santa Cruz Biotechnologies).

Measuring Intracellular ROS Production in Cells by H_2DCFDA and NO• Formation by DAF-2DA. As previously published,[31,33] we measured the total intracellular ROS production by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and intracellular NO• levels by diaminofluorescein diacetate (DAF-2DA).

Immunoprecipitation of GSNO-R and GSH/INOS. The cells were lysed directly in a buffer containing 100 mM tris base, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM sodium ortovanadate, and 1% Triton X-100. Samples were incubated with rabbit anti-GSNO-R (ADH5 polyclonal antibody; Protein Tech Group, Chicago, IL, USA) or mouse monoclonal anti-glutathione (GSH) antibody (Virogen, Watertown, MA, USA) overnight, followed by the addition of protein A Sepharose for 1 hour. After centrifugation, the pellets were washed in buffer (100 mM Tris Base, 2 mM sodium ortovanadate, 1 mM EDTA, and 0.5% Triton X-100). The immune-precipitated samples were prepared under reducing or nonreducing conditions as necessary and loaded onto SDS polyacrylamide gels. The membranes were blocked in nonfat milk and incubated with anti-GSNO-R or rabbit polyclonal anti-iNOS (Cell Signaling Technology) and subsequently incubated with appropriate secondary antibodies. Equal loading and transfer were ascertained by ponceau for GSNO-R.

Determination of Reduced Glutathione (GSH) Levels. Retinal GSH level was measured using the method described by Beutler et al.[34] with a few modifications.[27]

Determination of GSNO by Ultra High-Performance Liquid Chromatography (UHPLC). As described previously,[35] and with some adaptations, for the measurement of GSNO, the cells were washed with ice-cold PBS once before lysis with an extraction buffer (25 mM ammonia sulfamate dissolved in *o*-metaphosphoric acid 5%). The lysate was sonicated for 30 seconds. Samples were centrifuged, and the supernatant collected and filtrated. The GSNO measurement analyses were carried out on an Agilent 1290 Infinity UHPLC system (Agilent Technology, Waldbronn, Germany) by liquid chromatography with diode array detection (LC/DAD). Chromatographic separation was achieved on a 2.6 µm Kinetex-C18 column (50 × 2.1 mm; Phenomenex, Torrance, CA, USA), operating at 25°C. Mobile phases were constituted with 100% 20 mMKCl pH 2.5 (Ecibra, Curitiba, Brazil). The flow rate was 500 µL/min and injection volume was 3 µL. Chromatographic data were recorded and integrated using LCD ChemStation software (Agilent Technology). To confirm the GSNO identity in ARPE-19 cell samples, high-resolution electrospray ionization-MS analyses (MS/MS) were performed for GSNO in standard and in ARPE-19 cells exposed to normal glucose (see Supplementary Methods and Fig. S3).

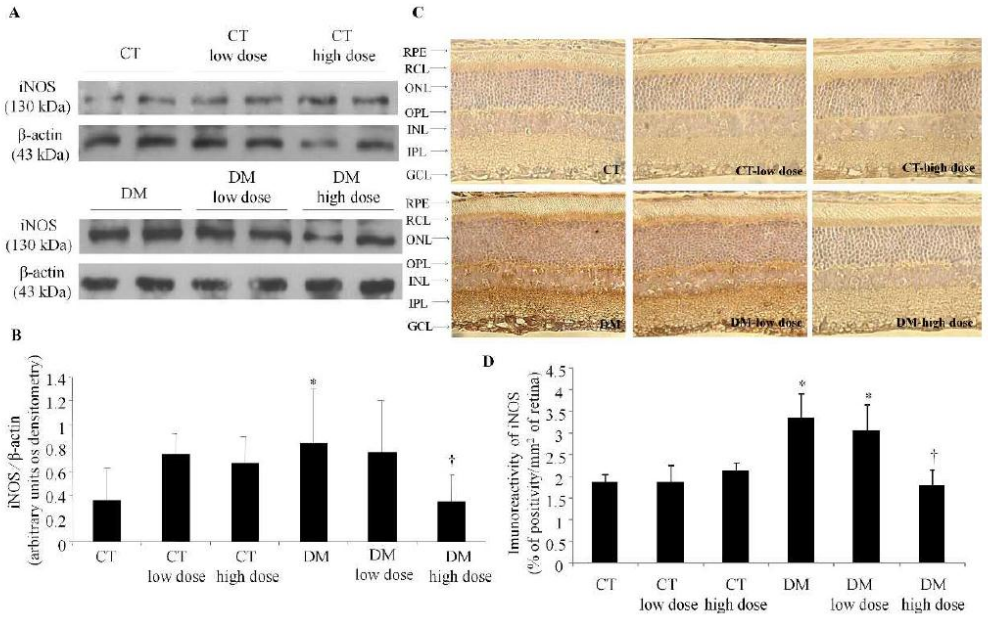

Figure 3. Evaluation of nitrosative stress of studied groups. **(A)** Western blot for iNOS expression in total retinal lysate. **(B)** Equal loading and transfer were ascertained by reprobing the membranes for β-actin. The *error bars* represent mean ±SD of band densities expressed in arbitrary units of densitometry. $^{*}P = 0.03$ versus CT group; $\dagger P = 0.04$ versus DM group. **(C)** Representative photomicrograph of iNOS immunoreactivity and localization on retinal tissue (magnification ×400). The presence of iNOS is shown in *brown* in all layers of the retina especially in RPE layer. **(D)** The *error bars* represent the mean ± SD of the percentage of positivity per retinal field (mm²). $^{*}P = 0.0005$ versus CT group; $\dagger P = 0.0004$ versus DM group. At least 3 independent experiments were performed for each assay.

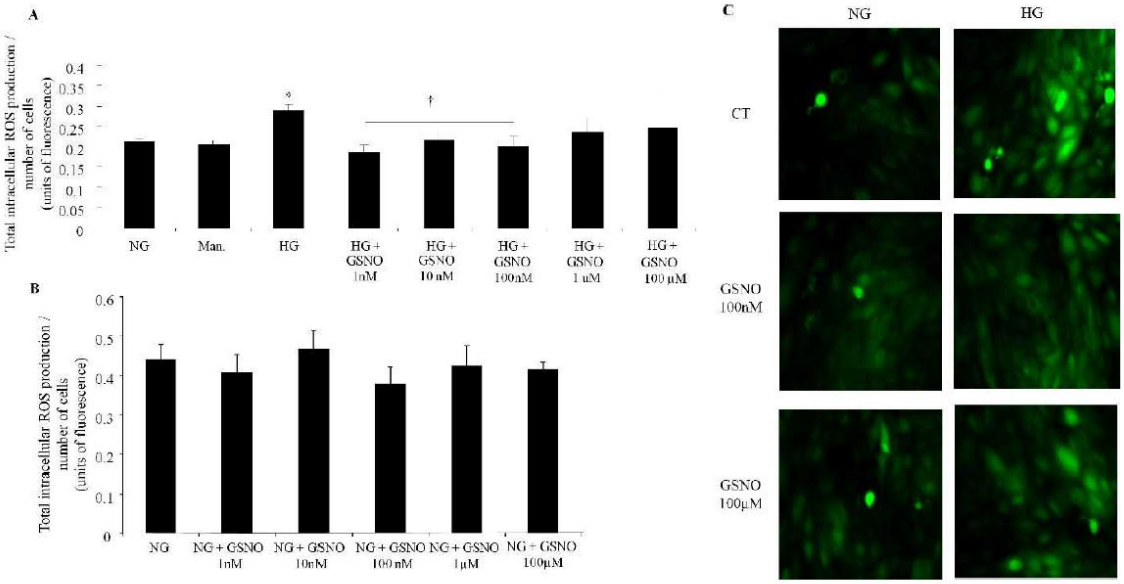

Figure 4. Total intracellular ROS production in ARPE-19 cells. Total ROS production was obtained by H_2DCFDA fluorescence. The ARPE-19 cell cultures at 80% of confluence were serum starved, then exposed to NG 5.5 mM; to NG+GSNO at 1 nM to 100 µM; to HG 30 mM; and to HG+GSNO at 1 nM–100 µM for 24 hours. NG + 24.5 mM of mannitol was used as an osmotic control. **(A)** *Error bars* represent the mean ± SD of fluorescence units obtained in ELISA reader and corrected by the number of cells at the end of each treatment. Mannitol was used for an osmotic control in this experiment to see if there is some effect of osmolarity. $^{*}P = 0.03$ versus NG; $\dagger P \leq 0.02$ versus HG group. **(B)** Total ROS production under NG condition. **(C)** Representative photomicrographs of qualitative H_2DCFDA assay indicating the levels of total ROS production in ARPE-19 cells using fluorescence microscope (Zeiss Axio Observer.A1 Inverted; Carl Zeiss Meditec, Jena, Germany).

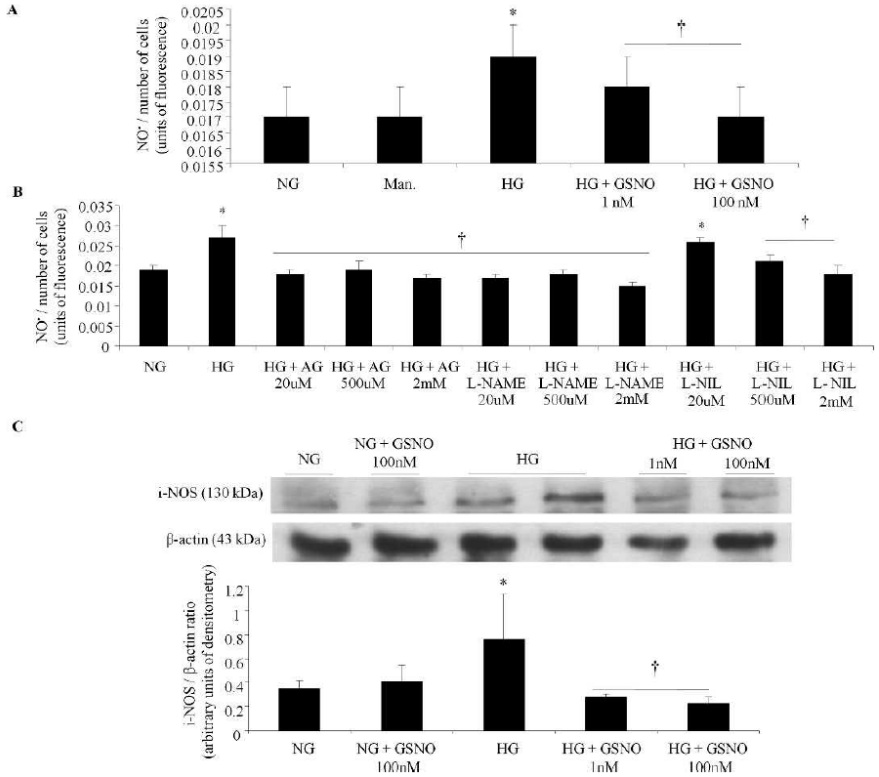

Figure 5. Intracellular NO• production and iNOS expression in ARPE-19 cells. ARPE-19 cell cultures at 80% of confluence were serum starved, then exposed to NG 5.5 mM; to NG+GSNO at 100 nM; to HG 30 mM; and to HG+GSNO at 1 nM and 100 nM for 24 hours. NG + 24.5 mM of mannitol was used as an osmotic control. (**A**) Detection of intracellular NO• by DAF-2DA fluorescence. *Error bars* represent the mean ± SD of the fluorescence units obtained via ELISA reader corrected by the number of cells at the end of each treatment. *$P = 0.0007$ versus NG condition; †$P \leq 0.008$ versus HG treatment. (**B**) NO• production in the presence of total NOS (L-NAME) and specific for iNOS (AG and L-NIL) inhibitors in cell culture. *$P < 0.0001$ versus NG; †$P < 0.0001$ versus HG. The mannitol treatment did not change the levels of NO• production ($P = 0.8$). (**C**) Western blot for iNOS expression on total cell lysate. Exposed films were scanned with a densitometer (Bio-Rad Laboratories, Inc., Hercules, CA) and analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems. Equal loading and transfer were ascertained by reprobing the membranes for β-actin. The *error bars* represent mean ± SD of band densities expressed in arbitrary densitometric units. *$P = 0.02$ versus NG condition; †$P \leq 0.009$ versus HG condition. At least 3 independent experiments were performed for each assay.

Statistical Analysis

The results were expressed as the means ± SD. The groups were compared by 1-way ANOVA, followed by the Fisher protected least-significant difference test. StatView statistics software (SAS Institute, Inc., Rockville, MD, USA) was used for all comparisons, with a significance value of $P < 0.05$.

