ALINE MACEDO FARIA

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Orientadora: Prof. Dr. José Butori Lopes de Faria

***Tutor: Associate Professor* José Butori Lopes de Faria**

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Provérbios 1:7

ÍNDICE

8-OHdG	8-hidroxi-2'-deoxiguanosina
AGE	produtos finais da glicosilação avançada
ANG II	angiotensina II
BH_4	tetrahidrobiopterina
BH_2	dihidrobiopterina
C	catequina
CaM	calmodulina
CG	catequina galato
cGMP	guanosina monofosfato cíclica
CTGF	fator de crescimento de conexão tecidual
CV	chá verde
DM	diabetes mellitus
DHFR	dihidrofolato redutase
EC	epicatequina
ECG	epicatequina galato
EGC	epigalo-catequina
EGCG	epigalo-catequina galato
ERC	espécies reativas de cloro
ERN	espécies reativas de nitrogênio
ERO	espécies reativas de oxigênio
FAD	flavina adenina dinucleotídeo
FMN	flavina adenina mononucleotídeo
GC	galo-catequina
GCG	galo-catequina galato
GTP	guanosina trifosfato
GTPCH I	guanosina trifosfato ciclohidrolase I
H_2O_2	peróxido de hidrogênio

HAS	hipertensão arterial sistêmica
IRC	insuficiência renal crônica
MGB	membrana basal glomerular
NAD+	nicotinamida adenina dinucleotídeo
NADPH	nicotinamida adenine dinucletídeo fosfato (forma reduzida)
ND	nefropatia diabética
NF-kβ	fator de transcrição nuclear-kB
NO	óxido nítrico
NOS	óxido nítrico sintase
O_2	oxigênio
$O_2^{\bullet-}$	superóxido
$OH^{\bullet-}$	radical hidroxila
$ONOO^{\bullet-}$	peroxinitrito
PKC	proteína quinase C
PTPS	6-piruioiltetrahidropterina sintetase
RAGE	receptor de produtos finais da glicosilação avançada
SHR	"Spontaneously hipertensive rats"
SR	sepiapterina redutase
STZ	estreptozotocina
TGF-β	fator de crescimento de transformação - β
WKY	Wistar Kyoto

No diabetes mellitus (DM), o estresse oxidativo e a redução do óxido nítrico (NO) contribuem para a patogênese da nefropatia diabética. O desacoplamento da NO sintase endotelial (eNOS) faz com que essa sintase produza superóxido ao invés de NO. O objetivo do presente estudo foi investigar o potencial do chá verde (CV) na melhora do desacoplamento da eNOS no DM. Em ratos com DM induzido por estreptozotocina, a biodisponibilidade NO estava reduzida pelo desacoplamento da eNOS, caracterizada pela redução nos níveis de BH_4 e pela redução da estrutura conformacional ativa da eNOS, avaliada pela expressão da razão dímero/monômero. O tratamento com chá verde foi capaz de reverter estas abnormalidades. Além disso, células mesangiais humanas imortalizadas (ihMCs) cultivadas sob condições de alta glicose (30mM) exibiram um aumento na produção de espécies reativas de oxigênio (ERO) e uma redução na biodisponibilidade de NO, que foram revertidos pelo CV. A produção de BH_4 e a atividade da guanosina trifosfato ciclohidrolase I (GTPCH I), enzima importante na formação do BH4, diminuíram em ihMCs expostas a alta glicose e foram normalizados pelo CV. Administração exógena de BH_4 nas ihMCs reverteu o aumento das ERO e declínio da produção de NO induzido pela alta glicose. Contudo, a co-administração de CV e BH_4 não resultou em uma redução adicional na produção de ERO, sugerindo que a redução na produção de ERO pelo CV é um efeito secundário ao desacoplamento da eNOS. Em resumo, CV reverte a redução dos níveis de BH_4, induzida pelo DM, melhorando o desacoplamento da eNOS, levando ao aumento da biodisponibilidade de NO e redução do estresse oxidativo, duas anormalidades que são envolvidas na patogêneses da nefropatia diabética.

The aim of the present study was to investigate the potential of green tea (GT) to improve uncoupling endothelial nitric oxide synthase (eNOS) in diabetic conditions. In rats with streptozotocin-induced diabetes mellitus (DM), nitric oxide (NO) bioavailability was reduced by uncoupling eNOS, characterized by a reduction in BH_4 levels and an increase in the eNOS dimer/monomer ratio. GT treatment ameliorated these abnormalities. Moreover, immortalized human mesangial cells (ihMCs) exposed to high glucose (HG) levels exhibited a rise in reactive oxygen species (ROS) and a decline in NO levels, which were reversed with GT. BH_4 and the activity of guanosine triphosphate cyclohydrolase I decreased in ihMCs exposed to HG and were normalized by GT. Exogenous administration of BH_4 in ihMCs reversed the HG-induced rise in ROS and decline in NO production. However, co-administration of GT with BH_4 did not result in a further reduction in ROS production, suggesting that reduced ROS with GT was indeed secondary to uncoupled eNOS. In summary, GT reversed the diabetes-induced reduction of BH_4 levels, ameliorating uncoupling eNOS, thus increasing NO bioavailability and reducing oxidative stress, two abnormalities that are involved in the pathogenesis of diabetic nephropathy.

CAPÍTULO 1

1. INTRODUÇÃO

1.1 Diabetes Mellitus

O *diabetes mellitus* (DM) é uma doença metabólica crônica caracterizada por hiperglicemia resultante, principalmente, de uma deficiência na secreção de insulina e/ou na inabilidade da insulina de exercer adequadamente seus efeitos (American Diabetes Association, 2004; Oliveira, et al., 2009). O DM é classificado em tipo 1, tipo 2, secundário e gestacional (The Expert Committee on the Diagnosis Classification of Diabetes Mellitus, 2003). O diabetes tipo 1 acomete de 5-10% dos pacientes com DM. No DM tipo 1 ocorre da destruição auto-imune das células beta no pâncreas, acarretando a deficiência absoluta de insulina, sendo necessário o uso de insulina exógena para regular o nível de glicose sanguínea (The Expert Committee on the Diagnosis Classification of Diabetes Mellitus, 2003). O diabetes tipo 2 é o tipo mais prevalente, acometendo cerca de 95% dos casos diagnosticados de diabetes. Neste caso, as células betas pancreáticas tornam-se progressivamente menos capazes de secretar insulina suficiente para manter a homeostase normal de carboidrato e lipídeo (Bell e Polonsky, 2001). Um aspecto muito importante do diabetes tipo 2 é usualmente caracterizado pela resistência à insulina, de maneira que os tecidos alvos não usam adequadamente a insulina por efeito da idade, obesidade, sedentarismo e estresse (Saltiel e Kahn, 2002). Um tipo também muito importante de DM é o secundário que se desenvolve como resultado de doenças pancreáticas, alcoolismo, má-nutrição entre outras doenças. Neste caso, ocorre à destruição das células β do pâncreas e a insulina não é produzida (American Diabetes Association, 2012). O diabetes gestacional desenvolve-se durante a gravidez desaparecendo após o parto. Esta condição patológica se desenvolve durante o segundo e terceiro trimestre da gestação em cerca de 2% das gestações (The Expert Committee on the Diagnosis Classification of Diabetes Mellitus, 2003).

O DM é um sério e crescente problema de saúde pública, com características epidêmicas, que resulta na redução da expectativa de vida e aumento da mortalidade devido às complicações vasculares específicas da doença (Ceriello et al., 2009).

A prevalência mundial do DM em 2000 foi de 171 milhões de pessoas (Wild et al., 2004) sendo que em 2011 foi de 366 milhões de pessoas, com previsões de atingir 552 milhões em 2030 (*International Diabetes Federation,* 2012). No Brasil, a freqüência do DM é comparável com países mais desenvolvidos e o Ministério da Saúde estima que o DM acometa aproximadamente 11 milhões de pessoas e a prevalência é de 11% para pessoas acima de 40 anos (Soc. Bras. de Diabetes, 2012).

O DM está relacionado a danos, disfunção e falência de vários órgãos, especialmente: rins, olhos, nervos, coração e vasos sangüíneos (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). Os DM do tipo 1, tipo 2 e secundário são caracterizados por danos microvasculares nos rins, na retina e nervos periféricos (Brownlee, 2001).

1.2 Nefropatia Diabética

Uma complicação importante no DM é a nefropatia diabética (ND). A ND constitui a principal causa de insuficiência renal crônica (IRC) na maior parte do mundo. Nos Estados Unidos, em 2008, cerca de 48.000 pessoas com diabetes iniciaram tratamento para IRC, e, 202 mil pessoas com IRC sobreviviam devido diálise ou transplante renal (American Diabetes Association, 2012). No Brasil estima-se que 25% dos pacientes dialíticos sejam diabéticos, sendo considerada a 2ª. causa de IRC (Soc. Bras. de Diabetes, 2012). A incidência de IRC por ND continua crescendo, sugerindo que as medidas adotadas para o tratamento e prevenção desta complicação do DM ainda sejam insuficientes (American Diabetes Association, 2012).

A ND é caracterizada pelo aumento na excreção urinária de albumina (albuminúria) parâmetro funcional utilizado como marcador precoce da doença. Além disso, a doença também apresenta alterações morfológicas como: espessamento da membrana basal glomerular, hipertrofia glomerular e expansão mesangial (Cooper, 1998). A alteração na expressão de proteínas de matriz extracelular como colágeno e fibronectina estão associadas ao declínio na filtração glomerular e à redução da superfície de filtração

glomerular. Essas alterações estruturais e funcionais podem causar o comprometimento da função glomerular e promover alterações progressivas culminando na IRC (Sheetz e King, 2002).

A patogênese da ND resulta do sinergismo entre alterações metabólicas e hemodinâmicas, desencadeadas pela interação entre susceptibilidade genética e fatores ambientais (Giunti, et al., 2006). Dentre os fatores ambientais pode-se citar: ativação da via do poliol e de produtos avançados da glicosilação não enzimática (AGE); o aumento do estresse oxidativo; o aumento da pressão arterial e a ativação do sistema renina-angiotensina-aldosterona, que promovem dano tecidual e podem contribuir para a progressão da ND (Cooper, 1998).

A albuminúria é resultante de anormalidades nas funções tubular e glomerular. O grau de proteinúria está relacionado à progressão da glomerulosclerose, bem como a fibrose tubulointersticial. Sabe-se que a ND é conseqüência de alterações na barreira de filtração glomerular que acarreta a perda da permeabilidade seletiva à passagem de proteínas (Wolf e Ziyadeh, 2007). Na progressão da nefropatia, a disfunção da barreira de filtração glomerular acarreta a sobrecarga de proteínas para as células epiteliais tubulares, ativação intrarenal do sistema complemento que é responsável pela expansão da lesão ao tubulointersticio, como inflamação tubulointersticial e fibrose tubular (Abbate et al.,2006).

As alterações morfológicas afetam a barreira de filtração glomerular, um complexo biológico de estruturas com propriedades que permitem a alta taxa de filtração de água, a passagem irrestrita de pequenas moléculas, e, restringem a passagem de albumina e proteínas maiores (Haraldsson et al., 2008). A barreira de filtração glomerular é composta pelo endotélio fenestrado glomerular, a membrana basal glomerular (MBG) e pelos podócitos. As principais lesões morfológicas da estrutura de filtração glomerular características da ND são a expansão da matriz mesangial e o aumento na espessura da MBG, o que acarreta em redução na área de filtração glomerular contribuindo, portanto, para a proteinúria, progressiva perda da função renal e consequente falência renal (Wolf, 2004). Essas alterações são causadas por aumento na deposição de matriz extracelular, principalmente colágeno IV e fibronectina, além da hipertrofia das células mesangiais

(Wolf e Ziyadeh, 1999). Tanto o aumento da síntese proteica pela matriz extracelular quanto à diminuição na degradação protéica contribuem para o espessamento da MBG. Em adição ao aumento de deposição de colágeno IV, ocorre a diminuição da expressão de heparan sulfato e proteoglicanos, demonstrando alterações na arquitetura e composição da MBG que acarretam perda na seletividade da filtração glomerular (Isogai et al., 1999; Wolf e Ziyadeh, 1999).