Results

In Vivo Study

The physiological characteristics of the study animals are shown in the Table.

GSNO Eye Drops Prevented DM Retinal Function Impairment and Early Markers of DR. A significant retinal function impairment was observed in *b*-waves among control (CT) high-dose and DM rats compared to those treated with vehicle ($P \leq 0.02$, Fig. 1B). To assess the RPE function, we acquired c-wave responses (Fig. 1C), and also a significant decrease in *c*-wave amplitude in the CT high-dose and DM rats compared to the CT group ($P \leq 0.02$) was observed. Both doses of GSNO eye drops prevented this impairment in DM animals compared to the nontreated DM and CT high-dose groups ($P < 0.01$, Fig. 1D).To evaluate early structural marker of DR, we assessed GFAP immunoreactivity (Fig. 2A). There was a clear increase in retinal GFAP positivity in the DM rats in all layers of the retina compared to the CT group ($P = 0.02$). The treatment with GSNO in both doses significantly decreased the GFAP expression in the DM groups ($P < 0.02$; Figs. 2A, 2B).

GSNO Eye Drop Reestablished the Nitrosative Status in Retinas of DM Rats. The nitrosative stress was estimated by nitrotyrosine expression, a product of tyrosine nitration mediated by RNS, such as peroxynitrite anion and nitrogen dioxide (Fig. 2C). We observed a GSNO-induced increase in NT expression in the CT-treated groups ($P \leq 0.03$ versus the CT group). The DM group also presented higher levels of NT compared to the CT group ($P = 0.007$). Treatment with GSNO in the DM groups prevented the increase in NT production ($P \leq 0.03$; Figs. 2C, 2D).

To better understand the nitrosative stress mechanisms involved in producing NT in retinal tissue among different conditions, we evaluated the expression of iNOS (Fig. 3A). There was no difference in iNOS expression among the CT

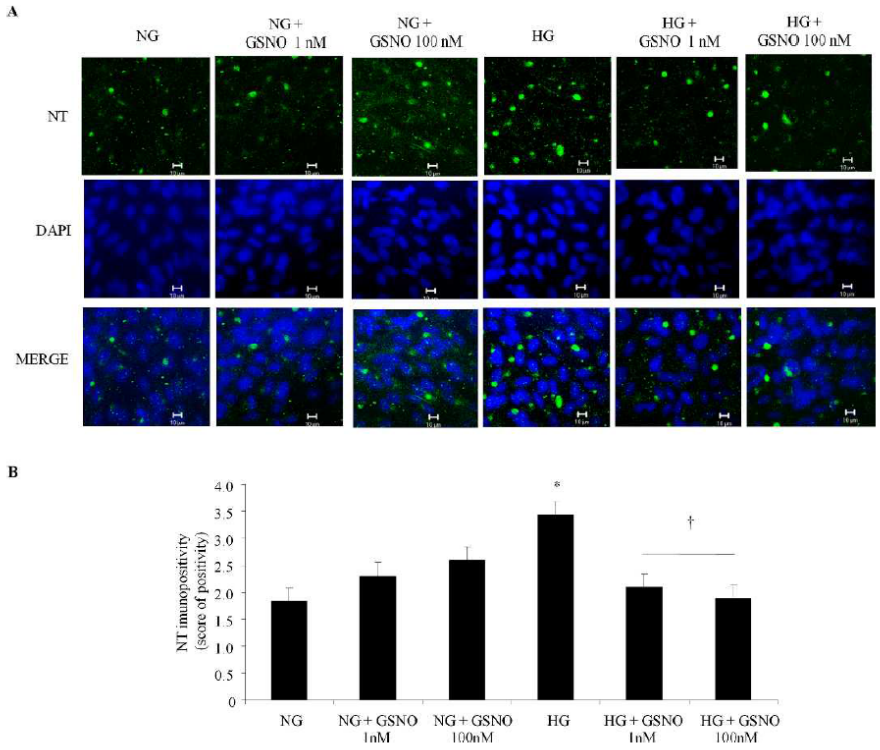

Figure 6. Immunofluorescence of nitrotyrosine in ARPE-19 cell lines. ARPE-19 cell cultures at 80% of confluence were serum starved, then exposed to NG 5.5 mM; to NG+GSNO at 1 and 100 nM; to HG 30 mM; and to HG+GSNO at 1 and 100 nM for 24 hours. (**A**) Confocal images showing NT positivity and localization. The positivity of NT is shown in *green* (localized on the cytoplasm) and the nucleus is indicated with nuclear dye (DAPI) under a confocal laser scanning microscope (×630; Carl Zeiss Meditec). (**B**) The *error bars* represent the mean ± SD of the score of positivity, from 0 for no positivity to 4 for ≥80% of positivity by blindness. *$P = 0.0006$ versus NG treatment; †$P \leq 0.003$ versus HG treatment. At least 3 independent experiments were performed.

low- and high-dose compared to the CT group ($P \geq 0.1$). Among the DM rats, there was a significant increase in iNOS expression compared to the CT groups ($P = 0.03$) and it was observed as an effective prevention in the DM high-dose group ($P = 0.04$; Figs. 3A, 3B). Immunoreactivity for iNOS localization showed diffuse positivity among all retinal layers, markedly in the RPE layer in the DM compared to the CT groups ($P = 0.0005$); in the DM high-dose group, the iNOS upregulation was prevented ($P = 0.0004$; Figs. 3C, 3D).

Collectively, these data suggested that the nitrosative stress observed in the CT animals treated with eye drops was GSNO-mediated. However, in the DM animals the nitrosative stress was associated with iNOS upregulation. The following in vitro experiments were designed to better understand the different effects of GSNO treatment eye drop in CT and DM animals. To assess whether GSNO inhibits NF-κB in retinal tissue, we measured the expression of retinal NF-κB (see Supplementary Fig. S2). There was no difference among the studied groups. This evidence support that the protective effect of GSNO demonstrated in diabetic retinal is not through NF-κB inhibition.

In Vitro Study

To better understand the role of the S-nitrosoglutathione/glutathione system in the diabetic setting, we conducted experiments with ARPE-19 cells, since RPE immunoreactivity for iNOS was highly expressed in diabetic tissue.

GSNO Counteracted the Upregulation of ROS and RNS in Cells Exposed to HG, but Promoted Nitrosative Stress in NG. We observed an increase in total ROS levels in response to HG compared to the NG condition ($P = 0.03$). The treatments with HG plus GSNO in nanomolar concentrations were effective in counteracting the upregulation of ROS production ($P \leq 0.02$), but not in micromolar concentrations ($P = 0.09$; Figs. 4A, 4C). Since nanomolar concentrations of GSNO were more efficient in protecting the cells against increased ROS production, the subsequent experiments were conducted only at 1 and 100 nM of GSNO. At NG condition, treatment with GSNO did not alter the levels of ROS production compared to NG ($P = 0.3$; Figs. 4B, 4C).

As detected in ROS production, the intracellular $NO^{\bullet}$ production was increased in HG compared to the NG condition ($P = 0.0007$); both doses of GSNO treatments prevented this increase in diabetic milieu condition ($P \leq 0.008$, Fig. 5A).

To investigate which isoform of NOS is the main source of the observed increased $NO^{\bullet}$ under the DM setting conditions, the cells cultured in the HG conditions were treated with L-NAME, a nonselective NOS inhibitor, and with specific blockers for the iNOS isoform, AG and L-NIL.[36] All treatments with AG or L-NAME similarly prevented the increase in $NO^{\bullet}$ production observed in the HG condition ($P < 0.0001$). The L-

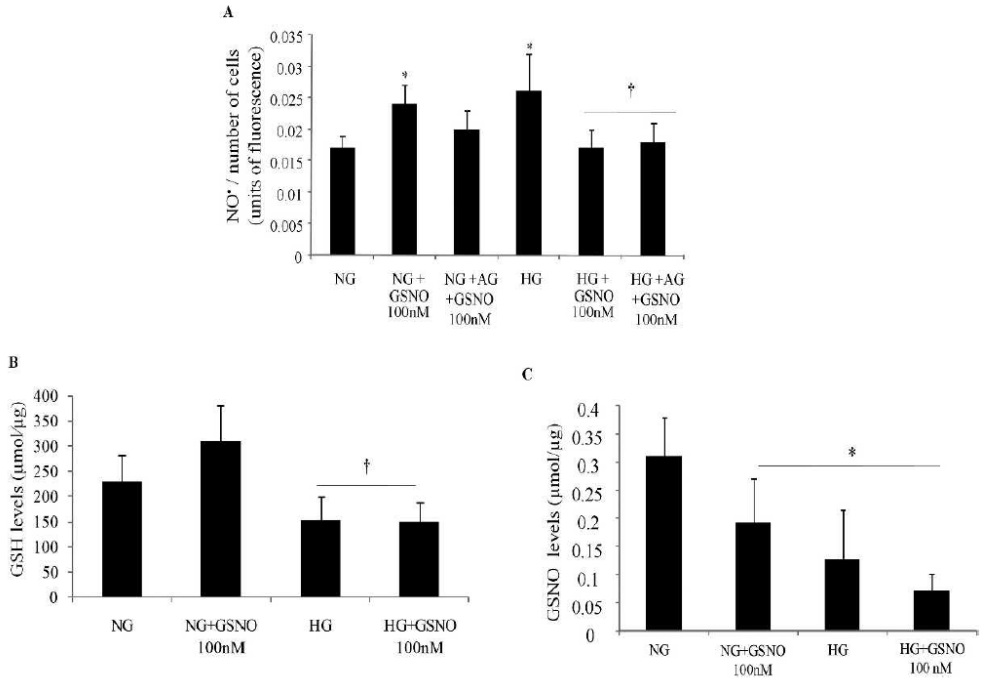

Figure 7. Effects of GSNO under NG and HG conditions in ARPE-19 cells. ARPE-19 cell cultures at 80% of confluence were serum starved, then exposed to NG 5.5 mM; to NG+GSNO at 100 nM; to HG 30 mM; and to HG+GSNO at 100 nM for 24 hours. (**A**) Detection of intracellular $NO^{\bullet}$ by DAF-DA fluorescence method in cells exposed to NG and HG in the presence or absence of specific iNOS inhibitor (AG). *Error bars* represent the mean ± SD of the fluorescence units corrected by the cell number. $^{*}P \geq 0.001$ versus NG treatment; $\dagger P = 0.002$ versus HG condition. (**B**) Measurement of GSH by colorimetric assay. Absorbance was read at 412 nm. GSH was used as an external standard for preparation of a standard curve. *Error bars* represent the mean ± SD of µmol of GSH corrected by protein cell lysate concentration (µg). $^{*}P < 0.04$ versus NG; $\dagger P < 0.03$ versus NG. (**C**) Measurement of endogenous GSNO by UHPLC method. Chromatography analyses of standard GSNO and levels of GSNO under different treatments, retention time = 0.6 minutes. *Error bars* represent the mean ± SD of µmol of GSNO levels corrected by protein cell lysate concentration (µg) under different conditions. $^{*}P \leq 0.02$ versus NG condition. At least 3 independent experiments were performed.

NIL at 500 µM and 2 mM concentrations also prevented these increases ($P < 0.0001$, Fig. 5B), suggesting that the main source of NO production under HG conditions is iNOS, since blocking all isoforms of NOS with L-NAME added no further effect to that observed with iNOS selective blockers. In agreement, GSNO treatments in NG conditions did not alter the iNOS expression compared to control NG ($P = 0.7$). In the HG condition, there was a significant increase in iNOS expression compared to the NG conditions ($P = 0.02$). Treatment with GSNO at 1 and 100 nM concentrations prevented this increment ($P \leq 0.009$, Fig. 5C).

To estimate the oxidative/nitrosative damage in ARPE-19 cells, we assessed NT (Fig. 6). Under NG conditions, the GSNO treatments (either 1 or 100 nM) did not significantly change the positivity of NT compared to NG ($P > 0.05$). However, in the highest dose of the GSNO treatment there was a strong tendency to increase compared to NG ($P = 0.07$), suggesting an action as a nitrosating inducer. Higher positivity was observed clearly in cells exposed to HG compared to the NG condition ($P = 0.0006$), and the presence of GSNO treatments counteracted this increase ($P \leq 0.003$; Figs. 6A, 6B).

These findings suggested that, under HG condition, there is an increase in RNS accompanied by upregulation of iNOS expression. The $NO^{\bullet}$ upregulation in HG was mediated by iNOS isoform and GSNO counteracted this effect. Based on this, these data indicated that GSNO under DM milieu is not a nitrosating agent but, instead, prevented RNS.

The Dual Effect of GSNO. To better understand whether GSNO itself can generate NO under NG conditions independently of iNOS, and whether in HG conditions GSNO can inhibit NO production via NOS system, we assessed $NO^{\bullet}$, GSNO, and GSH levels under NG and HG conditions.

In the NG+GSNO condition, the NO levels increased when compared to NG alone ($P = 0.01$). In the presence of iNOS inhibitor, the NO levels did not decrease compared to NG+GSNO ($P = 0.2$). These observations indicated that in NG, GSNO acts as an NO donor inducing nitrosative stress. Under the HG conditions, there was a marked increase in the NO levels compared to the NG condition ($P = 0.001$). In the presence of GSNO alone or associated with AG, we observed similar decreases in the $NO^{\bullet}$ intracellular levels compared to the HG conditions ($P = 0.002$). The combination of AG in the presence of GSNO treatment did not further decrease $NO^{\bullet}$ levels, demonstrating that in HG conditions GSNO counteracts $NO^{\bullet}$ upregulation through iNOS inhibition (Fig. 7A).

The redox state of the GSH/GSSG combination is an important indicator of the redox environment,[37] and glutathione dysregulation is linked with the etiology and progression of human diseases.[38] We quantified the GSH levels in ARPE-19 cells, and in the NG plus GSNO condition, we observed an increase in GSH levels compared to the NG condition ($P = 0.04$). In the HG condition, the GSH levels were lower when compared to NG ($P = 0.04$). The presence of GSNO in the HG conditions did not prevent this effect ($P = 0.9$, Fig. 7B). In the

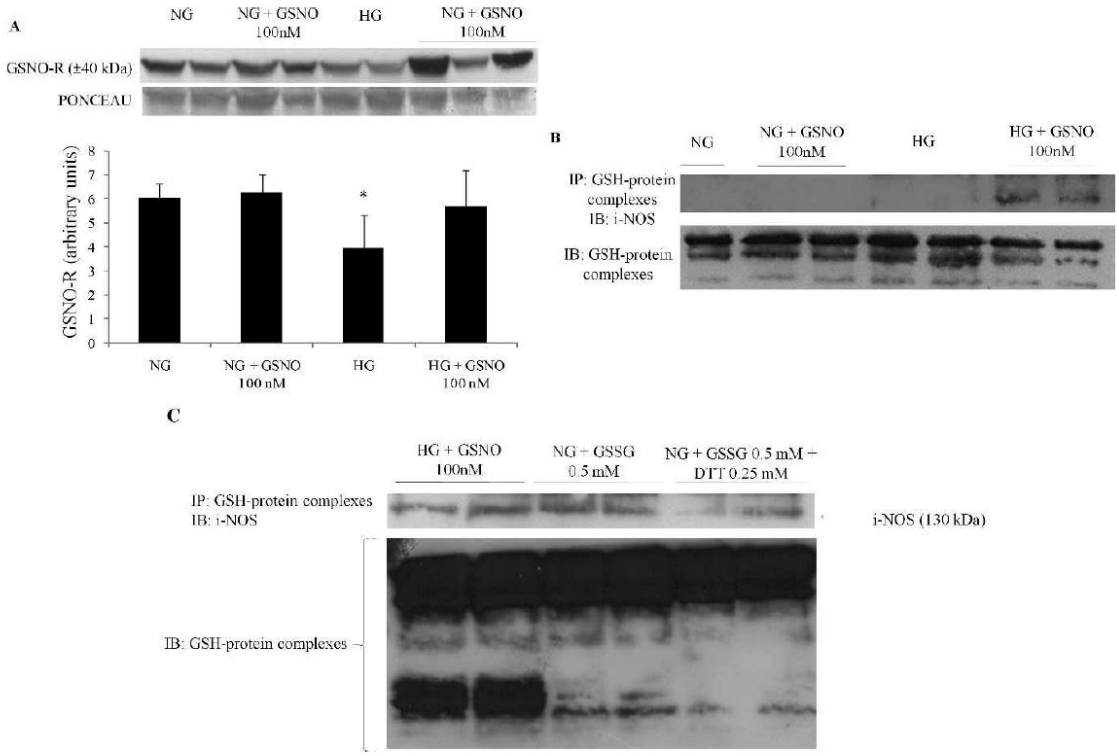

Figure 8. GSNO promotes S-glutathionylation of iNOS. ARPE-19 cell cultures at 80% of confluence were serum starved, then exposed to NG 5.5 mM; to NG+GSNO at 100 nM; to HG 30 mM; and to HG+GSNO at 100 nM for 24 hours. (**A**) Immunoprecipitation of cell lysate with GSNO-R antibody incubated with GSNO-R antibody. The GSNO-R protein expression was measured by Western blot. Equal loading protein and transfer were confirmed by Ponceau. *Error bars* represent the mean ± SD expressed in arbitrary units of densitometry. $^*P = 0.05$ versus NG. At least 3 independent experiments were performed for each assay. (**B**) Cell lysate was immunoprecipitated with the GSH protein complex antibody and immunoblotted against iNOS. The GSH-protein complexes were blotted against iNOS. Equal loading protein was ascertained by reprobing the membranes for total GSH complex proteins. (**C**) Controls for S-glutathionylation of iNOS were done by 0.5 mM GSSG (positive control) or GSSG plus the reduced agent DTT 0.25 mM (negative control) to reverse the reaction. The detection of S-glutathionylated iNOS at 130 kDa was present in cells exposed to HG treated with GSNO; in cells exposed to NG in the presence of GSSG, the expression of S-glutathionylated iNOS was equally increased as compared to HG plus GSNO, and DTT reversed this posttranslational iNOS modification.

NG conditions, the presence of GSNO resulting in GSH increase might be due to an increase in its synthesis through increased expression of γ-glutamylcysteine synthetase[39] promoted by $NO^\bullet$ generated by the GSNO+GSH = GSSG+$NO^\bullet$ reaction,[18] or might be metal-catalyzed[17] or thioredoxin-catalyzed[40] degradation-dependent.

We also evaluated the levels of endogenous GSNO in ARPE-19 cells with the UHPLC method. Curiously, in the NG condition treated with GSNO, the levels of GSNO decreased compared to the NG condition ($P = 0.02$). This intriguing observation indicated that exogenous GSNO is catalyzed rapidly by either by an enzymatic process or chemically reacting with thiol groups as GSH leading to total GSNO intracellular pool decreasing. The levels of GSNO are lower in the HG compared to NG condition ($P = 0.005$), and the treatment with GSNO under HG condition did not lead to an increase in GSNO levels ($P = 0.2$, Fig. 7C). In the HG condition, GSNO improved nitrosative stress, not through the reestablishment of GSH and/or GSNO levels.

GSNO Decreases $NO^\bullet$ Levels in HG by S-Glutathionylation of INOS. One possible mechanism by which GSNO displays different effects under NG or HG conditions is through GSNO-R, which reduces GSNO to GSSG. To address whether GSNO-R has a role in decreasing NO levels under the HG+GSNO treatment, we evaluated the expression of GSNO-R. There was no difference between NG and NG+GSNO treatment ($P = 0.8$); however, under HG, GSNO-R protein expression was markedly decreased ($P = 0.05$). The HG+GSNO treatment increased GSNO-R protein expression, but did not reach conventional statistical significance ($P = 0.09$, Fig. 8A). This increase in GSNO-R in cells exposed to HG treated with GSNO contributes to denitrosylation of GSNO leading to GSSG release. The decrease of endogenous GSNO under NG+GSNO treatment (Fig. 7C) also may be explained by the denitrosylation promoted by GSNO-R (Fig. 8A).

It was demonstrated previously that S-glutathionylation of eNOS regulates its activity.[41] To investigate whether similar posttranslational modification could be involved in inhibition of iNOS by GSNO in HG conditions, we addressed the S-glutathionylation of iNOS. We observed that there was no expression of S-glutathionylated iNOS in NG alone or in NG+GSNO. Under HG conditions, we observed a faint signal that was markedly increased in the presence of GSNO. This finding indicates that GSNO treatment promotes S-glutathionylation of iNOS (Fig. 8B). To verify and confirm the specificity of iNOS glutathionylation immunoblotting, we treated the cells

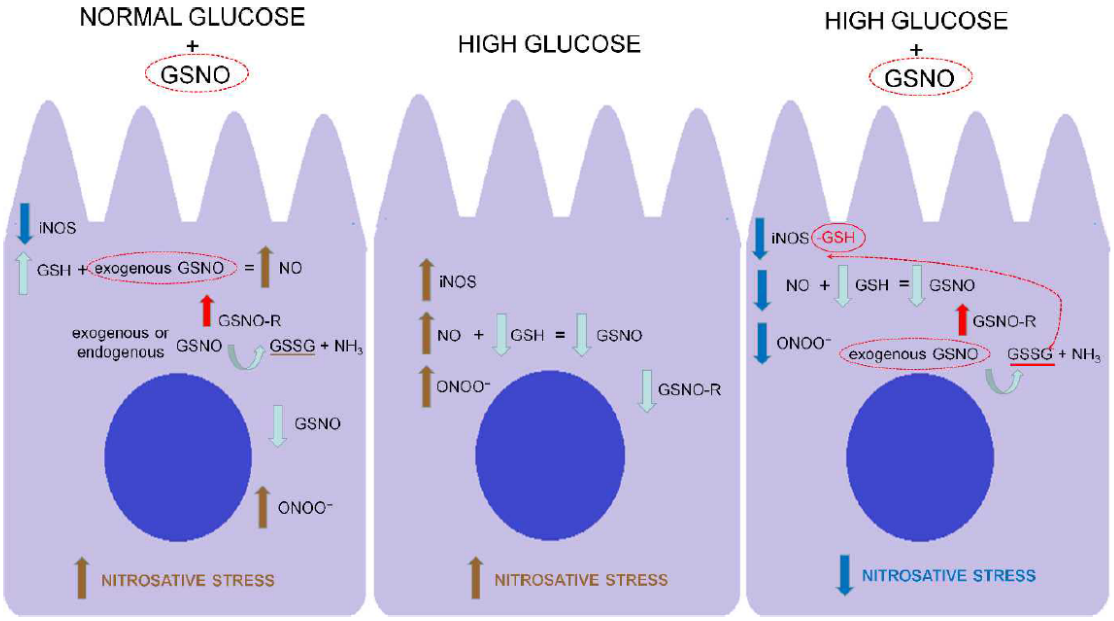

Figure 9. Schematic representation of possible mechanisms involved in nitrosative stress under normal or high glucose conditions in ARPE-19 cells. In NG+GSNO: The high content of intracellular GSH can react with exogenous GSNO generating NO, thus promoting nitrosative stress. The exogenous and/or endogenous GSNO may be converted by GSNO-R or metal or other enzyme-catalyzed, leading to low levels of GSNO. In HG condition: iNOS generates high levels ofNO accompanied by decrease of endogenous GSH, GSNO, and GSNO-R levels, resulting in nitrosative stress. The low content of GSH can explain the low levels of endogenous GSNO. In HG+GSNO: The effect of treatment was not due to endogenous GSH and GSNO reestablishment levels.GSNO-R expression is improved, which denitrosylates exogenous GSNO generating GSSG+NH_3 formation; GSSG S-glutathionylates iNOS, reducing NO production, thus, preventing nitrosative stress.

with GSSG 0.5 mM to induce S-glutathionylation or with the reducing agent dithiothreitol (DTT) 0.25 mM to reverse this reaction.[41] We observed that GSSG in NG conditions promoted S-glutathionylation of iNOS similar to that observed in the HG plus GSNO treatment; as expected, the presence of DTT reverses the S-glutathionylation of iNOS protein (Fig. 8C). Taken together, these results suggested that nitrosative stress was prevented by GSNO treatment through iNOS inhibition by S-glutathionylation. The posttranslational modification probably was promoted by the release of GSSG through GSNO denitrosylation via GSNO-R. In contrast, in the NG condition, GSNO treatment promoted nitrosative stress through NO formation. These findings showed the potential clinical implications of balancing the S-nitrosoglutathione/glutathione system in treating DR.

Discussion

In this innovative study, we described that GSNO eye drop mitigated nitrosative stress and slowed the early structural changes present in the retina, thus improving retinal function in an experimental model of diabetes. Of interest, iNOS upregulation in the RPE layer in the DM animals may reveal the role of RPE in the pathogenesis of DR In the ARPE-19 cells exposed to HG, treatment with GSNO alleviated oxidative and nitrosative stress by decreasing iNOS protein expression, thus reducing intracellular $NO^{\bullet}$ levels. In addition, GSNO-R expression was improved. Therefore, posttranslational modification (S-glutathionylation) of iNOS by GSNO in ARPE-19 cells under HG condition suggested its inhibition. This evidence further explains the protective mechanism of GSNO. Rosenfeld et al.[42] already have identified a GSH binding site adjacent to the N-NO-pterin of iNOS. However, under NG conditions, where the intracellular GSH pool is high, the GSNO compound acts as a donor of free NO.