1.2.1. Interações entre hipertensão arterial e nefropatia diabética

Já é bem conhecido que a presença da hipertensão arterial sistêmica (HAS) agrava a doença renal em modelo experimental de DM. Um modelo muito utilizado em estudos é o rato geneticamente hipertenso (*spontaneously hypertensive rat;* SHR), que tem o diabetes induzida pela injeção de estreptozotocina. Neste modelo foi demonstrado um agravamento de marcadores da nefropatia diabética quando comparados ao seu controle normotenso (Wistar Kyoto; WKY). Dentre os aspectos observados ocorreu um evidente aumento da albuminúria, bem como espessamento da MGB (Cooper et al., 1988). Cooper e colaboradores (1988) mostraram que a pré-existência da HAS pode ser um importante fator na progressão da nefropatia diabética. Estes resultados vão ao encontro com resultados que mostram que o tratamento da HAS foi capaz de corrigir alterações como: albuminúria, hipertrofia glomerular e aumento da expressão de fibronectina em animais SHR diabéticos (Amazonas e Lopes de Faria, 2006). Apesar dos resultados demonstrados suportarem o conceito de que a elevação da pressão sangüínea contribui para a ND em SHR eles não explicam o mecanismo fisiopatológico no desenvolvimento da nefropatia. Tem sido sugerido que reguladores do ciclo celular podem ser alterados pela presença do diabetes e que tais alterações contribuem para ND (Wolf, 2000). Mais especificamente tem sido demonstrado que o DM aumenta a expressão de inibidores de quinases dependente de ciclinas como p27 e p21, sinalizando hipertrofia ao invés de replicação das células mesangiais (Wolf, 2000). Tem sido sugerido também que o aumento inibidores de quinases dependente de ciclinas contribuem para a hipertrofia glomerular observada nos estágios iniciais da ND em modelo de diabetes desenvolvido geneticamente (db/db mouse)

(Wolf et al., 1998). Um estudo utilizando ratos SHR, com duração da diabetes de 10 dias, mostrou que ocorre hipertrofia renal acompanhada da diminuição da replicação celular e um aumento de inibidores de quinases dependente de ciclinas (Silveira et al., 2002). Além disso, interação entre HAS e a hiperglicemia pode promover danos por aumentar a produção de espécies reativas de oxigênio (ERO) e de AGE, estimular a ativação da via da proteína quinase C (PKC) e angiotensina II (ANG II), culminado na produção e ativação de vários fatores e citocinas que contribuem para a progressão da ND (Wolf, 2004).

1.3 Estresse Oxidativo

Dentre as fontes metabólicas que desencadeiam a ND está o estresse oxidativo sendo uma alteração crítica para o desenvolvimento da mesma. O estresse oxidativo convencionalmente definido como um desbalanço entre agentes pró-oxidantes e antioxidantes, em favor da oxidação, levando a uma alteração da sinalização redox e/ou dano molecular (Jones, 2006). Pró-oxidante é denominada qualquer substância com capacidade de gerar espécies reativas ou de induzir o estresse oxidativo. Já os antioxidantes são substâncias que reduzem ou impedem a oxidação, mesmo em baixas concentrações comparadas ao substrato (Halliwell e Whiteman, 2004).

Radicais livres são moléculas com capacidade oxidante, uma vez que, contém elétrons não pareados, o que faz com que estes elétrons sejam instáveis, com isso, capazes de reagir com outras moléculas. Portanto, um radical deve doar seu elétron não pareado para outra molécula, ou deve receber um elétron para se tornar estável (Halliwell, 1989). Entretanto se o radical livre doa seu elétron ou pega um elétron de outra molécula, esta outra molécula se tornará um radical. Este aspecto das reações que ocorrem com os radicais livres tendem a continuar como uma reação em cadeia (Halliwell, 1989).

Existem três diferentes classes de espécies reativas relevantes para a biologia e medicina são elas: ERO, espécies reativas de nitrogênio (ERN) e espécies reativas de cloro (ERC). ERO é um termo coletivo que inclui tanto oxigênios radicais e não radicais que são

agentes oxidativos e/ou são facilmente convertidos em radicais livres (Halliwell, 2006). (Tabela 1).

Dentre as ERO presentes nos sistemas biológicos o superóxido ($O_2^{\bullet-}$) é uma molécula de fundamental importância. Uma vez que é a primeira espécie produzida nas células, e, além disso, muitas outras espécies reativas, de importância fisiológica, são derivadas deste anion como por exemplo: o peróxido de hidrogênio (H_2O_2), radical hidroxila ($OH^{\bullet}$) e peroxinitrito ($ONOO^-$) (Munzel et al., 2002). O $O_2^{\bullet-}$ é produzido principalmente através da cadeia transportadora de elétrons mitocondrial, pelo complexo enzimático NADPH (nicotinamida adenina dinucletídeo fosfato) - oxidase, xantina oxidase, ciclooxigenase e lipooxigenase, óxido nítrico sintase desacoplado e citocromo P450 (Schnachenberg, 2002).

O desbalanço entre agentes pró-oxidantes e antioxidantes, em favor da oxidação, pode levar a sérias complicações como: promover danos no DNA, lipídios e proteínas (Halliwell e Whiteman, 2004). Existem várias vias que são induzidas pelas ERO levando a danos em biomoléculas. Dentre estas vias está a formação do radical $OH^{\bullet}$ que induz a peroxidação lipídica e a hidroxilação do DNA. A reação do hidroxila com o DNA forma o radical 8-hidroxi-2'-deoxiguanosina (8-OHdG) que causa alterações químicas no DNA, acarretando mutações, interrupção do ciclo celular ou apoptose (Evans et al., 2004). Outra complicação do estresse oxidativo é a formação do radical $ONOO^-$, o qual é formado da reação do $O_2^{\bullet-}$ com o óxido nítrico (NO). O $ONOO^-$ é um radical altamente reativo e instável que em pH fisiológico rapidamente é protonado a ácido peróxinitroso (ONOOH). Um agente com alta capacidade oxidante e de nitração, que danifica proteínas, lipídeos e DNA (Halliwell, 2006). A nitração de resíduos de tirosina das proteínas gera a nitrotirosina que vem sendo amplamente utilizado como biomarcador de estresse oxidativo e nitrosativo (Halliwell, 2006). A nitração de proteínas estruturais leva a modificação da conformação destas proteínas que muitas vezes perdem sua função como no caso da nitração de proteínas estruturais (Beckman and Koppenol, 1996) podendo levar a morte celular. Além disso, a formação de $ONOO^-$ leva ao consumo de óxido nítrico levando a diminuição da biodisponibilidade desta molécula, fator envolvido em diversas doenças (Thomas et al., 2008).

Tabela 1: Espécies reativas importantes no sistema biológico (Adaptado de Halliwell, 2006)

Radicais Livres	Não Radicais
Espécies Reativas de Oxigênio (ERO)	
Superóxido, $O_2^{\bullet-}$	Peróxido de hidrogênio, H_2O_2
Hidroxil, $OH^{\bullet}$	Ácido hipobromoso, HOBr
Hidroperoxil, $HO_2^{\bullet}$	Ácido hipocloroso, HOCl
Peroxil, $RO_2^{\bullet}$	Ozônio, O_3
Alcoxil, $RO^{\bullet}$	Oxigênio livre, $O_2{}^1\Delta g$
Carbonato, $CO_3^{\bullet-}$	Peróxidos orgânicos, ROOH
Dióxido de carbono, $CO_2^{\bullet-}$	Peroxinitrito, $ONOO^-$
	Ácido peroxinitroso, ONOOH
Espécies Reativas de Cloro (ERC)	
Átomo de cloro, $Cl^{\bullet}$	Ácido hipocloroso, HOCl
	Cloro Nitril (nitronium), NO_2Cl
	Cloraminas
	Gás Cloro, Cl_2
Espécies Reativas de Nitrogênio (ERN)	
Óxido Nítrico, $NO^{\bullet}$	Ácido nitroso, HNO_2
Dióxido de nitrogênio, $NO_2^{\bullet}$	Cátion nitrosil, NO^+
	Ânion nitroxil, NO^-
	Tetróxido de dinitrogênio, N_2O_4
	Trióxido de dinitrogênio, N_2O_3
	Peroxinitrito, $ONOO^-$
	Ácido peroxinitroso, ONOOH
	Cátion nitronio (nitril), NO_2^+
	Alquil peroxinitrito, ROONO
	Cloro Nitril (nitronium), NO_2Cl

1.3.1. Estresse oxidativo e nefropatia diabética

O estresse oxidativo é reconhecido como um dos principais componentes no desenvolvimento e progressão da ND (Giugliano et al., 1996; Baynes e Thorpe, 1999; Browlee, 2001). Estudos recentes revelam a importante contribuição do estresse oxidativo na patogênese da nefropatia diabética (Wardle, 2005). O aumento da glicose extracelular induz a glicação não enzimática de proteínas e subseqüente formação de AGEs que interagem com seus receptores RAGEs na membrana plasmática, ao mesmo tempo o aumento intracelular de glicose aumenta a atividade mitocondrial, assim como, estimula a atividade da PKC e a NADPH oxidase. Também ocorre um maior fluxo através da via do poliol todas estas alterações metabólicas levam ao aumento da produção de ERO e conseqüentemente aumento do estresse oxidativo, induzindo ao dano tecidual, como ocorre na ND (Bownlee, 2005 ; Calcutt et al., 2009) (Figura 1). Neste contexto, o ânion superóxido é o radical produzido de forma mais abundantemente, dentre as ERO no tecido renal (Wilcox, 2002).

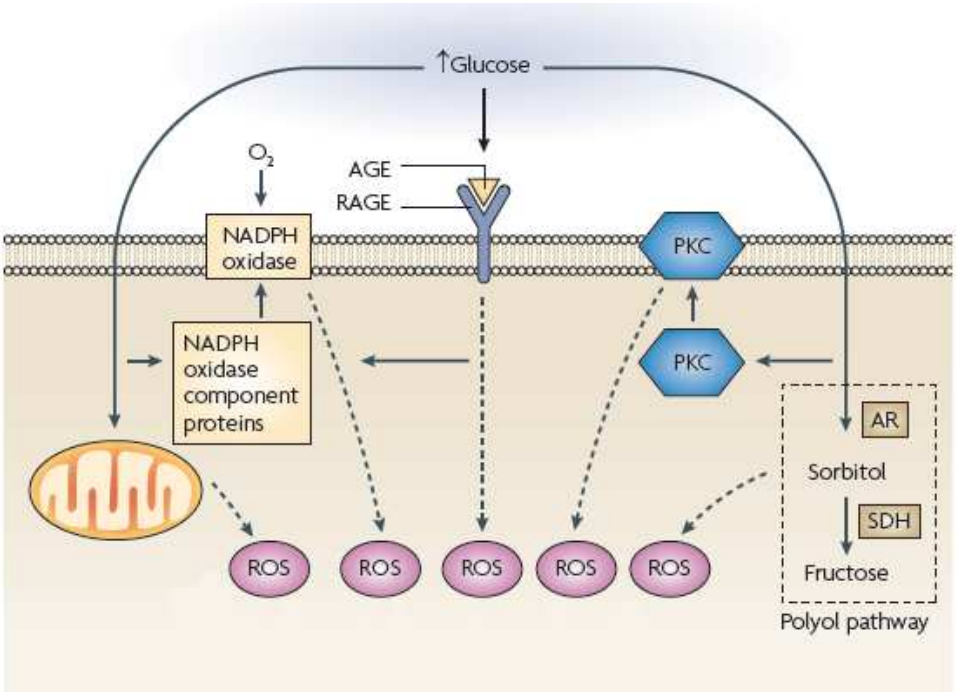

Figura 1: O aumento da glicose induz o aumento de ERO através de diferentes vias. Adaptado de Calcutt et al., Nat. Review Drug Disc. 2009; 8: 417-429.