Nitrosative stress is caused by overproduction of RNS. Diabetic stimuli may trigger generation of excess superoxide, which is converted rapidly to peroxynitrite (reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$), hydroxyl radicals (Fenton reaction or the iron-catalyzed Haber–Weiss reaction), and hydrogen peroxide (reaction catalyzed by superoxide dismutase). Peroxynitrite can modify tyrosine residues in proteins to form nitrotyrosine. Nitrotyrosine is a well-accepted indicator of RNS generation, and this stable end product is involved in inactivating mitochondrial and cytosolic proteins, resulting in damage to cellular constituents. Moreover, nitrotyrosine can initiate lipid peroxidation, increase DNA damage, deplete intracellular GSH levels, and induce overexpression of proinflammatory factors and adhesion molecules. Therefore, nitration is being increasingly proposed as a contributor to tissue injury in human diseases.[43,44]

It is becoming increasingly clear that iNOS activity is induced in rats and humans with DR.[13,45,46] Previous studies showed that $iNOS^{-/-}$or the inhibition of iNOS in the ischemic retina prevented angiogenesis locally in the avascular retina, mediated at least in part by downregulation of VEGF receptor 2 (VEGFR2). At the same time, pathological retinal neovascularization was considerably stronger in iNOS-expressing animals, showing that iNOS plays a crucial role in retinal neovascular disease.[47] It also was shown that $iNOS^{-/-}$ mice display protection from retinal cell apoptosis in an ischemic proliferative retinopathy model.[48] Diabetic $iNOS^{-/-}$mice showed downregulation of several inflammatory factors, nitration of

proteins, superoxide production, and leucostasis, thus preventing the formation of acellular capillaries and pericyte ghosts.[49] Our data showed that the upregulation of iNOS expression in diabetes or HG conditions led to excessive NO generation. Furthermore, treatment with GSNO was highly associated with S-glutathionylation of iNOS, thus decreasing NO levels. When NO generation by iNOS is pharmacologically inhibited by the iNOS-specific inhibitor, AG, NO levels were significantly decreased in the presence or absence of GSNO. The use of AG in this present study used as a selective iNOS inhibitor aimed to assess the mechanism by which GSNO exerted the protective effects. By doing this, we could demonstrate that this protective mechanism is through iNOS inhibition.

In NG situations, treatment with GSNO evoked an increase in nitrosative stress in vitro with higher levels of NO, although the GSH levels were upregulated in the ARPE-19 cells. Therefore, the presence of GSNO leads to GSH and NO production. This might be either spontaneous or metal-catalyzed,[17] or thioredoxin-catalyzed[40] degradation-dependent. Under HG conditions, we observed an increase in oxidative/nitrosative stress and higher levels of $NO^{\bullet}$ accompanied by decreases in the GSH and GSNO levels. The low GSH content in these cells may contribute to these effects. Cells exposed to HG showed decreased levels of GSNO-R protein expression, and the supplementation of GSNO at 100 nM improved GSNO-R protein expression and possibly increased the denitrosylation of GSNO, leading to GSSG generation (Fig. 9). A previous work reported that GSSG induces S-glutathionylation of eNOS protein under oxidative stress, uncoupling it and, thus, altering its function,[41] and other investigators demonstrated that this process was described in streptozotocin-induced animals.[50] The S-glutathionylation of NOS isoforms shows to be protective or not, dependent of the physiology function of the isoform. In this present work, S-glutathionylation of iNOS isoform is retinal protective.

The GSNO-R acts only on GSNO, meaning that SNO proteins are not substrates, and it controls protein *S*-nitrosylation by influencing the cellular equilibrium between SNO proteins and GSNO.[51] Others enzymatic systems, such as human carbonyl reductase 1 (hCBR1), an NADPH-dependent short chain dehydrogenase/reductase, has been demonstrated to reduce GSNO.[52] Previous studies using GSNO-$R^{-/-}$ mice showed increased levels of SNO proteins and decreased survival in mice when exposed to endotoxin, and these effects are attenuated by an inhibitor of iNOS.[53] Subsequent studies demonstrated that GSNO-R deficiency is linked to S-nitrosylation of the DNA repair enzyme.[7] In our in vitro model we described decreased expression of GSNO-R accompanied by reduction of GSNO levels in HG condition.

The present study showed, for the first time to our knowledge, the therapeutic effect of GSNO eye drop in counteracting nitrosative stress in an experimental model of DR, with consequent improved retinal function. The GSNO supplementation prevented nitrosative stress by reducing NO generation through iNOS inhibition by S-glutathionylation under diabetic milieu conditions. The regulation of the S-nitrosoglutathione/glutathione system with RNS-based signaling pathways might be potential therapeutic targets in ocular diabetic complications.

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References

1. Fong DS, Aiello L, Gardner TW, et al. American Diabetes Association. Retinopathy in diabetes. *Diabetes Care*. 2004; 1(suppl):S84–S87.
2. Zheng L, Kern TS. Role of nitric oxide, superoxide, peroxynitrite and PARP in diabetic retinopathy. *Front Biosci*. 2009;14:3974–3987.
3. Griffith OW, Stuehr DJ. Nitric oxide synthases: properties and catalytic mechanism. *Annu Rev Physiol*. 1995;57:707–736.
4. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J*. 1994;298:249–258.
5. Moncada S, Bolanos JP. Nitric oxide, cell bioenergetics and neurodegeneration. *Neurochem*. 2006;97:1676–1689.
6. Schmidt HH, Walter U. NO at work. *Cell*. 1994;78:919–925.
7. Wei W, Li B, Hanes MA, Kakar S, Chen X, Liu L. S-nitrosylation from GSNOR deficiency impairs DNA repair and promotes hepatocarcinogenesis. *SciTransl Med*. 2010;2:19ra13.
8. Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein S-nitrosylation purview and parameters. *Nat Rev Mol Cell Biol*. 2005;6:150–166.
9. Foster MW, McMahon TJ, Stamler JS. S-nitrosylation in health and disease. *Trends Mol Med*. 2003;9:160–168.
10. Du Y, Sarthy VP, Kern TS. Interaction between NO and COX pathways in retinal cells exposed to elevated glucose and retina of diabetic rats. *Am J Physiol Regul Integr Comp Physiol*. 2004;287:735–741.
11. Rosales MA, Silva KC, Lopes de Faria JB, Lopes de Faria JM. Exogenous SOD mimetic tempol ameliorates the early retinal changes reestablishing the redox status in diabetic hypertensive rats. *Invest Ophthalmol Vis Sci*. 2010;51:4327–4336.
12. Silva KC, Rosales MA, de Faria JB, de Faria JM. Reduction of inducible nitric oxide synthase via angiotensin receptor blocker prevents the oxidative retinal damage in diabetic hypertensive rats. *Curr Eye Res*. 2010;35:519–528.
13. Abu El-Asrar AM, Desmet S, Meersschaert A, Dralands L, Missotten L, Geboes K. Expression of the inducible isoform of nitric oxide synthase in the retinas of human subjects with diabetes mellitus. *Am J Ophthalmol*. 2001;132:551–556.
14. Hernández C, Lecube A, Segura RM, Sararols L, Simó R. Nitric oxide and vascular endothelial growth factor concentrations are increased but not related in vitreous fluid of patients with proliferative diabetic retinopathy. *Diabet Med*. 2002;19:655–660.
15. Liu Z, Rudd MA, Freedman JE, Loscalzo J. S-transnitrosation reactions are involved in the metabolic fate and biological actions of nitric oxide. *J Pharmacol Exp Ther*. 1998;284:526–534.
16. Stamler JS, Lamas S, Fang FC. Nitrosylation, the prototypic redox-based signaling mechanism. *Cell*. 2001;106:675–683.
17. Hogg N. The biochemistry and physiology of S-nitrosothiols. *Annu Rev Pharmacol Toxicol*. 2002;42:585–600.
18. Singh SP, Wishnok JS, Keshive M, Deen WM, Tannenbaum SR. The chemistry of the S-nitrosoglutathione/glutathione system. *Proc Natl Acad Sci USA*. 1996;93:14428–14433.
19. Zeng H, Spencer NY, Hogg N. Metabolism of S-nitrosoglutathione by endothelial cells. *Am J Physiol Heart Circ Physiol*. 2001;281:432–439.

20. Hedberg JJ, Griffiths WJ, Nilsson SJ, Hoog JO. Reduction of S-nitrosoglutathione by human alcoholdehydrogenase 3 is an irreversible reaction as analysed by electrospray mass spectrometry. *Eur J Biochem*. 2003;270:1249–1256.
21. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature*. 2001;410:490–494.
22. Khan M, Sekhon B, Giri S, et al. S-Nitrosoglutathione reduces inflammation and protects brain against focal cerebral ischemia in a rat model of experimental stroke. *J Cereb Blood Flow Metab*. 2005;25:177–192.
23. Haq E, Rohrer B, Nath N, Crosson CE, Singh I. S-nitrosoglutathione prevents interphotoreceptor retinoid-binding protein (IRBP(161-180))-induced experimental autoimmune uveitis. *J Ocul Pharmacol Ther*. 2007;23:221–231.
24. Holtkamp GM, Kijlstra A, Peek R, de Vos AF. Retinal pigment epithelium-immune system interactions: cytokine production and cytokine-induced changes. *Prog Retin Eye Res*. 2001;20:29–48.
25. Goureau O, Lepoivre M, Becquet F, Courtois Y. Differential regulation of inducible nitric oxide synthase by fibroblast growth factors and transforming growth factor b in bovine retinal pigmented epithelial cells: inverse correlation with cellular proliferation. *Proc Natl Acad Sci U S A*. 1993;90:4276–4280.
26. Liversidge J, Grabowski P, Ralston S, Benjamin N, Forrester JV. Rats retinal pigment epithelial cells express an inducible form of nitric oxide synthase and produce nitric oxide in response to inflammatory cytokines and activated T cells. *Immunology*. 1994;83:404–409.
27. Pinto CC, Silva KC, Biswas SK, Martins N, de Faria JB, de Faria JM. Arterial hypertension exacerbates oxidative stress in early diabetic retinopathy. *Free Radic Res*. 2007;41:1151–1158.
28. Silva KC, Pinto CC, Biswas SK, de Faria JB, de Faria JM. Hypertension increases retinal inflammation in experimental diabetes: a possible mechanism for aggravation of diabetic retinopathy by hypertension. *Curr Eye Res*. 2007;32:533–541.
29. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth*. 1983;65:55–63.
30. Bui BV, Armitage JA, Tolcos M, Cooper ME, Vingrys AJ. ACE inhibition salvages the visual loss caused by diabetes. *Diabetologia*. 2003;46:401–408.
31. Silva KC, Rosales MA, Hamassaki DE, et al. Green tea is neuroprotective in diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2013;54:1325–1336.
32. Garcia-Ramírez M, Villarroel M, Corraliza L, Hernández C, Simó R. Measuring permeability in human retinal epithelial cells (ARPE-19): implications for the study of diabetic retinopathy. *Methods Mol Biol*. 2011;763:179–194.
33. Faria AM, Papadimitriou A, Silva KC, Lopes de Faria JM, Lopes de Faria JB. Uncoupling endothelial nitric oxide synthase is ameliorated by green tea in experimental diabetes by re-establishing tetrahydrobiopterin levels. *Diabetes*. 2012;61:1838–1847.
34. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. *J Lab Clin Med*. 1963;16:882–888.
35. Yap, LP, Sancheti H, Ybanez MD, Garcia J, Cadenas E, Han D. Determination of GSH, GSSG, and GSNO using HPLC with electrochemical detection. *Methods Enzymol*. 2010;473:137–147.
36. Moore WM, Webber RK, Jerome GM, Tjoeng FS, Misko TP, Currie MG. L-N6-(1-iminoethyl) lysine: a selective inhibitor of inducible nitric oxide synthase. *J Med Chem*. 1994;37:3886–3888.
37. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med*. 2001;30:1191–1212.
38. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. *Biol Chem*. 2009;390:191–214.
39. Moellering D, McAndrew J, Patel RP, et al. Nitric oxide-dependent induction of glutathione synthesis through increased expression of gamma-glutamylcysteine synthetase. *Arch Biochem Biophys*. 1998;358:74–82.
40. Nikitovic D, Holmgren A. S-Nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J Biol Chem*. 1996;271:19180–19185.
41. Chen CA, Wang, TY, Varadharaj, S, et al. S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. *Nature*. 2010;468:1115–1118.
42. Rosenfeld RJ, Bonaventura J, Szymczyna BR, et al. Nitric-oxide synthase forms N-NO-pterin and S-NO-cys: implications for activity, allostery, and regulation. *J Biol Chem*. 2010;285:31581–31589.
43. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*. 2007;87:315–424.
44. Pacher P, Obrosova IG, Mabley JG, Szabo C. Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. *Curr Med Chem*. 2005;12:267–275.
45. Kowluru RA, Engerman RL, Kern TS. Abnormalities of retinal metabolism in diabetes or experimental galactosemia VIII. Prevention by aminoguanidine. *Curr Eye Res*. 2000;21:814–819.
46. do Carmo A, Lopes C, Santos M, Proença R, Cunha-Vaz J, Carvalho AP. Nitric oxide synthase activity and L-arginine metabolism in the retinas from streptozotocin induced diabetic rats. *Gen Pharmacol*. 1998;30:319–324.
47. Sennlaub F, Courtois Y, Goureau O. Inducible nitric oxide synthase mediates the change from retinal to vitreal neovascularization in ischemic retinopathy. *J Clin Invest*. 2001;107:717–725.
48. Sennlaub F, Courtois Y, Goureau O. Inducible nitric oxide synthase mediates the retinal apoptosis in ischemic proliferative retinopathy. *J Neurosci*. 2002;22:3987–3993.
49. Zheng L, Du Y, Miller C, et al. Critical role of inducible nitric oxide synthase in degeneration of retinal capillaries in mice with streptozotocin-induced diabetes. *Diabetologia*. 2007;50:1987–1996.
50. Schuhmacher S, Oelze M, Bollmann F, et al. Vascular dysfunction in experimental diabetes is improved by pentaerithrityltetranitrate but not isosorbide-5-mononitrate therapy. *Diabetes*. 2011;60:2608–2616.
51. Foster MW, Liu L, Zeng M, Hess DT, Stamler JS. A genetic analysis of nitrosative stress. *Biochemistry*. 2009;48:792–799.
52. Bateman RL, Rauh D, Tavshanjian B, Shokat KM. Human carbonyl reductase 1 is an S-nitrosoglutathione reductase. *J Biol Chem*. 2008;283:35756–35762.
53. Liu L, Yan Y, Zeng M, et al. Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell*. 2004;116:617–628.