A principal fonte produtora superóxido, no rim, é a NADPH oxidase (Gill e Wilcox, 2006; Yang et al., 2006), A NADPH oxidase é um complexo enzimático que consiste de seis subunidades: subunidades de membrana $p22^{phox}$ e $gp91^{phox}$, componentes citosólicos como $p40^{phox}$, $p47^{phox}$ e $p67^{phox}$, e uma proteína G denominada Rac1 ou Rac2 (Yang et al., 2006). Existe uma similaridade dos componentes da NADPH oxidase com a família Nox, por exemplo, na terminologia Nox, $gp91^{phox}$ é denominado Nox 2 (Bedard e Krause, 2007). Pelo menos três diferentes isoformas de Nox são expressas no córtex renal: Nox 1, Nox 2 e Nox 4 (Bedard e Krause, 2007). Trabalhos demonstram que a expressão da NADPH oxidase está aumentada em modelos experimentais de DM, reforçando a contribuição do estresse oxidativo na ND (Jones et al., 1995; Kitada et al., 2003; Frecker et al., 2005; Tojo et al., 2007). Ribaldo e colaboradores (2009) observaram um aumento da atividade da NADPH oxidase bem como da expressão da subunidade de membrana Nox 4 no tecido renal de animais diabéticos. Pessôa e colaboradores (2012) por sua vez observaram um aumento da expressão da subunidade citosólica $p47^{phox}$. Células mesangiais também têm sido implicadas no estresse oxidativo, induzido pelo DM, uma vez que, a expressão das subunidades $p22^{phox}$ e $p47^{phox}$ são alteradas por altas concentrações de glicose (Jones et al., 1995; Kitada et al., 2003; Frecker et al., 2005).

O estresse oxidativo induzido pelo DM acarreta injurias teciduais como o dano causado no DNA, que leva a morte celular avaliada pelo 8-OhdG (Halliwell e Whiteman, 2004). A expressão de 8-OHdG tem mostrado a associação entre os marcadores de estresse oxidativo e a presença da ND. Estudos experimentais, avaliando este parâmetro, mostraram um aumento da expressão no tecido renal (Peixoto et al., 2009 ; Ribaldo et al., 2009) e na urina (Pessôa et al., 2012) em ratos SHR diabéticos. Em humanos foi observado que pacientes diabéticos tipo 2 com ND apresentavam níveis significativamente mais elevados de 8-OHdG do que pacientes que não possuíam esta complicação (Hinokio et al., 2002 ; Nishikawa et al., 2003). Além disso, foi demonstrado que os níveis de 8-OHdG urinário em pacientes, com diabetes tipo 2, foram associados com a progressão da nefropatia diabética.

Estudos da ND mostraram que o estresse oxidativo induzido pelo DM leva a alterações morfológicas e fisiológicas em diferentes tipos celulares. Inicialmente

pesquisadores mostraram as alterações nas células mesangiais causada pela exposição à alta glicose. Dentre estes fatores podemos citar o aumento da produção de matriz extracelular levando a uma progressiva perda da capacidade filtrante do glomérulo (Banas et al., 1999). Outro fator envolvido na perda da seletividade glomerular é o processo degenerativo que ocorre em podócitos devido às alterações induzidas pelo estresse oxidativo gerado pelo DM. Neste processo ocorrem alterações moleculares na estrutura dos podócitos como perda de proteínas estruturais. A diminuição da expressão destas proteínas leva a alterações na morfologia celular com conseqüente perda de funcionalidade seguida da morte celular por apoptose (Susztak, et al., 2006). Ao mesmo tempo ocorre um progressivo processo de dano das células tubulares (Oldfield, et al., 2001).

Recentes estudos têm explorado os potenciais mecanismos bioquímicos e moleculares que poderiam ser responsáveis pela progressão das lesões renais causadas pelo DM. Nestes estudos pode-se constatar que a ligação AGE/RAGE ativa a produção de espécies reativas de oxigênio, PKC, citocinas inflamatórias, como NF-kB, e fatores de crescimento como TGF-β e CTGF (Kelly et al., 2001; Wendt et al., 2003; Flyvbjerg et al., 2004). Por sua vez TGF-β (Ziyadeh et al., 2000) e CTGF (Burns et al., 2006) promovem o acúmulo de matriz extracelular, acarretam lesão glomerular e fibrose túbulo-intersticial (Browlnee et al., 1988; Jerums et al., 2003).

Outra via de importância fundamental na progressão da ND é a via da PKC. Em células mesangiais a PKC é ativada indiretamente por meio de receptores de AGE e aumento da via do poliol. Esta ativação leva ao aumento da geração de ERO (Inoguchi et al., 2003; Brownlee, 2001). A PKC tem demonstrado exercer importante papel na ativação da NADPH oxidase (Inoguchi et al., 2000). A ativação de PKC juntamente com a sinalização da ANG II renal induz ativação da NADPH oxidase aumentando a translocação de p47phox do citosol para a membrana e, portanto, há a produção de pró-oxidantes e estresse oxidativo no DM (Onozato e Tojo, 2005; Tojo et al., 2007). A ativação de PKC está ligada à patogênese da ND por meio da indução de TGF-β, que estimula o aumento da síntese de fibronectina e colágeno IV levando ao acúmulo de matriz extracelular e conseqüente redução na área de filtração glomerular e albuminúria. A PKC diminui a produção de NO, pela diminuição da expressão de eNOS, e aumenta a atividade de

endotelina-1, levando a alterações na pressão intra-glomerular renal com o DM (Brownlee, 2001).

Tem se tornado aparentemente crescente que vias metabólicas e hemodinâmicas não somente interagem através de mediadores comuns, como moléculas de sinalização intracelular e fatores de crescimento, mas também uma com a outra. Como exemplo a ANG II pode aumentar acúmulo de AGE no rim, e a AGE por sua vez pode modular diretamente componentes do sistema renina-angiotensina (Thomas et al., 2005). Deste modo, estímulos metabólicos e dinâmicos desencadeados pelo DM interagem de modo a amplificar a lesão e perpetuar o dano renal no DM.

1.4 Óxido Nítrico

O NO é um radical livre gasoso que foi largamente estudado pelos químicos inorgânicos em reações na atmosfera. É um gás potencialmente tóxico, incolor e poluente, presente em processos industriais. Mas só recentemente foi descoberta a importância do NO para os organismos vivos (Moncada e Higgs, 1993). Dentre as funções descobertas do NO podemos citar: vasodilatação, inibição da agregação plaquetária, inibição da aderência de leucócitos, inibição da proliferação de células musculares lisas vasculares e manutenção do tônus vascular. No sistema nervoso central age como neurotransmissor, e, no sistema nervoso periférico o NO participa da regulação de funções nos tratos gastrointestinal, respiratório e genitourinário (Moncada e Higgs, 1993; Singh et al., 1999). Estas ações são mediadas pela ativação da guanilato ciclase solúvel e conseqüente aumento na concentração de guanosina monofosfato cíclica (cGMP) nas células-alvo (Moncada e Higgs, 1993).

O NO é um composto presente em quase todos os tipos celulares. Sua produção ocorre a partir de O_2 e L-arginina, sendo necessária a ação da enzima óxido nítrico sintase (NOS). Existem três isoformas conhecidas da enzima NOS: a óxido nítrico sintase induzida (iNOS; NOS1), a óxido nítrico sintase neuronal (nNOS; NOS2) e a óxido nítrico sintase endotelial (eNOS; NOS3) (Moncada, 1997). A iNOS é produzida através da indução de

estímulos como produção de citocinas inflamatórias. A eNOS e nNOS são formas ditas constitutivas encontradas em condições fisiológicas. A eNOS é produzida por diferentes tipos celulares e a nNOS produzida principalmente por células de origem neuronal. Todas as NOS compartilham a mesma arquitetura (Mayer e Hemmens, 1997). São enzimas diméricas, com uma região oxigenase N-terminal, ocorrendo nesta região a ligação do substrato L-arginina, heme e do cofator tetahidrobiopterina (BH_4). Na região C-terminal redutase contém locais para ligação para NADPH, flavina adenina dinucleotídeo (FAD) e flavina adenina mononucleotídeo (FMN) (Aoyagi et al., 2003) (Figura 2). Apesar de apresentarem a mesma conformação cada isoforma é regulada de forma única. A eNOs (Pollock et al., 1991) e a nNOS (Bredt e Snyder, 1990) são ativadas pela ligação Ca^{2+}/ Calmodulina (CaM), já a iNOS (Stuehr et al., 1991) apresenta atividade independente da Ca^{2+}/ CaM.

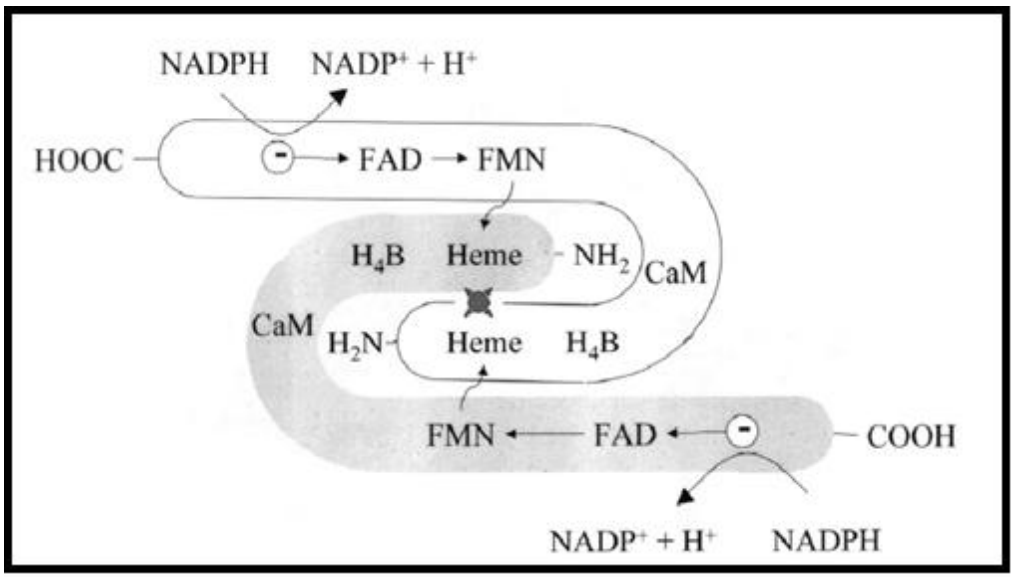

Figura 2: Modelo proposto para enzima NOS na conformação dimérica mostrando a transferência de elétrons. Apresentando sua interação com os cofatores FMN, FAD, BH_4 e CaM. Adaptado de Fleming e Busse. Cardiovasc Res. 1999; 43: 532-541.

Sabe-se que o NO atua como mecanismo fundamental de sinalização nos sistemas cardiovascular e nervoso e desempenha a função de defesa do hospedeiro. Uma das funções

fisiológicas do NO foi inicialmente descoberta na vasculatura, quando foi demonstrado que o fator de relaxamento derivado do endotélio, descrito por Furchgott e Zawadzki, em 1980, podia ser explicado pela formação de NO nas células endoteliais. O NO compartilha várias propriedades com o O_2, em particular sua alta afinidade pelo grupo heme e por outros grupos de ferro-enxofre. Isto é importante para a ativação da guanilato ciclase, que contém um grupo heme, e, para a inativação do NO pela hemoglobina (Moncada e Higgs, 1993).

Evidências suportam que a disfunção endotelial, caracterizada pela diminuição da biodisponibilidade de NO estão presentes em várias desordens vasculares como arteriosclerose, hipertensão e diabetes. A acredita-se que haja uma intrínseca relação da disfunção endotelial com o aumento do estresse oxidativo (Thomas et al., 2008).

1.4.1 Óxido nítrico na nefropatia diabética

No rim o NO desempenha importantes funções fisiológicas que incluem a regulação da hemodinâmica renal e glomerular (Majid e Navar, 2001); manutenção da perfusão medular (Mattson et al., 1992); mediação da pressão natriurética (Majid et al., 1993); inibição da reabsorção de sódio pelos túbulos (Ortiz & Garvin 2002) e modulação atividade simpática neural no rim (Eppel et al., 2003). O diabetes experimental está associado com complexas alterações na biodisponibilidade e sinalização do NO renal (Komers et al., 2003). Este fato é demonstrado por estudos que observaram a diminuição de NO, no rim, durante o DM (Komers et al., 2003; Mohamadin et al., 2007).

Os estudos da associação da patogênese da ND com a diminuição da biodisponibilidade do NO ganharam grande força quando Nakagawa (2007) avaliou as complicações renais gerados pelo DM em animais "knockout" para eNOS. Sendo observado um agravamento da ND quando comparado a animais diabéticos. Neste modelo animal foi possível observar alterações morfológicas como mesangiólise, lesão acelular nodular, microaneurisma glomerular, que até o momento, só eram observados em biopsias renais de pacientes com ND. Além disso, havia um exacerbado aumento da matriz extracelular quando comparado a animais diabéticos selvagens (Nakagawa, 2007).

O diabetes leva a disfunção endotelial, que tem como principal característica a diminuição dos níveis de NO. Isto ocorre devido a: glicotoxicidade direta, alterações na via dos polióis, aumento da atividade da PKC, aumento da produção de radicais livres com conseqüente inativação do NO e formação de AGEs. A hiperglicemia induz a todas estas alterações descritas acima que, por sua vez, estão ligados um ao outro, resultando em uma via comum que leva ao aumento da produção de $O_2^{\bullet-}$ (Ohtake et al., 2007). O radical $O_2^{\bullet-}$ é responsável por inativar rapidamente o NO, elevando a resistência vascular e promovendo a proliferação e migração celular, a agregação de plaquetas e leucócitos. O papel do estresse oxidativo na diminuição da biodisponibilidade de NO tem sido objeto de intensas pesquisas nos últimos anos. Curiosamente, a segunda maior fonte geradora de superóxido é a NOS em seu estado desacoplado (Satoh et al., 2005), mostrado mais uma vez a íntima relação entre o estresse oxidativo e a biodisponibilidade de NO.

No rim estão presentes as 3 isoformas da NOS: a iNOS é expressa principalmente nas células tubulares, a nNOS nas células da mácula densa e a eNOS é expressa por vários tipos celulares, sendo a principal enzima óxido nítrico sintase presente no rim (Mount e Power, 2006). Assim como descrito previamente, a eNOS contém dois domínios funcionais distintos. Um N-terminal oxigenase, onde se ligam a BH_4 e a L-arginina, e, o C-terminal redutase que apresenta sítios de ligação para vários cofatores imprescindíveis para formação de NO, como a NADPH e flavina (FAD e FMN). Durante a síntese de NO, a eNOS recebe e estoca elétrons para transformar os co-substratos O_2 e L-arginina em NO e L-citrulina. Na ausência de substrato ou co-fatores, a eNOS ativada não catalisa a oxidação de L-arginina em NO. Apesar disso, a enzima ainda é capaz de receber e estocar elétrons em seu domínio redutase, doando-os um a um ao seu substrato O_2. Conseqüentemente, em seu estado desacoplado, a eNOS gera superóxido ao invés de NO (Moncada e Higgs, 1993).

A ausência do cofator BH_4 ou do substrato L-arginina, leva a redução do grupo heme na enzima e conseqüente desacoplamento da NOS, levando a produção de $O_2^{\bullet-}$ em lugar de NO (Satoh et al., 2005). Por vezes não há diminuição dos níveis de NO e sim aumento da biodegradação do NO, reduzindo sua biodisponibilidade. A presença de superóxido no leito vascular determina uma reação rápida com o NO local, formando $ONOO^-$, levando assim ao consumo de NO, além de induzir o desacoplamento da eNOS via

oxidação do cofator BH_4 (Beckman et al., 1990). Esta última ação determina uma produção adicional de superóxido e perpetuação do dano endotelial.

No diabetes, a presença de altos níveis de marcadores de peroxidação lipídica (malondialdeído) e dano ao DNA (8-OHdG), têm sido relatados juntamente com o aumento da oxidação de BH_4 resultando na formação de 7,8-dihidrobiopterina (BH_2) (Satoh et al., 2005) (Figura 3). Este resultado sugere que a redução na biodisponibilidade de BH_4, induzido pelo estresse oxidativo, desacopla a eNOS, levando a diminuição da biodisponibilidade de NO e conseqüente aumento na produção de $O_2^{\bullet-}$ no glomérulo de animais diabéticos (Satoh et al., 2005).

BH_4 é um cofator de crucial importância para a manutenção da conformação dimérica da eNOS, bem como em seu estado acoplado. Este cofator é produzido através de duas vias enzimáticas; sendo uma via conhecida como a síntese *de-novo* e a outra como via de reciclagem. O primeiro passo envolvido na síntese *de-novo* da formação do BH_4 envolve a enzima guanosina trifosfato ciclohidrolase I (GTPCH I). A GTPCH I catalisa a formação de dihidroneopteria trifosfato a partir de guanosina trifosfato (GTP). O BH_4 e formado após mais dois passos que envolvem as enzimas 6-piruioiltetrahidropterina sintetase (PTPS) e sepiapterina redutase (SR). Uma via de produção alternativa do BH_4 ocorre pela via de reciclagem onde a BH_2 é reduzida a BH_4 via a ação da enzima dihidrofolato redutase (DHFR) (Schimidt e Alp, 2007) (Figura 4).

Um recente estudo mostrou que a razão intracelular BH_4:BH_2, mais que BH_4 depleção por si, é o ativador molecular da insuficiência do NO no diabetes (Noguchi et al., 2010). Crabtree e colaboradores (2011), utilizando um modelo *in vivo* de camundongo deficiente em BH_4, mostraram que na presença de pequenas quantidades de BH_4 total, a enzima DHFR é responsável regular a razão BH_4:BH_2 e conseqüentemente o acoplamento da eNOS.

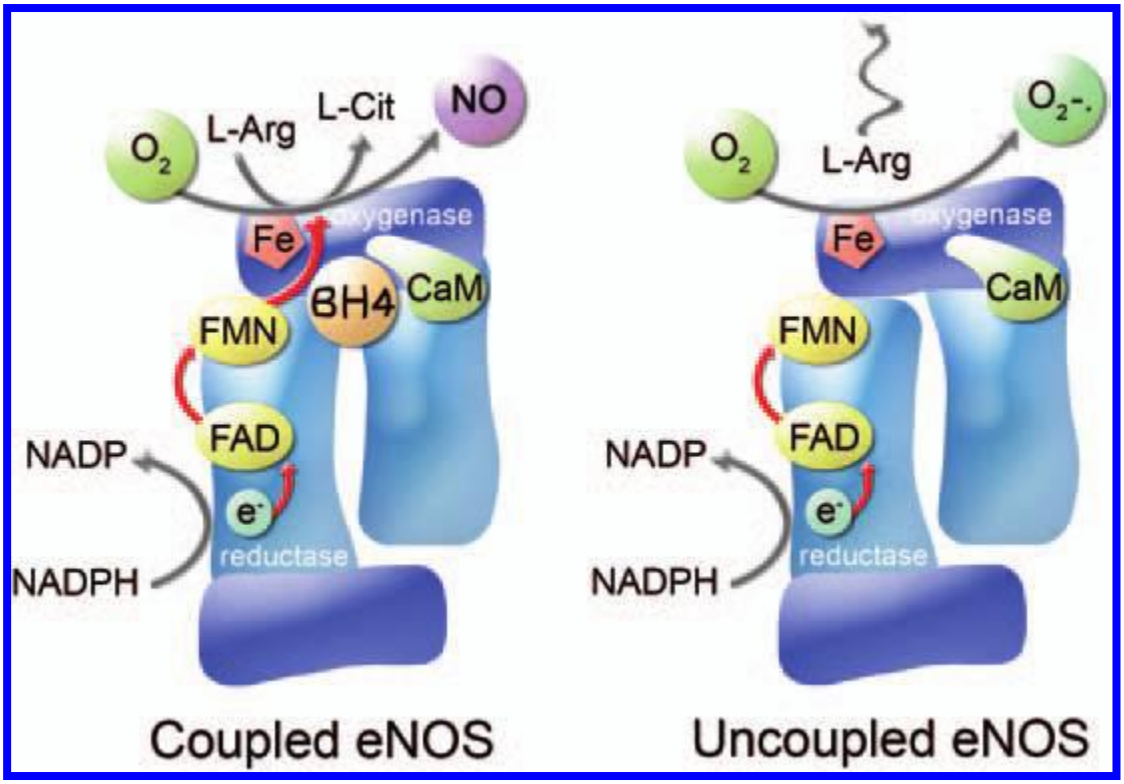

Figura 3: Ilustração que demonstra a enzima eNOS em seu estado acoplado ("coupled" eNOS) com a presença de BH_4 e formação de NO, bem com a eNOS em seu estado desacoplado ("uncoupled" eNOS) sem o cofator BH_4 e consequente formação de $O_2^{\bullet-}$ ao invés de NO. Adaptado de Schulz et al., Antioxid. Redox Signal. 2008; 10: 1115-1126.

Foi sugerido que a diminuição dos níveis de BH_4 pode ocorrer devido à degradação da enzima GTPCH I via proteossomo (Xu et al., 2007). Outra hipótese é que a GTPCH durante o DM possa estar ligada a caveolina-1 permanecendo em um estado inativado (Peterson et al., 2009). Estas hipóteses são reforçadas pelo fato do tratamento com BH_4 prevenir a diminuição da biodisponibilidade do NO (Satoh et al., 2005; Werner et al., 2011). Deste modo manobras que levem ao restabelecimento de BH_4 com conseqüente acoplamento da eNOS e diminuição da formação de superóxido podem ser úteis no tratamento da ND, uma doença caracterizada pela disfunção endotelial (Nakagawa et al., 2011).