Investigative Ophthalmology & Visual Science

Retinal Cell Biology

Endocytosis of Tight Junctions Caveolin Nitrosylation Dependent Is Improved by Cocoa Via Opioid Receptor on RPE Cells in Diabetic Conditions

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MABR and KCS contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Purpose. Retinal pigment epithelium cells, along with tight junction (TJ) proteins, constitute the outer blood retinal barrier (BRB). Contradictory findings suggest a role for the outer BRB in the pathogenesis of diabetic retinopathy (DR). The aim of this study was to investigate whether the mechanisms involved in these alterations are sensitive to nitrosative stress, and if cocoa or epicatechin (EC) protects from this damage under diabetic (DM) milieu conditions.

Methods. Cells of a human RPE line (ARPE-19) were exposed to high-glucose (HG) conditions for 24 hours in the presence or absence of cocoa powder containing 0.5% or 60.5% polyphenol (low-polyphenol cocoa [LPC] and high-polyphenol cocoa [HPC], respectively).

Results. Exposure to HG decreased claudin-1 and occludin TJ expressions and increased extracellular matrix accumulation (ECM), whereas levels of TNF-α and inducible nitric oxide synthase (iNOS) were upregulated, accompanied by increased nitric oxide levels. This nitrosative stress resulted in S-nitrosylation of caveolin-1 (CAV-1), which in turn increased CAV-1 traffic and its interactions with claudin-1 and occludin. This cascade was inhibited by treatment with HPC or EC through δ-opioid receptor (DOR) binding and stimulation, thereby decreasing TNF-α–induced iNOS upregulation and CAV-1 endocytosis. The TJ functions were restored, leading to prevention of paracellular permeability, restoration of resistance of the ARPE-19 monolayer, and decreased ECM accumulation.

Conclusions. The detrimental effects on TJs in ARPE-19 cells exposed to DM milieu occur through a CAV-1 S-nitrosylation–dependent endocytosis mechanism. High-polyphenol cocoa or EC exerts protective effects through DOR stimulation.

Keywords: RPE, high glucose, tight junction, nitrosative stress, cocoa

Diabetic retinopathy (DR) is the most serious complication of diabetic (DM) eye disease and is one of the most common causes of irreversible blindness worldwide.[1] The RPE is a monolayer of pigmented cells that separates the neural retina from a network of fenestrated vessels called the choriocapillaris, which serves as the major blood supply for the photoreceptors, and therefore the RPE constitutes the outer blood-retinal barrier (BRB). Impairment of the outer BRB is increasingly recognized to play an important role in the initiation and progression of early DR.[2,3] The outer BRB is responsible for transport of nutrients, ions, and water; absorption of light and protection against photo-oxidation; the visual cycle; phagocytosis of shed photoreceptor membranes; and secretion of essential factors for preservation of the structural integrity of the retina. It also contributes to the immune-privileged status of the eye.[4]

Apart from these functions, the RPE stabilizes the ion composition in the subretinal space, which is crucial for the maintenance of photoreceptor excitability. Any disturbance in function of these cells necessarily has detrimental consequences for the retina.[2] Defects in RPE function are well documented to underlie a number of sight-threatening conditions, such as AMD,[5] proliferative vitreoretinopathy,[6] and DR.[2,3,7,8] The functioning of the RPE layer, measured by c-waves in ERGs, is substantially reduced in experimental DM models.[9,10] However, direct data for the effects of high glucose (HG) or hyperglycemia on the tight junction (TJ) integrity and transport functions at the outer BRB are not completely understood. Tight junctions expressed in the outer BRB control fluid and solute entry into the retina, and this sealing function, which is essential to retinal homeostasis, is impaired in DR.[11] Our previous work showed that ARPE-19 cells exposed to HG displayed a decrease in claudin-1 expression,[12] but the mechanisms were not addressed.

Tumor necrosis factor-α (TNF-α) was shown to induce a focal intrajunctional concentration of occludin followed by caveolin-1(CAV-1)–dependent endocytosis in the intestinal epithelial cells.[13] Caveolin-1, the main scaffolding protein of caveolae, consists of a lipophilic, hairpin-shaped, helical sequence embedded in the inner leaflet of the plasma membrane, together with both N- and C-terminal cytoplasmic domains. The N-terminus binds to signaling molecules that are

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required for CAV multimerization. Caveolae have been implicated in endocytosis, transcytosis, calcium signaling, and numerous other signal transduction events. An understanding of CAV trafficking and caveola formation is therefore crucial to understanding the possible roles of CAV and caveolae.[14]

Tight junctions are relatively cholesterol-rich,[15] and the cholesterol-binding protein, CAV-1, was identified as a component of TJ membrane microdomains more than a decade ago by Nusrat and coworkers.[16] Many studies have provided compelling evidence that CAV-1 is involved in regulating endothelial permeability.[17] Cavelon-1 can be precipitated and it binds independently to claudin-2 and occludin in MDCK II cells, suggesting a potential mechanism for selective retrieval of TJ components.[18] Thus, CAV-1 might have a more general role in regulating cell junctions, but its molecular regulation of epithelial cell adhesion and barrier function needs to be defined.

The RPE contributes to the immune-privileged status of the eye as part of the blood-eye barrier and by the secretion of immunosuppressive/inflammatory factors inside the eye.[19] Rat RPE cells express inducible nitric oxide synthase (iNOS) and produce nitric oxide (NO) in response to inflammatory cytokines and activated T cells.[20,21] In addition, 48 hours of HG exposure causes an increase in iNOS expression in ARPE-19 cells.[22] Recently, activation of opioid receptors, particularly the δ-opioid receptor (DOR), was demonstrated to block proinflammatory cytokines, such as TNF-α in the retina under ischemia/reperfusion conditions.[23]

Epicatechin (EC), the predominant flavonoid present in dark chocolate, is a well-known antioxidant.[24] Structure-activity relationships of flavonoids with opioid receptor ligands show binding activity in vitro.[25,26] The purpose of this study was to evaluate the mechanism by which TJs are decreased in ARPE-19 cells under HG conditions and to determine whether cocoa powder, through its EC content, could prevent this effect. Our data revealed that HG promotes an increase in TNF-α levels and iNOS expression, accompanied by an increased production of NO and a resulting nitrosative imbalance. Consequently, CAV-1 is S-nitrosylated, thereby modulating the claudin-1/occludin CAV-1 interactions and CAV-1 traffic. Epicatechin, through its opioid receptor binding capacity, activates DOR and decreases TNF-α–induced CAV-1 endocytosis. As a result, the claudin-1 and occludin levels are restored in the TJs, leading to reestablishment of paracellular permeability and restoration of the resistance of ARPE-19 monolayers.

Materials and Methods

Characteristics of Low- and High-Polyphenol Content Cocoa Powder

Cocoa powders with different amounts of polyphenol were provided by Barry Callebau. The composition of cocoa was the same in both preparations, with the only difference being in the amounts of polyphenol: 0.5% for low-polyphenol cocoa (LPC) and 60.5% for high-polyphenol cocoa (HPC). The quantitative analysis of HPC composition is shown in Supplementary Figure S1.

The ARPE-19 Cell Line Culture

The human RPE cell line (ARPE-19) was obtained from the Federal University of Rio de Janeiro (RJCB Collection) at passage 28. Cell cultures at 70% to 90% confluence were serum starved with a fetal bovine serum (FBS) concentration of 1%, and then were exposed to normal glucose (5.5 mM = NG[27]) or HG (30 mM = HG); or HG plus 100 ng/mL LPC (HG+LPC) or HPC (HG+HPC) for 24 hours in the presence or absence of the NOS nonselective inhibitor L-NAME and the iNOS-specific inhibitor aminoguanidine (AG) (2 μM–2 mM), EC (0.38 nM–380 nM), naltrindole (Nalt) (10 nM–100 μM) (Sigma-Aldrich, St. Louis, MO, USA); S-nitrosoglutathione (GSNO) (10 nM–10μM) (synthesized at the Chemistry Institute, State University of Campinas [UNICAMP] as previously described[28]), and TNF-α (10-100ng/ml) (Calbiochem-Novabiochem, La Jolla, CA, USA). The potential interference of glucose osmolarity in ARPE-19 cells was checked by treating cells with 30 mM mannitol for 24 hours. The cytotoxicity of treatments on ARPE-19 cells was determined by a thiazolyl blue tetrazolium bromide (MTT) colorimetric assay.[29] Concentrations that caused less than 10% cell toxicity were chosen for the experimental treatments (Supplementary Fig. S2).

Immunofluorescence Assays

The immunofluorescence assays in ARPE-19 cells were performed as previously published.[30] The cover glasses with fixed cells were incubated with the appropriate primary antibodies: anti-claudin-1, occludin, ZO-1 (Zymed Lab Gibco; Invitrogen, Carlsbad, CA, USA); FN (Calbiochem-Novabiochem); Col-IV (Southern Biotech, Birmingham, AL, USA), CAV-1 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and Cellular retinaldehyde-binding protein (CRALBP; Thermo Scientific, Waltham, MA, USA) overnight at 4°C, and the appropriate secondary antibodies were applied for 1 hour at room temperature.

Western Blotting Analysis

Western blotting was performed as previously described.[12] Membranes were incubated overnight at 4°C with the appropriate primary antibodies: anti-claudin-1, occludin-1, ZO-1 (Zymed Lab Gibco; Invitrogen); FN (Calbiochem-Novabiochem); Col-IV (Southern Biotech) and iNOS (Cell Signaling Technology, USA) and the appropriate secondary antibodies were applied for 1 hour at room temperature. Equal loading and transfer were ascertained by reprobing the membranes for ß-actin. Exposed films were scanned with a densitometer (Bio-Rad, Hercules, CA, USA) and analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems (Hercules, CA, USA). The arbitrary unit of densitometry was transformed to fold increment.

Measurement of Permeability to Dextran and Paracellular Epithelial Electrical Resistance

The integrity of TJs in cell culture is generally measured using transepithelial electrical resistance (TER) and/or paracellular tracer flux,[30] as previously described with some modifications. The ARPE-19 cells were placed on a Transwell-Clear Polyester Membrane Insert (HTS, Costar; Corning, Inc., Corning, NY, USA). At day 5, a monolayer structure was observed and the complete medium was replaced by a medium with the treatments. The permeability of the RPE cells was determined by measuring the apical-to-basolateral movements of FITC dextran (40 kDa; 100 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA). Sample fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm with a microplate fluorescence reader (SynergyMx; Biotek, Winooski, VT, USA). For dextran permeability, at least three samples of each condition in four independent experiments were considered for area under the curve (AUC) analysis at 30, 60, 120, and 240 minutes. The AUC values were calculated by adding the areas under the graph between each pair of consecutive observations, as follows: $(T_2 - T_1)(Y_1 + Y_2)/2$.[31] To compare independent experiments, AUC values were calculated and expressed by fold increment.