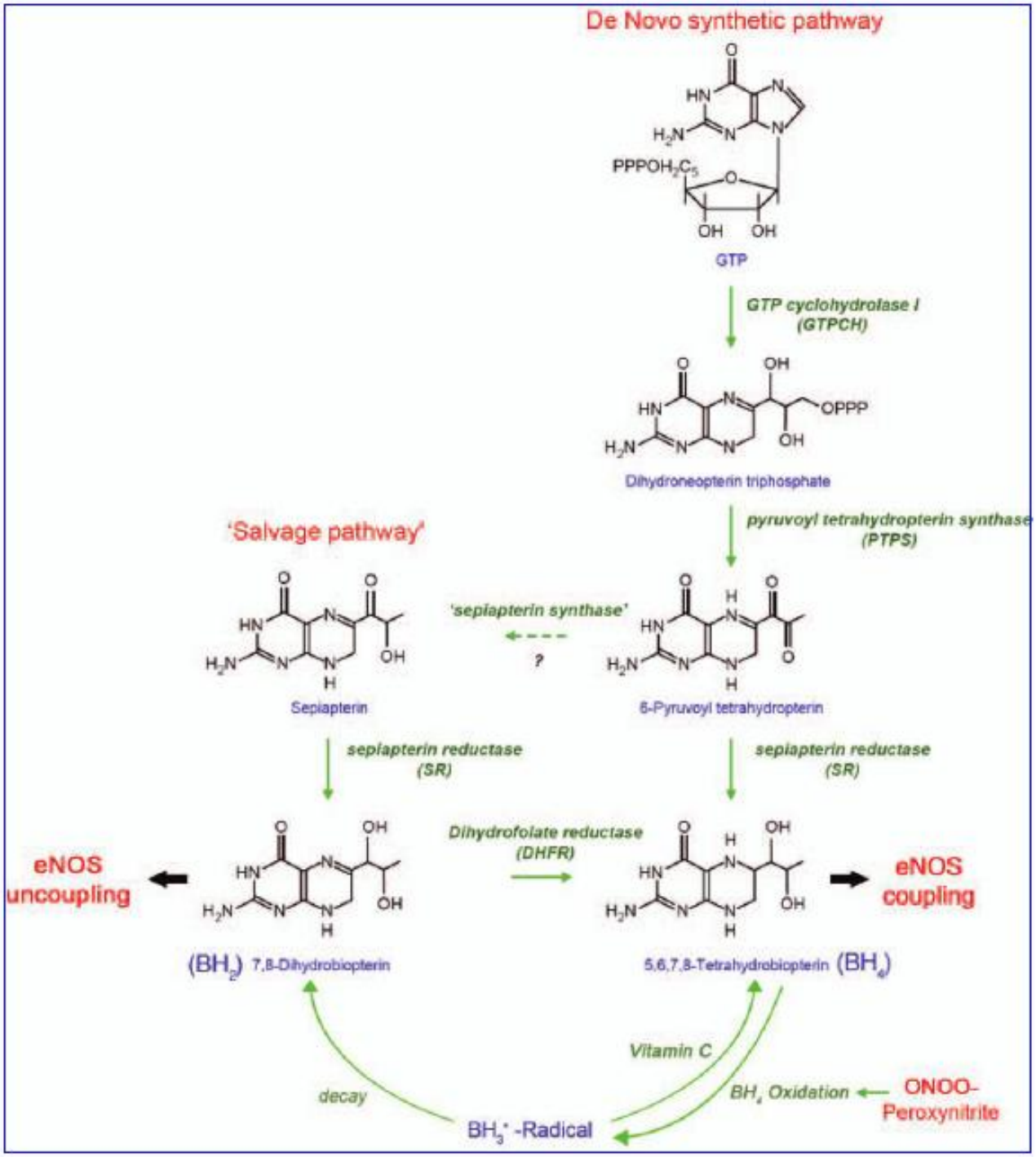

Figura 4: Vias de produção do cofator BH_4 : via da síntese *de-novo* (*De Novo synthetic pathway*) e via da reciclagem (*Salvage pathway*), bem como as enzimas envolvidas neste processo. Adaptado de Schulz et al., Antioxid Redox Signal. 2008; 10: 1115-1126.

1.5 Células mesangiais

As células mesangiais constituem um tipo celular de crucial importância do glomérulo. As células mesangiais têm como funções o provimento do suporte estrutural para os capilares glomerulares além de controlar a produção e modulação da matriz extracelular. Também influenciam na regulação do fluxo sangüíneo do capilar glomerular, da filtração glomerular e servem como uma fonte de fatores de crescimento (Schlöndorff e Banas, 2009).

Células mesangiais constituem o pivô central do glomérulo estando em continuidade com a matriz extracelular, que juntas constituem o mesângio. As células mesangiais apresentam propriedades contráteis geradas por filamentos ancorados na membrana basal, sendo esta propriedade envolvida no suporte e manutenção da organização capilar. Células mesangiais estão em contado direto com as células endoteliais sem a presença de membrana basal, na face do lúmem capilar. A interação entre as próprias células mesangiais e entre podócitos e células mesangiais é de extrema importância para a manutenção da homeostase e filtração glomerular (Figura 5). No glomérulo, a alteração patológica de um tipo celular repercute por vias de sinalizações para outros tipos celulares induzindo alterações no processo de filtração (Schlöndorff e Banas, 2009).

A matriz mesangial é constituída por: colágeno tipo IV e V, laminina A, B1 e B2, fibronectina, heparam sulfato, condroitina, proteoglicanos sulfatados, entactina e nidogênio. A composição e quantidade de matriz mesangial é altamente controlada em condições fisiológicas normais, mas pode ser drasticamente alterada em processos patológicos (Schlondorff, 1987; Couchman et al., 1994). Alguns dos componentes da matriz não só proporcionam um suporte estrutural para o mesângio, mas também contribuiem para a sinalização matriz-célula. Componentes da matriz mesangial ainda têm a capacidade de influenciar o crescimento e proliferação de células mesangiais. Um método pelo qual a matriz controla a proliferação celular é através da sua ligação a fatores de crescimento influenciando, deste modo, a liberação destes fatores. A ativação de metaloproteinases leva a liberação de TGF-β da matriz que, por sua vez, contribui para a esclerose glomerular (Mason e Wahab, 2003).

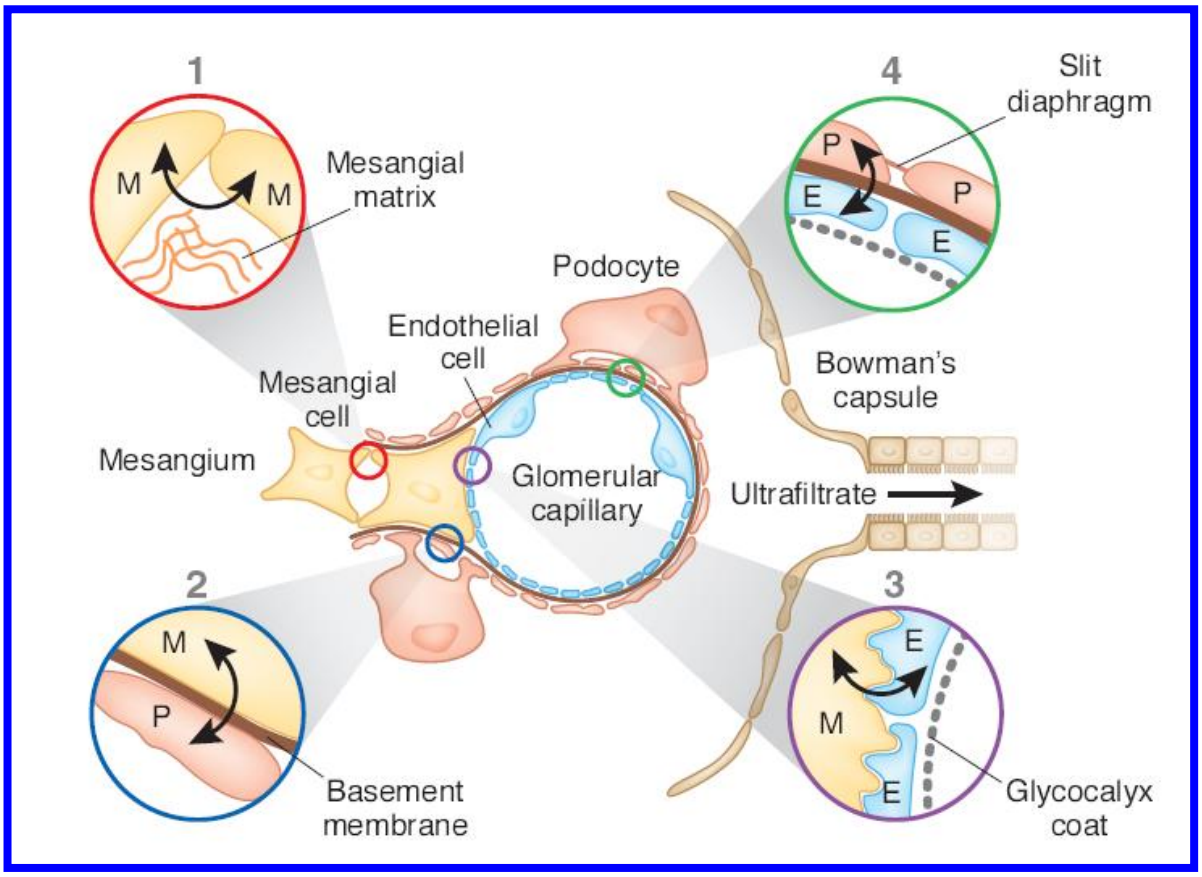

Figura 5: Sinalização celular entre células glomerulares intrínsecas. 1) Comunicação inter-celular entre células mesangiais – modulação da matriz extracelular; 2) Comunicação entre células mesangiais e pódocitos ; 3) Comunicação direto entre células mesangiais e células endoteliais sem a presença de membrana basal. Todas estas interações estão envolvidas para a manutenção da homeostase e filtração glomerular. Adaptado de Schlöndorff e Banas. J Am Soc Nephrol 20:1179-1187,2009.

Sabe-se que o DM induz a alterações morfológicas e moleculares nas células mesangiais como hipertofia celular, proliferação, expansão da matriz extracelular, além da produção de quimiocinas que contribuem para o influxo de leucócitos (Schmid et al., 2006). Acredita-se que tais alterações possam ser causadas pelo aumento da expressão protéica e gênica de GLUT-1, um receptor de membrana com a função de modular o influxo de glicose para o meio intracelular (Heilig et al., 1997). O aumento intracelular de glicose nas células mesangiais leva a ativação da PKC. Que, por sua vez, levará a alteração da regulação das subunidades $p22^{phox}$ e $p47^{phox}$, induzindo a ativação da NADPH oxidase seguido do aumento na produção de ERO e do estresse oxidativo no tecido renal (Xia et al., 2006). Flutuações nos níveis de glicose, nas células mesangiais, induzem ao aumento da

absorção de glicose induzido por GLUT-1 (de Lima et al., 2009). Vidotti e colaboradores (2004) observaram que em células mesangiais cultivadas em alta glicose ocorre um aumento na geração de Ang II resultante de uma aumento intracelular da atividade da renina que por sua vez é mediada por 3 fatores: aumento da transcrição gênica, redução da secreção de enzima pró-renina e um aumento na taxa de conversão de pró-renina em renina, provavelmente mediada pela catepsina B .

O glomérulo apresenta uma estrutura complexa com a interação de diferentes tipos celulares cruciais para a manutenção da homeostase no processo de filtração. Células mesangiais apresentam-se sensíveis à alterações na taxa de glicose com aumento do desenvolvimento do estresse oxidativo, provavelmente outras vias metabólicas também estejam sendo afetadas pela hiperglicemia, como a via do óxido nítrico. Estudos avaliando a biodisponibilidade do NO, no tecido renal, têm focado principalmente alterações causadas nas células endoteliais, mas a avaliação da via do NO nas células mesangiais cultivadas sob alta glicose podem trazer novas informações sobre a ND.

1.8 Chá Verde

O Chá é preparado a partir de folhas da planta *Camélia sinensis*, sendo segunda bebida mais consumida mundialmente superada somente pela água (Schmitt e Dirsch, 2009). Originário da China o chá tem sido consumido a mais de 2000 anos (Cabrera et al., 2006). Baseado no processo de produção, os chás são classificados em 3 principais tipos: chá verde (CV) que é um chá não fermentado utilizando o processo de secagem e cozimento das folhas frescas que impedem a oxidação dos polifenóis; o oolong é um chá semi-fermentado produzido com folhas frescas submetidas a um processo de fermentação parcial; e o terceiro tipo é o chá preto - fermentado - onde ocorre o processo de oxidação dos polifenóis (McKay e Blumberg, 2002).

Atualmente o chá preto é o chá mais consumido mundialmente, mas o CV tem apresentado maiores benefícios à saúde segundo estudo recente (Cabrera et al., 2006).

Acredita-se que estes benefícios sejam devido à presença de grande quantidade de polifenóis, particularmente flavonóis (Vison et al., 1995).

Os flavonois são compostos derivados dos fenóis sintetizados em quantidade substanciais (0,5-1,5%) e em grande variedade (mais de 4000 identificados) sendo amplamente distribuídos entre as plantas (Vison et al., 1995). Os principais flavonois presentes no CV são as catequinas, que são monômeros de flavonóides de potente ação antioxidante, e que têm demonstrado possuir um efeito antioxidante consideravelmente maior que as vitaminas C e E (Mustata et al., 2005). Além disso, têm sido observado efeitos positivos na modulação do óxido nítrico endotelial (Schmitt e Dirsch, 2009).

As principais catequinas naturalmente encontradas no CV são: epigalo-catequina galato (EGCG) que representa aproximadamente 59% do total de catequinas, epigalo-catequina (EGC; 19% aproximadamente), epicatequina galato (ECG; 13,6% aproximadamente), epicatequina (EC; 6,4% aproximadamente) (McKay e Blumberg, 2002) (Figura 6). Em menor concentração, também, estão presentes a galo-catequina (GC), catequina (C), galo-catequina galato (GCG) e catequina galato (CG) (Nishitani et al., 2004).

A EGCG constitui a catequina com maior capacidade antioxidade no CV, em decorrência do maior número de radicais hidroxilas, que são doadores de elétrons e também por ser a catequina mais abundante no CV, o que faz do CV um dos principais antioxidantes entre os chás. (Nishitani et al., 2004).

A tradicional medicina chinesa tem recomendado o uso medicinal do CV para tratamentos de dores de cabeça, dores no corpo, má digestão, depressão, detoxificação, sendo também utilizado como energético e para prolongar a vida (Cabrera et al., 2006). Estudos também têm comprovado a ação benéfica deste alimento funcional. Dentre estas ações podemos destacar a capacidade antioxidante do CV avaliado por uma variedade de métodos, como o desenvolvido por Cao e colaboradores (1996) que avaliaram a capacidade de absorção de espécies reativas de oxigênio e observaram que o CV apresenta uma atividade antioxidante contra o radical peróxido muito maior que a encontrada em vegetais como o alho, o espinafre e a couve de Bruxelas. Por sua vez, Langley-Evans (2000)

observou que em termos de capacidade antioxidante o chá verde apresentou-se mais potente que do chá preto. Esta capacidade antioxidante também foi observada em ensaios *in vitro,* nos quais, a EGCG mostrou a capacidade de se ligar ao radical peróxido inibindo a peroxidação lipídica (Saffari e Sadrzadeh, 2004).

A capacidade antioxidante do CV também foi observada em seres humanos, sendo reportado que o consumo de CV, e, de extrato de chá verde encapsulado demonstrou uma diminuição de biomarcadores de oxidação (McKay e Blumberg, 2002). Do mesmo modo, um estudo com fumantes demonstrou redução no dano oxidativo, na peroxidação lipídica e na geração de radicais livres após o consumo de 6 copos de CV por dia durante 7 dias (Klaunig et al., 1999).

O CV tem se mostrado efetivo no tratamento de doenças como: hipertensão arterial, obesidade, doenças coronarianas, câncer, diabetes entre outras doenças (Moore et al., 2009). Estudos de metánalise mostraram que o consumo de uma xícara ao dia de CV reduziu o risco de desenvolvimento doença arterial coronariana em até 18% (Wang et al., 2011). Esses efeitos benéficos têm sido freqüentemente atribuídos aos flavanóis, abundantemente presentes em frutas e vegetais (Grassi et al., 2005).

A administração de EGCG, em ratos SHR, foi capaz de melhorar a função endotelial, a sensibilidade a insulina, além de reduzir a pressão arterial (Potenza et al., 2007). Em ratos diabéticos induzidos por estreptozotocina (STZ), a administração de CV na água de beber melhorou a função renal (baseada na proteinúria e no “clearance” de creatinina), e, reduziu os níveis de glicose sanguínea e glicação de proteínas (Renno et al., 2008).

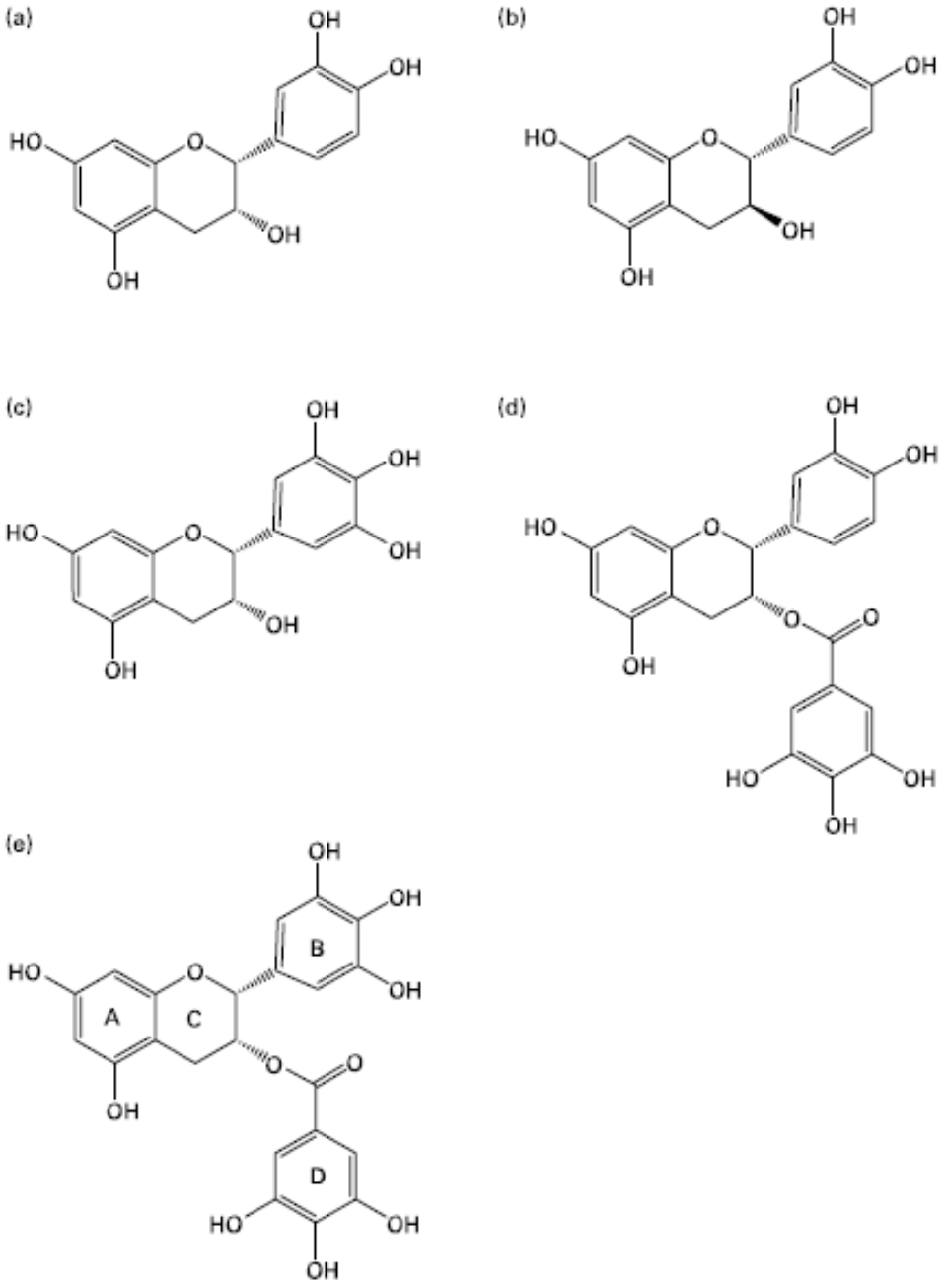

Figura 6: Principais catequinas encontradas na infusão de CV (a) epicatequina (EC); (b) catequina (C); (c) epigalo-catequina (EGC), (d) epicatequina galato (ECG) e (e) epigalo-catequina galato (EGCG). A EGCG apresenta uma maior quantidade de hiroxilas (-OH) em sua composição molecular possibilitando uma maior capacidade antioxidante desta catequina. Moore et al. Br J Nutr. 2009; 102: 1790-1802.

Em um estudo prévio desenvolvido em nosso laboratório, utilizando ratos SHR diabéticos, foi observado a melhora do estresse oxidativo via diminuição da expressão da subunidade Nox 4 do complexo enzimático NADPH oxidase, conjuntamente a diminuição da formação de superóxido produzido pelo mesmo complexo enzimático. A redução do estresse oxidativo como resultado do tratamento com CV ainda levou há uma melhora dos parâmetros de danos renais como: excreção urinária de albumina e acúmulo de colágeno tipo IV (Ribaldo et al., 2009). Entretanto, o papel do CV na biodisponibilidade do NO na ND ainda não foi avaliado. Portanto, no presente estudo, buscamos entender o mecanismo que induz ao desacoplamento da eNOS, que por sua vez, concorre para a diminuição da biodisponibilidade de NO concomitante ao aumento do estresse oxidativo. Neste contexto avaliamos o tratamento do chá verde na nefropatia causada pelo DM.

2. HIPÓTESE E OBJETIVO

Hipótese: O aumento do estresse oxidativo e a redução da biodisponibilidade do óxido nítrico participam da lesão renal em ratos espontaneamente hipertensos e com diabetes mellitus induzido por estreptozotocina. A suplementação dietética com chá verde pode melhorar a nefropatia pelo restabelecimento da biodisponibilidade do óxido nítrico e redução do estresse oxidativo, através do aumento nos valores de tetrahidrobiopterina.

Objetivos específicos:

1. Investigar se o aumento do estresse oxidativo renal, em ratos SHR diabéticos, leva a redução da biodisponibilidade do NO.

2. Investigar as alterações renais que induzem a redução da disponibilidade do NO concomitante ao aumento do estresse oxidativo. Fazendo uso de estudos *in vivo* com ratos SHR, bem como estudos *in vitro* utilizando células mesangiais humanas imortalizadas.

3. Testar os efeitos da suplementação com chá verde na produção de óxido nítrico e no estresse oxidativo renal em ratos SHR diabéticos.