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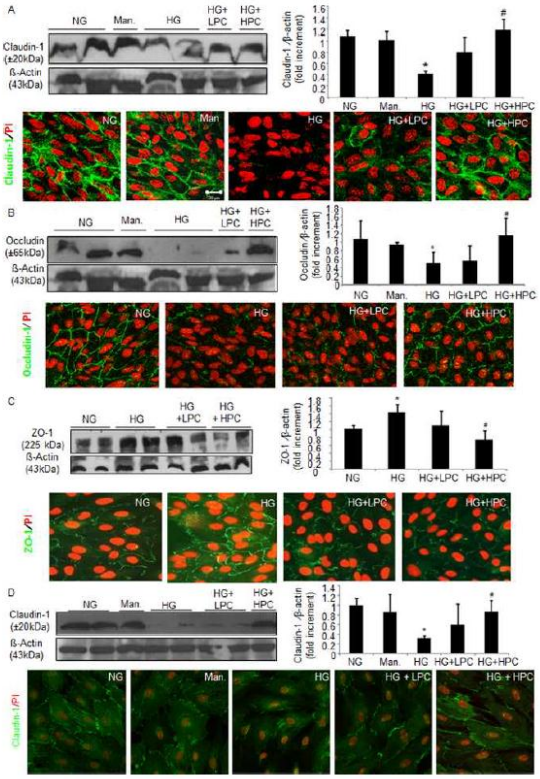

Figure 1. Expression of claudin-1, occludin, and ZO-1 TJ proteins in ARPE-19 and primary pRPE cells under the HG condition and the effects of LPC and HPC treatments. The expression of TJs was evaluated after 24 hours in NG, HG, HG+HPC, or LPC (100 ng/mL) conditions. Mannitol (Man.) was used as an osmotic control. (**A**, **B**, **C**) Western blot for claudin-1, occludin, and ZO-1 expressions, respectively, in total ARPE-19 cell lysates. Equal loading and transfer were confirmed by reprobing the membranes for ß-actin. The arbitrary unit of densitometry was transformed to a fold increment. The *bars* represent mean ± SD. $^{*}P \leq 0.03$ versus NG; $^{\#}P \leq 0.03$ versus HG. Confocal immunofluorescence images showing claudin-1, occludin, and ZO-1 immunolocalization. The marked TJs are shown in *green* (located on cell membrane) and the nucleus is indicated in *red* in the confocal microscopic field (magnification ×630). At least three independent experiments were performed for each assay. (**D**) Western blot and immunofluorescence for claudin-1 in pRPE cells. Equal loading and transfer were confirmed by reprobing the membranes for ß-actin.

High-Polyphenol Cocoa Preserved the Integrity of TJs of ARPE-19 Cells Exposed to Diabetic (DM) Milieu Conditions

We investigated the integrity of the monolayer of ARPE-19 cells by evaluating the expression of the TJ proteins claudin-1, occludin, and ZO-1. The ARPE-19 cells exposed to HG conditions for 24 hours showed a decrease in the expression of claudin-1 and occludin proteins when compared with the NG control ($P \leq 0.03$). Immunofluorescence assays revealed less staining of these TJ proteins on the cell membrane under HG conditions. This decrease was prevented by HPC ($P \leq 0.03$) but not by LPC ($P > 0.2$). Immunofluorescence measurements for cells exposed to mannitol, used as an osmotic control, were not significantly different from the NG values ($P = 0.7$) (Figs. 1A, 1B).

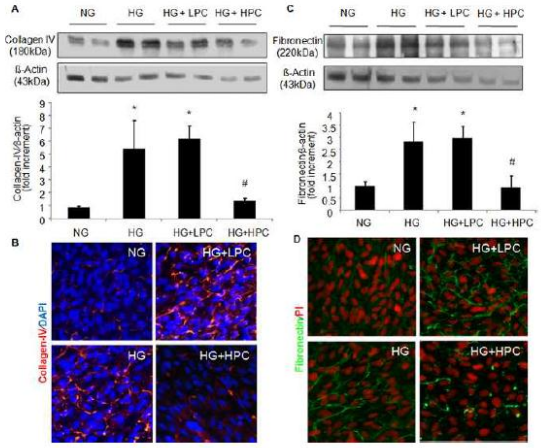

Figure 2. High-polyphenol cocoa prevents the ECM accumulation in ARPE-19 cells under HG conditions. The expression of ECM materials was evaluated after treating with NG, HG, HG+HPC, or LPC (100 ng/mL) for 24 hours. (**A**, **C**) Western blot for fibronectin and collagen-IV expression, respectively, in total cell lysates. Equal loading and transfer were ascertained by reprobing the membranes for ß-actin. The arbitrary unit of densitometry was transformed to fold increments in relation to the NG treatment in each experiment to compare independent experiments. The *bars* represent mean ± SD. $^{*}P \leq 0.02$ versus NG; $^{\#}P \leq 0.05$ versus HG. (**B**, **D**) Immunofluorescence images showing collagen-IV and fibronectin expression and localization. Collagen-IV is marked in *red* and the nuclei by 4′,6-diamidino-2-phenylindole (DAPI), and fibronectin is marked in *red green* and the nuclei with propidium iodide (PI). Both are localized on the membrane under a microscopic field (magnification ×630). At least three independent experiments were performed for each assay.

In contrast, ZO-1 expression was increased in response to HG, compared with NG conditions ($P = 0.03$); the treatment with HPC significantly prevented this increase compared with HG ($P = 0.001$) (Fig. 1C). The LPC treatment showed a tendency toward a decrease compared with HG, but the difference did not reach statistical significance ($P = 0.09$).

In addition, the expression of claudin-1 in pRPE cells (Fig. 1D) showed significant decreases compared with NG medium ($P = 0.01$) and in the presence of HPC, this alteration was prevented ($P = 0.03$ versus HG).

High-Polyphenol Cocoa Prevented Extracellular Matrix Accumulation in Cells Exposed to HG Conditions

The RPE cells play a crucial role in the survival of photoreceptors, the choriocapillaris, and the choroid through the release of growth factors and production of extracellular matrix (ECM).[4,38] The changes and disorganization of TJ proteins observed under HG conditions means that the ARPE-19 monolayer barrier must depend on ECM accumulation. After 24 hours under HG, the cells showed upregulated production of collagen IV and fibronectin proteins when compared with NG conditions ($P \leq 0.02$), and the treatment with HPC prevented this alteration ($P \leq 0.05$) (Fig. 2). Thus, the ARPE-19 cells exposed to HG for 24 hours showed a profound disturbance of TJ protein expression accompanied by accumulation of ECM proteins, but HPC prevented these abnormalities. Therefore, we assessed the functional features (permeability and resistance) of this monolayer barrier.

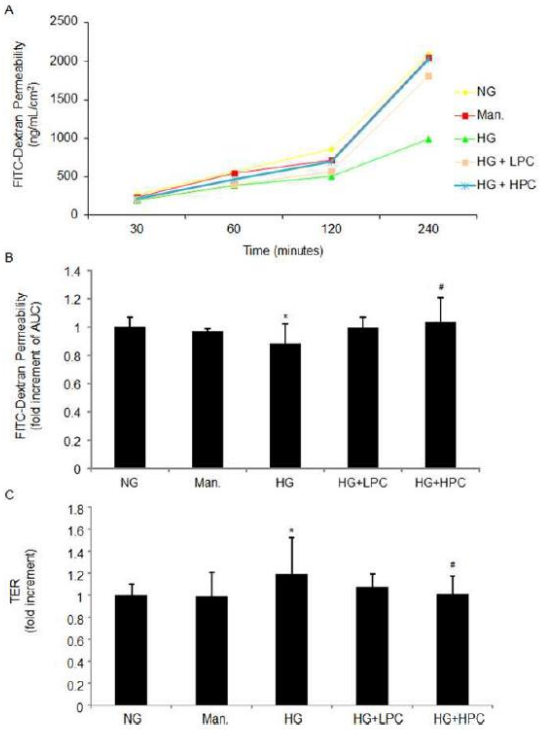

Figure 3. High-polyphenol cocoa protection against RPE dysfunction in ARPE-19 cells under HG conditions. The permeability and TER of the RPE cells were determined after 24 hours in NG, Man., HG, HG+HPC, or LPC (100 µg/mL) conditions. Mannitol was used as an osmotic control. **(A)** Permeability was measured by the apical-to-basolateral movements of FITC dextran (40 kDa). Samples (200 µL) were collected from the basolateral side at 30, 60, 120, and 240 minutes after adding the molecules. **(B)** The *bars* represent fold increment (mean ± SD) of AUC of ARPE-19 cells' monolayer permeability at each condition. $^{*}P = 0.05$ versus NG; $^{\#}P = 0.02$ versus HG. **(C)** Transepithelial electrical resistance measurements expressed in fold increment of resistance values (mean ± SD). $^{*}P = 0.008$ versus NG; $^{\#}P = 0.01$ versus HG.

High-Polyphenol Cocoa Protected the Barrier Features of the ARPE-19 Cell Monolayer Under HG Conditions

We investigated whether the observed changes could influence the functional features of the ARPE-19 monolayer barrier by measuring its paracellular permeability (by assessing apical-basolateral movements of FITC-dextran) and its TER. The cells grown under HG conditions showed significantly lower dextran diffusion accompanied by higher TER, when compared with the NG condition ($P = 0.05$ and $P = 0.008$, respectively). Similar to the previous results, only HPC significantly protected the normal barrier function ($P = 0.02$ and $P = 0.01$ for dextran permeability and TER, respectively) (Fig. 3). The ARPE-19 cells showed no differences from the NG control when grown in mannitol ($P = 0.7$).

One possible mechanism by which the TJ claudin-1 and occludin expressions are decreased in the HG condition could involve a CAV-1 endocytosis phenomenon. In vitro studies have reported occludin endocytosis via macropinocytosis, clathrin-coated pits, and caveolae.[39–41] We tested the potential involvement of endocytosis by examining the formation of

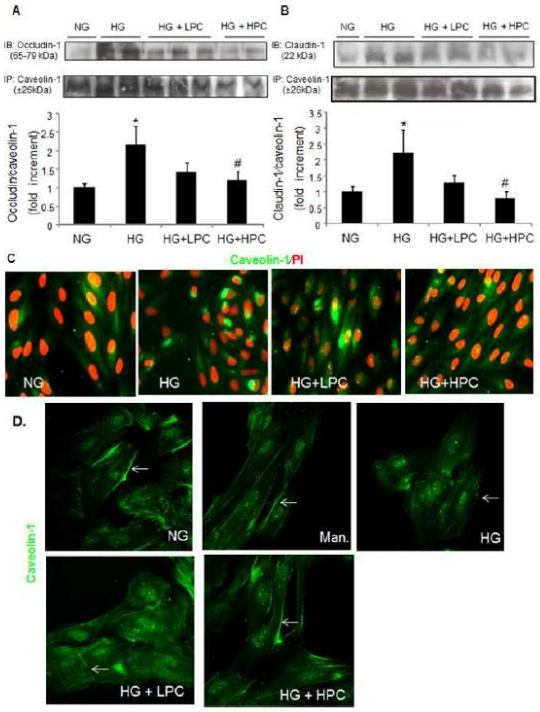

Figure 4. High-polyphenol cocoa prevented CAV-1/claudin-1 and occludin complexes and CAV-1 internalization. The expressions of CAV-1/claudin or occludin complexes and CAV-1 were evaluated after a 24-hour treatment with NG, HG, HG+HPC, or HG+LPC (100 ng/mL). **(A, B)** Immunoprecipitation of cell lysate with CAV-1 antibody incubated with claudin-1 or occludin antibodies, respectively. The CAV-1/claudin or occludin complex expressions were measured by Western blotting. Equal loading and transfer were ascertained by reprobing the membranes for CAV-1.The arbitrary unit of densitometry was transformed to fold increments in relation to the NG data in each experiment to compare independent experiments. The *bars* represent mean ± SD in both experiments. $^{*}P \leq 0.04$ versus NG; $^{\#}P \leq 0.04$ versus HG. **(C)** Immunofluorescence images showing CAV-1 expression and localization. Caveolin-1 was marked in *green* and the nuclei with PI under the microscopic field (magnification ×630). At least three independent experiments were performed for each assay. **(D)** Immunofluorescence images showing CAV-1 expression and localization in pRPE cells marked in *green* (magnification ×630).

CAV-1/claudin-1 and CAV-1/occludin complexes by immunocytochemistry.

High-Polyphenol Cocoa Prevented the Formation of CAV-1/Claudin-1 and CAV-1/Occludin Complexes in ARPE-19 Cells Under HG Conditions

In the HG condition, a clear augmentation of CAV-1/claudin-1 and CAV-1/occludin binding occurred when compared with the NG condition ($P \leq 0.04$); the presence of HPC suppressed these interactions when compared with the HG condition ($P \leq 0.04$) (Figs. 4A, 4B). Immunolocalization of the CAV-1 protein on the ARPE-19 monolayer revealed a uniform distribution of CAV-1 in all parts of the cell, membrane, cytoplasm, and nucleus in the NG condition. Exposure of the cells to HG caused a massive internalization of CAV-1, which formed roundish structures in the cytoplasm compatible with caveo-

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somes. These structures are associated with endocytosis. The LPC seemed to attenuate this process, and HPC clearly prevented this endocytosis (Fig. 4C). In agreement with this finding, pRPE cells also displayed a similar translocation of CAV-1 from the membrane to the nucleus and HPC treatment prevented this change (Fig. 4D).

Collectively, these results suggest that the HG condition promotes a decrease in expression of TJ claudin-1 and occludin in ARPE-19 cells that is associated with caveosome formation. The protective effect of cocoa powder was at least partly dependent on the amounts of polyphenols present in the cocoa treatment.

Epicatechin, the Main Polyphenol in Cocoa, Counteracts Nitrosative Stress and Prevents CAV-1 S-Nitrosylation

Tumor necrosis factor-α has been shown to stimulate occludin endocytosis via caveolin in intestinal epithelial cells,[42] but the mechanism by which it increases internalization and trafficking of CAV-1 is unclear. Post-translational modifications of CAV-1, such as ubiquitination[43] and phosphorylation[33] at the N-terminal near the scaffolding domain, are involved in the increased trafficking. Tumor necrosis factor-α is also known to increase NO production by regulating iNOS; therefore, we examined the direct modification of the assembly and mobility of CAV-1 by S-nitrosylation as a potential mechanism for regulation of vesicular trafficking in ARPE-19 cells.

We first evaluated the levels of oxidative and nitrosative stress in our system. Cells exposed to HG showed an increase in total ROS production compared with the NG condition ($P \leq 0.0001$). This effect was suppressed by LPC treatment ($P = 0.01$) and was strongly suppressed by HPC treatment when compared with HG ($P \leq 0.0001$) or LPC conditions ($P = 0.01$). We tested the effect of EC, the most abundant polyphenol present in cocoa, using the corresponding percentages found in the LPC and HPC powders (0.15% and 12%, respectively). Only the higher EC concentration was effective in suppressing the HG-induced increase in ROS production ($P = 0.0005$) (Fig. 5A). This demonstrates that the high polyphenol levels present in the HPC counteracted the increase in ROS production in ARPE-19 cells exposed to HG.

The NO pathways potentially involved in ROS production in HG conditions were then investigated by the colorimetric method for detection of intracellular ROS, H_2DCFDA. The presence of HPC or the unspecific NOS blocker, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), prevented the increase in ROS levels seen with HG ($P \leq 0.02$). Concomitant treatments of L-NAME+HPC or L-NAME+EC under HG conditions showed no additive effects. The significant difference observed between HG+L-NAME+HPC and HG+L-NAME+EC ($P = 0.05$) suggests that other cocoa compounds might exert a protective effect through different pathways (Fig. 5B).

We then evaluated the production of intracellular NO by the DAF-2DA method and the specific effect of EC on this production. We observed an increase in NO production in cells exposed to HG compared with the NG condition ($P = 0.008$). Treatment with HPC or the corresponding EC percentage prevented this increase ($P \leq 0.005$) (Fig. 5C), suggesting a specific effect of EC on the NO system. We also confirmed the source of NO by assessing iNOS expression. As expected, iNOS was upregulated under HG conditions ($P = 0.003$), and this upregulation was abolished in the presence of HPC ($P = 0.007$) (Fig. 5D).

The presence of nitrosative stress induced by iNOS in our system prompted us to investigate whether CAV-1 was nitrosylated by the excessive amounts of NO. The nitrosylation

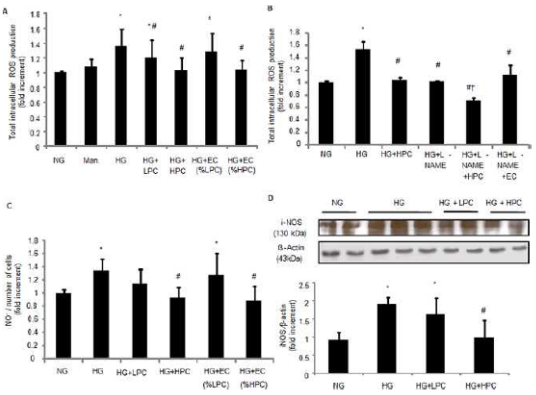

Figure 5. Epicatechin, the main phenolic compound in HPC, counteracts nitrosative stress. Measurements of intracellular ROS and NO production after treatment with NG, HG, HG+HPC, or LPC (100 ng/mL) for 24 hours; the EC content in the HG+EC treatment corresponds to the percentage of EC found in the LPC and HPC (0.25% and12%, respectively). (**A**, **B**) Total intracellular ROS production determined by the H_2DCFDA fluorescence method. (**A**) The effects of EC amounts on ROS production. *Bars* represent the mean ± SD of fluorescence units obtained with an ELISA reader and corrected by the number of cells at the end of each treatment. Mannitol was used as an osmotic control. $^{*}P \leq 0.0001$ versus NG; $^{\#}P \leq 0.01$ versus HG group. (**B**) The NOS nonselective inhibitor L-NAME (2 mM) was used to test the role of EC in downregulating ROS production via the NO system. *Bars* represent the mean ± SD of fluorescence units obtained with an ELISA reader and corrected by the number of cells at the end of each treatment. $^{*}P \leq 0.03$ versus NG; $^{\#}P \leq 0.02$ versus HG; $^{\dagger}P = 0.05$ versus HG+L-NAME+EC. (**C**) Intracellular NO production measured by the DAF-2DA method. *Bars* represent the mean ± SD of fluorescence units obtained in ELISA reader and corrected by the number of cells at the end of each treatment. $^{*}P = 0.008$ versus NG; $^{\#}P \leq 0.005$ versus HG. (**D**) Western blot of iNOS expression in total cell lysates. Equal loading and transfer were confirmed by reprobing the membranes for ß-actin. The arbitrary unit of densitometry was transformed to fold increments in relation to the NG treatment in each experiment to compare independent experiments. The *bars* represent mean ± SD. $^{*}P = 0.003$ versus NG; $^{\#}P = 0.007$ versus HG. At least three independent experiments were performed for each assay.

of CAV-1 was assessed by immunoprecipitation experiments and analyzed by spectrofluorometry with the DAN method and Western blots (Figs. 6A, 6B). Figure 6A shows the NO release from S-nitrosylated CAV-1 detected by DAN. Cells incubated with an NO donor (NG+GSNO) as a positive control or in HG showed a clear induction of S-nitrosylation of the CAV-1 molecule ($P < 0.0001$). Treatment with HPC, EC, or the iNOS inhibitor AG equally prevented this process ($P \leq 0.002$). The combination of AG and HPC showed an additional effect compared with HPC alone ($P = 0.05$), suggesting that other cocoa compounds might act in this pathway. However, the HG+AG+EC treatment did not show any additional effects compared with HG+EC ($P = 0.6$), indicating that the main action of EC is in the prevention of CAV-1 S-nitrosylation via iNOS downregulation (Fig. 6A).

In line with the fluorometric assays, HG treatment caused a significantly increased expression of S-nitrosylated CAV-1 compared with the NG condition ($P < 0.0001$). The HPC and EC were equally effective at suppressing this expression when compared with HG ($P = 0.5$). The combined HG+AG+HPC treatment again indicated an additional effect when compared with HG+HPC ($P = 0.04$), and no difference was noted between the HG+EC and HG+AG+EC treatments ($P = 0.2$) (Fig. 6B).

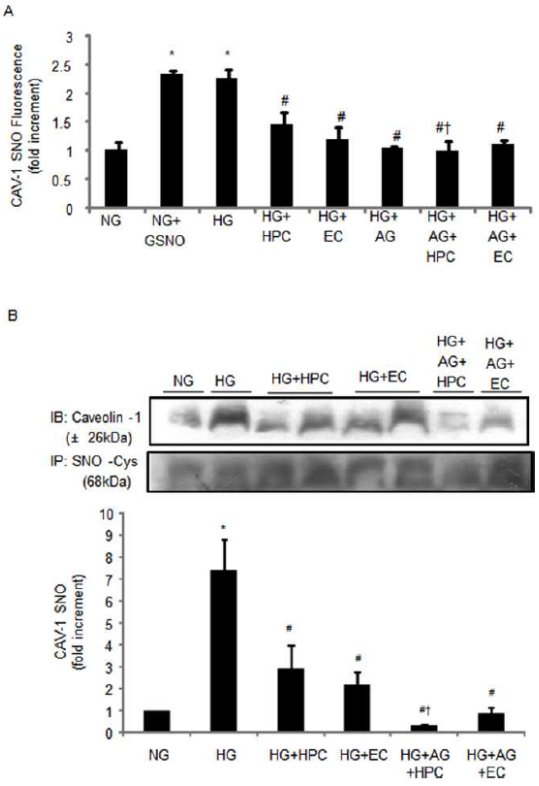

Figure 6. Epicatechin counteracts nitrosative stress and prevents S-nitrosylation of CAV-1. Measurement of CAV-1 S-nitrosylation after treatment with NG, HG, HG+HPC (100 ng/mL), or HG+EC (12 ng/mL) for 24 hours. The specific iNOS blocker aminoguanidine (AG) was used at a concentration of 2 mM to assess the protective effects of EC via downregulation of iNOS. (**A**) Fluorometric measurement of CAV-1 S-nitrosylation using the diaminonaphthalene (DAN) assay. S-nitrosoglutathione (1 μM) was used as a positive control for the induction of CAV-1 S-nitrosylation. The fluorescence units obtained via ELISA reader were transformed to fold increments in relation to the media of NG in each experiment to compare independent experiments. *Bars* represent the mean ± SD. $^{*}P < 0.0001$ versus NG conditions; $^{\#}P \leq 0.002$ versus HG treatment; $^{\dagger}P = 0.05$ versus HG+HPC. (**B**) Expression of S-nitrosylated CAV-1. Western blot of cell lysate immune precipitated with S-Nitroso-Cysteine (SNO-Cys) antibody and immunoblotted for anti-CAV-1. Equal loading and transfer were ascertained by reprobing the membranes for SNO-Cys antibody. The arbitrary unit of densitometry was transformed to fold increments in relation to the NG treatment in each experiment to compare independent experiments. The *bars* represent mean ± SD in both experiments. $^{*}P < 0.0001$ versus NG; $^{\#}P \leq 0.002$ versus HG; $^{\dagger}P = 0.04$ versus HG+HPC. At least three independent experiments were performed for each assay.

We next investigated whether the nitrosylation of the CAV-1 molecule interferes in the interaction between CAV-1 and the TJs and the CAV-1 internalization by studying the endocytosis of CAV-1.

Epicatechin Prevents CAV-1 Endocytosis and This Effect is Dependent on TNF-α–iNOS Upregulation Via the δ-Opioid Receptor (DOR)

We measured the TNF-α levels in the supernatant of these cells and observed an increase in cells exposed to HG compared with NG ($P = 0.002$), and both HPC and EC were equally effective at preventing this increase ($P \leq 0.03$) (Fig. 7A). Activation of opioid receptors is known to reduce TNF-α production in the retina model of ischemic/reperfusion[23]; therefore, we used the DOR blocker naltrindole (Nalt) to test whether opioid receptors were involved in the HG effects on ARPE-19 cells. The addition of Nalt abolished the effects of HPC or EC compared with controls ($P \leq 0.0008$) (Fig. 7A), indicating that EC modulates TNF-α via DOR receptor.