CAPÍTULO 2

1. Artigo Publicado

Uncoupling endothelial nitric oxide synthase is ameliorated by green tea in experimental diabetes mellitus by reestablishing BH_4 levels

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ABSTRACT

The aim of the present study was to investigate the potential of green tea (GT) to improve uncoupling endothelial nitric oxide synthase (eNOS) in diabetic conditions. In rats with streptozotocin-induced diabetes mellitus (DM), nitric oxide (NO) bioavailability was reduced by uncoupling eNOS, characterized by a reduction in BH_4 levels and an decrease in the eNOS dimer/monomer ratio. GT treatment ameliorated these abnormalities. Moreover, immortalized human mesangial cells (ihMCs) exposed to high glucose (HG) levels exhibited a rise in reactive oxygen species (ROS) and a decline in NO levels, which were reversed with GT. BH_4 and the activity of guanosine triphosphate cyclohydrolase I decreased in ihMCs exposed to HG and were normalized by GT. Exogenous administration of BH_4 in ihMCs reversed the HG-induced rise in ROS and decline in NO production. However, co-administration of GT with BH_4 did not result in a further reduction in ROS production, suggesting that reduced ROS with GT was indeed secondary to uncoupled eNOS. In summary, GT reversed the diabetes-induced reduction of BH_4 levels, ameliorating uncoupling eNOS, thus increasing NO bioavailability and reducing oxidative stress, two abnormalities that are involved in the pathogenesis of diabetic nephropathy.

INTRODUCTION

Oxidative stress, has been seen as a critical underlying mechanism causing the microvascularcomplications of diabetes, including diabetic nephropathy (DN) (1-3). Hyperglycemia is known to increase oxidative stress via the activation of multiple pathways, leading to the generation of superoxide anions and other reactive oxygen species (ROS) in different renal cell types, which thus contributes to renal damage (1, 2). Some of these pathways include enhanced activity of the mitochondrial electron transport chain (3), activation of NADPH-oxidase enzyme-induced superoxide formation (2-6), and uncoupling of endothelial nitric oxidase synthase (eNOS) (6). Uncoupled eNOS is a phenomenon characterized by the diversion of electron transfer within the eNOS molecule from L-arginine oxidation, resulting in a reduction of molecular oxygen to form superoxide instead of NO (7). Therefore, uncoupled eNOS contributes not only to increases in ROS formation but also to decreases in NO bioavailability, two conditions involved in the pathogenesis of DN (8). Indeed, eNOS uncoupling has been seen as a major source of local superoxide production in diabetic kidneys (6). Three main pathways have been identified as the mechanism for uncoupling eNOS: oxidation of tetrahydrobiopterin (BH_4), depletion of L-arginine, and accumulation of methylarginines (9). A recent study has suggested that the balance between NO and superoxide production by eNOS is determined by the levels of BH_4 at its production and stability level (10). BH_4 is synthesized via two main pathways—the *de novo* synthesis and salvage pathways. The first step involved in the *de novo* synthesis of BH_4 formation includes a rate-limiting enzyme, such as guanosine triphosphate cyclohydrolase I (GTPCH I), which catalyzes the formation of BH_4 from guanosine triphosphate (GTP) via a series of enzymatic reactions (11). An alternative pathway for BH_4 synthesis has been documented, whereby 7,8-dihydrobiopterin (BH_2) is reduced to BH_4 via dihydrofolate reductase (DHFR), the so-called "salvage pathway" (12). A recent study indicated that increased BH_4 oxidation, rather than BH_4 depletion, is the molecular trigger for NO insufficiency in high glucose conditions (13). Researchers have proposed that the mechanism of decreased BH_4 in diabetes is proteasome-dependent degradation of GTPCH I in BH_4 synthesis (14). To this end, there is evidence that the administration of BH_4 may prevent endothelial dysfunction (15). Therefore, maneuvers that

reestablish BH_4 bioavailability with consequent eNOS coupling may be useful in treating DN, a disease characterized by endothelial dysfunction (16).

Tea is considered the second most frequently consumed beverage worldwide, after water (17). Green tea (GT; *Camellia sinensis*) is a rich source of polyphenols, particularly flavonoids, which have been shown to positively affect the modulation of endothelial nitric oxide (17). In a double blind, placebo-controlled study, one of the main components of GT, (-)-epigallo-catechin gallate (EGCG), acutely improved flow-mediated dilation, an estimation of endothelial function in humans (18). In a recent study, GT ameliorated oxidative stress in diabetic rat kidneys via reduced expression of NOX4, and hence superoxide formation (5). The reduction in oxidative stress as a result of GT also contributed to the amelioration of indices of renal injury, such as albuminuria and renal accumulation of collagen IV (5). However, GT's role in BH_4 synthesis, coupling eNOS, and hence, NO bioavailability under diabetic conditions, has not been evaluated. Therefore, the aim of the present work was to assess GT's potential to ameliorate BH_4 levels, uncoupling eNOS, and NO bioavailability in diabetic conditions using an *in vivo* model of diabetic spontaneously hypertensive (SHR) rats and an *in vitro* system of human kidney mesangial cells.

RESEARCH DESIGN AND METHODS

Reagents. All reagents were purchased from Sigma, St. Louis, MO unless otherwise stated.

Animals and study design. This study's protocol was approved by the local committee for ethics in animal research (CEEA/IB/Unicamp). The SHR rats used in the study were provided by Taconic (Germantown, NY). Experimental diabetes was induced in 12-week-old SHR rats via a single intravenous injection of streptozotocin (50 mg/kg in sodium citrate buffer, pH 4.5). Following the day of diabetes induction, the diabetic rats were randomly assigned to either receive or not receive GT (7g/kg body weight/day) instead of drinking water. Japanese GT (Midori Industria de Chá) was prepared daily as we have described before (5). During the study, the diabetic rats received two units of insulin

(human insulin HI-0310; Lilly), three times per week, subcutaneously. The control rats only received the vehicle. We have chosen to induce diabetes in SHR because these rats present a more progressive form of renal disease (19), we have a vast experience with this model (2,5,20), and also because of the frequent association of diabetes with hypertension in human diabetic renal disease. After 12 weeks of diabetes induction, the rats were killed, the kidneys were decapsulated and removed, and a piece of the cortex was used for protein isolation. The remaining kidney cortex was snap-frozen at -80^{o}C for future assays.

Renal histopathology: The kidney was embedded in paraffin. Three-micrometer sections were cut and stained with PAS. Matrix mesangial expansion (MME) The analyses were determined using Leica Application Suite (LAS Image Analysis). MME was derived from assessment of 30 glomeruli from each rat.

Human mesangial cell culture: Immortalized human mesangial cells (ihMCs) (passage 10 to 20) from Dr. Bernhard Banas (Nephrology Center, Medical Policlinic, Ludwig-Maximilian University of Munich, Germany) were kindly provided by Dr. Nestor Schor (Department of Medicine, Nephrology Division, Federal University of São Paulo, Brazil). The ihMCs were cultured as described previously (21). The cells were kept without serum in normal glucose (NG, 5.5 mM) and high glucose (HG, 30 mM) mediums in the presence of various treatments for an additional 24 h. The concentrations of treatments used in the HG medium in all experiments were chosen after carrying out a thiazolyl blue tetrazolium bromide (MTT) assay (data not shown).

Western blotting analysis. The kidney cortex or ihMCs were lysed in a RIPA buffer supplemented with a protease inhibitor cocktail (Complete; Boehringer-Mannheim, Indianapolis, IN). The samples and Western blot were prepared as previously described (5). Bradford method (22) was used for protein quantification. The following primary antibodies were used: rabbit polyclonal anti-eNOS (Santa Cruz) rabbit polyclonal p-eNOS Thr495 and rabbit polyclonal p-eNOS Ser1177 (Cell Signaling Tech). Equal loading and transfer was achieved by reprobing the membranes for β-actin. To determine eNOS

dimer/monomer the sample preparing and Western blot was performed as previously described (23).

NO_X^- analysis by Griess reaction. The analysis of NO end products, such as nitrate and nitrite (NO_X^-), was evaluated by the Griess reaction, as previously described (24). Briefly, the renal cortex was lysed in 300 μL of extraction buffer (50 mmol/l Tris-HCl; pH 7.4, 1 mmol/l EDTA, 10 mmol/l DTT). The kidney cortex lysate was then deproteinized by 0.6 M of trichloroacetic acid (TCA) for 1h at 4°C, and the samples were incubated with chloride vanadium (VCl_3) in 1:1 proportion for 15 min. Then, 0.1% of N-naftil-etilenodiamine (NED) and 2% of sulfanilamide were added to the samples for 30 min in the dark, at room temperature. The samples were read by a spectrophotometer (Powerwave XS2, Biotek, USA) at 540 nm absorbance.

Measurement of intracellular levels of biopterins. Oxidized and reduced forms of biopterins were analyzed by the differential oxidation method and were determined according to a previously described method (25), with some modifications (26). The renal cortex and ihMCs were lysed in a 500 μL extraction buffer (50 mmol/l Tris-HCl; pH 7.4, 1 mmol/l EDTA, 10 mmol/l DTT). The samples were injected into an ultra-performance liquid chromatography system (UPLC; Waters, USA). BH_4 concentration, expressed as picomoles or nanomoles per milligram of protein, was calculated by subtracting BH_2 plus biopterin from total biopterins. Percentage of BH_4 oxidation was calculated using the following formula:

$$\text{Percentage of } BH_4 \text{ oxidation} = 100 - (BH_4 \text{ levels} * 100/\text{biopterin levels})$$

Measurement GTPCH activity: GTPCH activity was measured using the high performance liquid chromatography (HPLC) method, as described previously (27).

DCF measurement of ROS production. Intracellular ROS levels were measured by 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). Qualitative assessment of ROS was carried out in the ihMCs after they were kept for 24-h with the NG and HG mediums alone or in the presence of GT (100 μg/ml) and different treatments. To quantify the ROS levels,

the same procedure used for the qualitative analysis of ROS was applied. Relative fluorescence was measured using a fluorescence plate reader (SynergyMx, Biotek, USA) at excitation and emission wavelengths of 485 and 528 nm, respectively. The relative fluorescence values were corrected by the number of cells in each treatment.

DAR-4M AM and DAF-2DA measurement of NO. Intracellular NO was measured in ihMCs via diaminorhodamine-4M AM (DAR-4M AM; Alexis Biochemicals, Switzerland). To quantify NO levels, the same procedure used for the qualitative analysis of NO was applied, but using the probe 4,5-diamino-fluoresceindiacetate (DAF-2DA). Relative fluorescence was measured using a fluorescence plate reader (SynergyMx, Biotek, USA) at excitation and emission wavelengths of 495 and 515 nm, respectively. The relative fluorescence values were corrected by the number of cells in each treatment.

Estimation of epigallocatechin gallate (EGCG) in GT. The estimation of EGCG in GT was assessed by HPLC (Waters, USA) using an EGCG standard, as previously reported (28).

Statistical analysis. All experiments were independently performed three times and the results are expressed as means ± SD. One-way analysis of variance (ANOVA), followed by the Bonferroni test, were used to compare the groups. A value of $p < 0.05$ was considered significant. All analyses were performed using statistical software StatView (SAS Institute Inc, NC, USA).

RESULTS

Physiological characteristics. Body weight gain was lower in the diabetic rats than in the control rats. The kidney to body weight ratio was significantly less in the diabetic animals than in the control animals. SBP was similar in all groups. Blood glucose concentration was greater in the diabetic rats than in the control rats, but it was not affected by GT (Table 1).

Renal histopathology. MME was greater in SHR DM rats than control rats, and this abnormality was reversed by GT treatment ($p = 0.03$) (Figure S1A-B).

NO_X^- levels and eNOS expression in renal cortical tissue. Kidney homogenates from the diabetic rats had significantly lower levels of NO_X^- compared to the control rats ($p = 0.02$), which was reversed by GT treatment ($p = 0.05$) (Figure 1A).