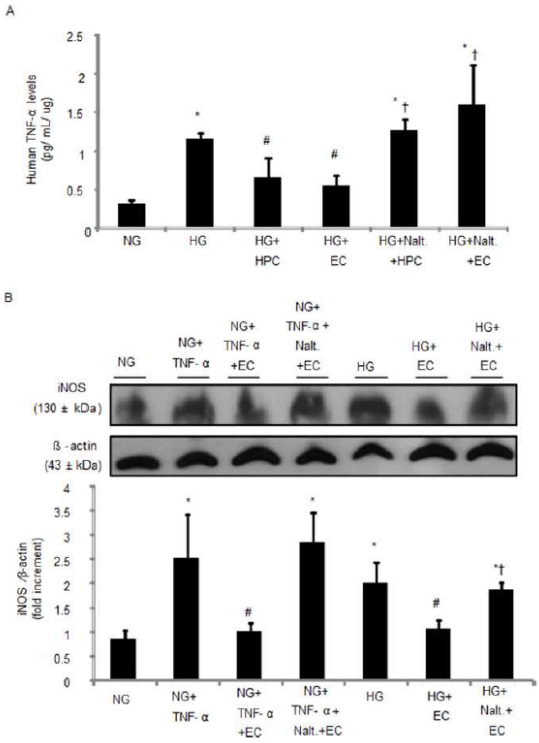

Figure 7. Epicatechin prevents TNFα-iNOS upregulation through DOR. (**A**) Measurement of TNF-α levels in the supernatant of ARPE-19 cells by ELISA. We used a specific DOR blocker, naltrindole (Nalt), at a concentration of 10 μM to investigate whether the effects of EC are dependent on the DOR receptor. The absorbance values were corrected for protein concentration and expressed as pg/mL/μg. $^{*}P = 0.002$ versus NG; $^{\#}P \leq 0.03$ versus HG; $^{\dagger}P \leq 0.0008$ versus HG+HPC and HG+EC, respectively. (**B**) Western blot for iNOS expression in total cell lysates. Tumor necrosis factor-α, at a concentration of 40 ng/mL, was used to induce iNOS expression. Equal loading and transfer were confirmed by reprobing the membranes for ß-actin. The arbitrary units of densitometry were transformed to fold increments in relation to the NG treatment in each experiment to compare independent experiments. The *bars* represent mean ± SD. $^{*}P \leq 0.01$ versus NG; $^{\#}P \leq 0.02$ versus; $^{\dagger}P = 0.04$ versus HG+EC. At least three independent experiments were performed for each assay.

We then assessed whether TNF-α could induce iNOS upregulation in the ARPE-19 cells and if the DOR was involved in this modulation. As expected, TNF-α treatment increased iNOS expression compared with the NG condition ($P = 0.0006$) and treatment with EC prevented this stimulation ($P = 0.001$). The addition of Nalt abrogated this effect when compared with the NG+TNF-α+EC treatment ($P = 0.0002$). As expected, ARPE-19 cells under HG conditions showed increased iNOS expression ($P = 0.008$ versus NG conditions),

sponse to the proinflammatory mediator thrombin. This weakens the association of catenin with VE-cadherin and the junction-associated actin filaments are lost, thereby compromising the barrier function.[57]

Abnormalities in gene expression of CAV-1 have been linked to DR,[58] but the possible involvement of the CAV-1/caveolae in the outer retina needs to be better understood. The caveolae of RPE cells have a unique bipolar distribution,[59] but their functions in either the apical or basolateral RPE membrane domains have not been elucidated. Previous work showed that ablation of CAV-1 resulted in reduced inner and outer retinal functions, whereas *Cav-1* knockout retinas also displayed unusually tight adhesion with the RPE, suggesting alterations in outer retinal fluid homeostasis. These findings demonstrate that the reduced retinal function resulting from CAV-1 ablation involves impairment of subretinal and/or RPE ion/fluid homeostasis.[60] Posttranslational modifications of CAV-1, such as ubiquitination[61] and phosphorylation[33] of the N-terminal near the scaffolding domain, resulted in exacerbated trafficking. In line with our present data, these authors demonstrated that CAV-1 SNO is an important regulatory mechanism controlling caveolar trafficking in endothelial cells.

The opioid receptor family comprises three members, the μ-, δ-, and k-opioid receptors, which respond to classical opioid alkaloids, such as morphine and heroin, as well as to endogenous peptide ligands like endorphins. They belong to the G-protein-coupled receptor superfamily, and are recognized as excellent therapeutic targets for pain control.[62] Activation of one or more opioid receptors by morphine can reduce ischemic/reperfusion injury by the suppression of TNF-α production in the retina. Naloxone, an opioid antagonist, can reverse the morphine-induced suppression of TNF-α production in vitro.[23] Epicatechin, the predominant flavonoid component present in cocoa and dark chocolate, is a well-known antioxidant associated with a lower risk of stroke and heart failure.[63–65] Moreover, EC-induced cardiac protection has shown a dependence on DOR stimulation.[26] In the present study, we demonstrated that the increase in TNF-α levels in ARPE-19 cells exposed to HG is abolished when the cells were treated with HPC or the corresponding amount of EC, which effectively neutralized the HG effect; this action was abrogated in the presence of Nalt, a DOR blocker. This set of experiments clearly demonstrated that EC protects the ARPE-19 monolayer barrier/permeability through stimulation of DOR, thereby modulating TNF-α action. The crystal structure of the mouse DOR, bound to the subtype-selective antagonist Nalt,[66] has indicated that blocking the DOR with oral administration of Nalt resulted in a decrease of the cardiac protective effect of EC on mitochondrial structure in mice.[67]

In conclusion, we identified EC as a negative regulator of the CAV-1 nitrosylation that occurs in retinal pigmented epithelium cells under DM milieu conditions due to activation of DOR. Caveolin-1 plays an important role in major diseases, such as cancer,[68] atherosclerosis,[69] DM complications,[70] and inflammation[71]; therefore, our findings might provide insights into the regulation of claudin-1 and occludin by CAV-1 internalization in RPE cells exposed to HG conditions, as well as in other pathological conditions.

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References

1. Frank RN. Diabetic retinopathy. *N Engl J Med*. 2004;350:48–58.
2. Simo R, Villarroel M, Corraliza L, Hernandez C, Garcia-Ramirez M. The retinal pigment epithelium: something more than a constituent of the blood retinal barrier—implications for the pathogenesis of diabetic retinopathy. *J Biomed Biotechnol*. 2010;2010:190724.
3. Xu HZ, Le YZ. Significance of outer blood-retina barrier breakdown in diabetes and ischemia. *Invest Ophthalmol Vis Sci*. 2011;52:2160–2164.
4. Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev*. 2005;85:845–881.
5. Eagle RC Jr. Mechanisms of maculopathy. *Ophthalmology*. 1984;91:613–625.
6. Dorey CK, Wu G, Ebenstein D, Garsd A, Weiter JJ. Cell loss in the aging retina: relationship to lipofuscin accumulation and macular degeneration. *Invest Ophthalmol Vis Sci*. 1989;30:1691–1699.
7. Vinores SA, Gadegbeku C, Campochiaro PA, Green WR. Immunohistochemical localization of blood-retinal barrier breakdown in human diabetics. *Am J Pathol*. 1989;134:231–235.
8. Hewitt AT, Adler R. *Retina: Basic Science and Inherited Retinal Disease*. 1st ed. St. Louis: Mosby; 1989;58–71.
9. Pautler EL, Ennis SR. The effect of induced diabetes on the electroretinogram components of the pigmented rat. *Invest Ophthalmol Vis Sci*. 1980;19:702–705.
10. Samuels IS, Lee CA, Petrash JM, Peachey NS, Kern TS. Exclusion of aldose reductase as a mediator of ERG deficits in a mouse model of diabetic eye disease. *Vis Neurosci*. 2012;29:267–274.
11. Villarroel M, García-Ramírez M, Corraliza L, Hernández C, Simó R. High glucose concentration leads to differential expression of tight junction proteins in human retinal pigment epithelial cells. *Endocrinol Nutr*. 2009;56:53–58.
12. Silva KC, Rosales MA, Hamassaki DE, et al. Green tea is neuroprotective in diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2013;54:1325–1336.
13. Marchiando AM, Shen L, Graham WV, et al. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. *J Cell Biol*. 2010;189:111–126.
14. Parton RG, Simons K. The multiple faces of caveolae. *Nat Rev Mol Cell Biol*. 2007;8:185–194.
15. Feltkamp, CA, Van der Waerden AW. Junction formation between cultured normal rat hepatocytes. An ultrastructural study on the presence of cholesterol and the structure of developing tight-junction strands. *J Cell Sci*. 1983;63:271–286.
16. Nusrat A, Parkos CA, Verkade P, et al. Tight junctions are membrane microdomains. *J Cell Sci*. 2000;113:1771–1781.
17. Schubert W, Frank PG, Razani B, Park DS, Chow CW, Lisanti MP. Caveolae-deficient endothelial cells show defects in the uptake and transport of albumin in vivo. *J Biol Chem*. 2001;276:48619–48622.
18. Itallie CM, Anderson JM. Caveolin binds independently to claudin-2 and occludin. *Ann N Y Acad Sci*. 2012;1257:103–107.
19. Holtkamp GM, Kijlstra A, Peek R, de Vos AF. Retinal pigment epithelium-immune system interactions: cytokine production

ARPE-19 cells under endoplasmic reticulum stress. *Curr Eye Res*. 2011;36:1153–1163.

56. Zheng L, Du Y, Miller C, et al. Role of inducible nitric oxide synthase in degeneration of retinal capillaries in mice with streptozotocin-induced diabetes. *Diabetologia*. 2007;50: 1987–1996.
57. Kronstein R, Seebach J, Grossklaus S, et al. Caveolin-1 opens endothelial cell junctions by targeting catenins. *Cardiovasc Res*. 2012;93:130–140.
58. Klaassen I, Hughes JM, Vogels IMC, Schalkwijk CG, Van Noorden CJF, Schlingemann RO. Altered expression of genes related to blood–retina barrier disruption in streptozotocin induced diabetes. *Exp Eye Res*. 2009;89:4–15.
59. Mora RC, Bonilha VL, Shin BC, et al. Bipolar assembly of caveolae in retinal pigment epithelium. *Am J Physiol Cell Physiol*. 2006;290:832–843.
60. Li X, McClellan ME, Tanito M, et al. Loss of caveolin-1 impairs retinal function due to disturbance of subretinal microenvironment. *J Biol Chem*. 2012;287:16424–16434.
61. Kirchner P, Bug M, Meyer H. Ubiquitination of the N-terminal region of caveolin-1 regulates endosomal sorting by the VCP/p97 AAA-ATPase. *J Biol Chem*. 2013;288:7363–7372.
62. Pradhan AA, Befort K, Nozaki C, Gaveriaux-Ruff C, Kieffer BL. The delta opioid receptor: an evolving target for the treatment of brain disorders. *Trends Pharmacol Sci*. 2011;32:581–590.
63. Engler MB, Engler MM. The emerging role of flavonoid-rich cocoa and chocolate in cardiovascular health and disease. *Nutr Rev*. 2006;64:109–118.
64. Ramiro-Puig E, Castell M. Cocoa: antioxidant and immunomodulator. *Br J Nutr*. 2009;101:931–940.
65. Steffen Y, Schewe T, Sies H. Myeloperoxidase-mediated LDL oxidation and endothelial cell toxicity of oxidized LDL: attenuation by (–)-epicatechin. *Free Radic Res*. 2006;40: 1076–1085.
66. Granier S, Manglik A, Kruse AC, et al. Structure of the δ-opioid receptor bound to naltrindole. *Nature*. 2012;485:400–404.
67. Panneerselvam M, Ali SS, Finley JC, et al. Epicatechin regulation of mitochondrial structure and function is opioid receptor dependent. *Mol Nutr Food Res*. 2013;57:1007–1014.
68. Yu S, Fan J, Liu L, Zhang L, Wang S, Zhang J. Caveolin-1 upregulates integrin α2,6-sialylation to promote integrin α5β1-dependent hepatocarcinoma cell adhesion. *FEBS Lett*. 2013; 587:782–787.
69. Rodriguez-Feo JA, Hellings WE, Moll FL, et al. Caveolin-1 influences vascular protease activity and is a potential stabilizing factor in human atherosclerotic disease. *PLoS One*. 2008;3:e2612.
70. Cassuto J, Dou H, Czikora I, et al. Peroxynitrite disrupts endothelial caveolae leading to eNOS uncoupling and diminished flow-mediated dilation in coronary arterioles of diabetic patients. *Diabetes*. 2014;63:1381–1393.
71. Wang Y, Wang X, Lau WB, et al. Adiponectin inhibits TNF-α-induced vascular inflammatory response via caveolin-mediated ceramidase recruitment and activation. *Circ Res*. 2014;28: 792–805.

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