Modulation of NO bioavailability was also assessed via analysis of eNOS expression and its phosphorylation status. Renal cortical expression of eNOS did not differ between the studied rats (Fig. 1B-C). Diabetic SHR rats demonstrated increased phospho-Thr495 eNOS expression ($p = 0.002$), which was reversed by GT treatment ($p = 0.005$) (Figure 1B and D). Thr495 represents the major negative regulatory site of eNOS, and it is constitutively phosphorylated in cultures in many endothelial cell types (29). On the other hand, the expression of phospho-Ser1177 eNOS in renal cortical homogenates increased in the diabetic SHR rats ($p = 0.03$), and GT consumption reduced its expression ($p = 0.05$) (Fig 1B and E). Ser1177 thus appears to be the most important positive regulatory domain in eNOS. Finally, the percentage of dimer/monomer ratio, an indicative of eNOS uncoupling, showed a significant decrease in diabetic rats ($p = 0.02$), which was reestablished by GT treatment ($p = 0.05$) (Figure 1F-G).

Urinary and renal cortical levels of total biopterin and BH_4, and the percentage of BH_4 oxidation in the control and diabetic animals. BH_4 and total biopterin levels in renal cortical homogenates ($p < 0.002$) and urine ($p < 0.0001$) decreased in the DM SHR rats compared to the control SHR rats (Figure 2A-B). GT significantly abrogated the reduction of BH_4 in the renal cortex ($p = 0.0008$), and the urinary levels of BH_4 with GT tended to increase in the diabetic SHR rats (Figure 2A-B). Similar results were obtained for total biopterin levels (Figure 2A-B). Moreover, the percentage of oxidation of BH_4 to BH_2 was increased in the diabetic rats compared to the control rats ($p = 0.03$ in the renal cortex and $p = 0.05$ in urine), whereas GT treatment abolished the oxidation of BH_4 (Figure 2C-D).

Effects of high glucose and green tea on NO production in ihMCs. Qualitative and quantitative analyses of NO production showed that the ihMCs kept in the HG medium for 24-h had a significant reduction ($p = 0.03$) in fluorescence intensity compared to cells kept

in the NG medium, whereas GT (100 μg/ml) reversed the high glucose-induced reduction in NO ($p = 0.02$) (Figure 3A-B). In Western blot analysis the eNOS expression not change between the groups in ihMCs (Figure 3C-D). But the eNOS Dimer/monomer ratio was decreased in ihMCs cultured under high glucose conditions and improved by GT treatment (Figure 3E-F).

Effects of high glucose in ROS production in ihMCs. Qualitative and quantitative analyses (Figure 4A-B) showed a significant rise in ROS production after exposing the ihMCs to HG levels for 24-h ($p = 0.0001$) in comparison to NG levels. To evaluate the sources involved in the HG-induced ROS production in the ihMCs, we analyzed ROS production at HG in the presence of DPI (50 nM, a blocker of NADPH-oxidase), L-NAME (100 μM, an inhibitor of NOS enzymes) and rotenone (10 μM, an inhibitor of mitochondria electron transport complex I). Fluorescent microscope (Figure 4A) and fluorometer (Figure 4B) data showed that DPI reversed HG-induced ROS production to levels lower than those of NG ($p = 0.03$), suggesting that NADPH oxidase is one main source of superoxide in ihMCs. DPI reduces ROS production even in cells cultured in NG. This observation may explain the reduction of ROS in cells under HG to below control levels (Figure 4B). Furthermore, incubation of ihMC with L-NAME significantly reduced HG-induced superoxide production ($p = 0.04$), suggesting that eNOS uncoupling is an important source of ROS production. Finally, incubation with rotenone reduced HG-induced superoxide production, though it failed to reach statistical significance ($p=0.07$). Mannitol (30 mM), which was used as an osmotic control, did not alter ROS production. These results imply that both NADPH-oxidase and uncoupling eNOS are important sources in the HG-induced ROS production in ihMCs.

Effects of BH_4 in ROS and NO production at HG in ihMCs. We observed a significant reduction in total biopterin and BH_4 levels in ihMCs kept in the HG medium ($p = 0.05$) in comparison to cells in the NG medium (Figure 5A), which was reversed by GT ($p = 0.04$). Furthermore, there was an increase in oxidation of BH_4 to BH_2 in cells kept in the HG medium ($p = 0.01$), and this also was reversed after GT treatment ($p = 0.01$) (Figure 5B). We next assessed the role of BH_4 in ROS and NO production in ihMCs. Exogenous

administration of BH_4 in ihMCs kept in the HG medium (Figure 5C) reduced the HG-induced rise in ROS production in a concentration-dependent manner, although this only reached significance at 100 μM ($p = 0.004$). Moreover, measurement of NO via DAF-2DA (Figure 5D) showed that BH_4 at all concentrations used ($p = 0.001$) reversed the HG-induced decline in NO production ($p = 0.007$). These findings may imply that a reduction in BH_4 levels may be involved in HG-induced ROS production and a decline in NO formation.

Effects of HG and GT in GTPCH I activity and DHFR function in ihMCs. To investigate the mode of action of HG-induced reduction in BH_4 levels, we next assessed the activity of GTPCH I by HPLC. Our results showed that there was a significant reduction in GTPCH I activity ($p = 0.03$) in ihMCs kept in the HG medium (Figure 6A) in comparison to cells kept in the NG medium. Interestingly, GT reversed the HG-induced decline in GTPCH I activity ($p = 0.009$) (Figure 6A). To further assess the hypothesis that GT reestablished GTPCH I activity in ihMCs, we cultured these cells in HG with GT (100 μg/ml), and after 1h, we supplemented the medium with DAHP, an inhibitor of GTPCH I. We observed that the presence of the GTPCH I inhibitor abrogated the beneficial effect of GT, i.e., it increases ROS production (Figure 6E) and decreases NO bioavailability (Figure 6F), in a concentration dependent manner. These observations further support the concept that GT improved NOS uncoupling by reestablishing the GTPCH I activity and synthesis of BH_4. To test the integrity of BH2-BH4 recycling in ihMC under HG, the cells were kept with HG in the presence of BH_2, and ROS production was evaluated. Analysis (Figure 6B) of ROS production showed that BH_2 reduced the HG-induced ROS production in a concentration-dependent manner, although this was only significant at 10 μM and 100 μM ($p = 0.0001$). Analysis of NO production showed that the exogenous administration of BH_2 significantly blunted the HG-induced decline of NO production ($p = 0.03$) (Figure 6C). These data may further suggest that HG in ihMCs reduces the production of BH_4 via a decline in GTPCH I activity at the *de novo* synthesis pathway of BH_4 formation. On the other hand, HG does not affect BH_4 production via the recycling pathway, since BH_2 ameliorated both the high glucose-induced rise in ROS production and the decline in NO formation.

Effects of GT in ROS production at high glucose in ihMCs. In ihMCs, assessment of ROS production showed that GT reversed the HG-induced rise in ROS formation to NG levels (Figure 7A-B) ($p = 0.0001$). Co-treatment of ihMCs with GT and BH_4 (Figure 7C) did not confer an additional reduction in ROS production in comparison to GT treatment alone. This suggests that GT may inhibit HG-induced ROS production through a rise in BH_4 levels.

Content of EGCG in GT and the EGCG effect in ihMC. When compared with the EGCG standard, we identified this polyphenol as an important constituent of GT. The EGCG retention time was 8.891 minutes (Figure S2A), consistent with the retention time 8.894 minutes, the main peak observed in GT (Figure S2B). Assessment of the effect of EGCG in ihMC exposed to HG has shown that this component of GT can reduce ROS production (Figure S2C) and increase NO bioavailability (Figure S2D). These observations suggest that the main effect of GT may be attributed, at least partly, to EGCG.

DISCUSSION

The present study aimed to explore GT's potential to ameliorate kidney-uncoupling eNOS in diabetic conditions. We also investigated the mechanisms by which GT reversed uncoupling eNOS. We observed, both *in vivo* and *in vitro*, an uncoupling of eNOS secondary to a reduction of BH_4 and elevation of its oxidized form with a consequent decrease in NO and increase in oxidative stress. GT reestablished the levels of BH_4, reduced its oxidized form, coupled eNOS, and consequently increased NO bioavailability and reduced oxidative stress. Our *in vitro* data also suggest that the reestablishment of GTPCH I activityis the main mechanism by which GT increased BH_4 and coupled eNOS. These observations are of great interest. First, they reinforce the importance of uncoupling eNOS and BH_4 synthesis/oxidation in reducing NO bioavailability, and increase oxidative stress, two conditions involved in the pathogenesis of DN (1-8). In addition, for the first time, the mechanism by which GT can couple eNOS in DM is described. Together with previous observations that GT can reduce oxidative stress and improve indices of diabetic

nephropathy in rats (5), data from this translational study demonstrate the possible beneficial use of GT or its main flavonol, EGCG, in patients with DN.

Bioavailability of nitric oxide in the diabetic kidney has been a subject of major controversy (7,8). Earlier studies have suggested that NO production increases and contributes to glomerular hyperfiltration in short-term diabetes (30,31). More recently, it has been suggested that endothelial dysfunction, which is often defined as a decrease in the bioavailability of endothelial-derived NO, is a preponderant factor in diabetes and contributes to the pathogenesis of DN (6-8,17). To this end, researchers have demonstrated that the knockout of eNOS in diabetic mice leads to severe histological lesions in the kidney, which resemble the lesions seen in human DN, but which are not seen in the control wild type diabetic mice (32). In addition, hypertensive type 2 individuals subjected to an acute reduction in NO bioavailability, by means of blockade of NO synthesis with L-NAME, displayed an elevated albumin excretion rate, a hallmark of DN (33). Therefore, it seems that a reduction in NO bioavailability is the predominant abnormality in DM. It has become apparent that eNOS phosphorylation at Thr495 or Ser1177, rather than eNOS expression, are crucial parameters in estimating NOS production by eNOS (7,8). Interestingly, in our diabetic rats, we observed an increase in inactivation of eNOS via a rise in phosphorylated eNOS at Thr495, which is known to lead to a reduction in electron transfer in eNOS, thus diminishing NO production (34). Surprisingly, in the same model we also observed a rise in the expression of phosphorylated eNOS at Ser1177, which is known to enhance eNOS activity (8). These observations are in agreement with a recent study showing that phosphorylation at Thr495 results in a less active eNOS, even in the context of phosphorylation of eNOS at Ser1177 (35). In our study, GT reversed diabetes-induced alterations in phosphorylated Th495, Ser1177 eNOS and dimer/monomer ratio and hence improved NO bioavailability.

It is well known from previous studies that enzymatic coupling of eNOS by BH_4 plays a critical role in the maintenance of NO bioavailability (6,12.16,36,37). For example, endothelial function improved after the exogenous administration of BH_4 in stroke-prone SHR rats via an improvement of eNOS coupling, and hence, a rise in NO bioavailability (37). In our studies, we observed a reduction in BH_4 levels and a rise in the oxidation rate of BH_4 to BH_2 in both the renal cortex and urine of diabetic SHR rats. A recent study suggested that BH_4 oxidation, rather than decreased BH_4, is the main determinant of uncoupling eNOS (11,38). In our study, GT treatment restored the levels of BH_4 and the diabetes-induced oxidation of BH_4 to BH_2 in the renal cortex and urine of diabetic SHR rats. Therefore, GT seems to reverse diabetes-induced eNOS uncoupling, thereby increasing NO bioavailability via a rise in BH_4 availability. This is further supported by our observation that co-treatment of GT and BH_4 did not confer additional protection against HG-induced ROS production in comparison to GT treatment alone. The ihMCs exposed to high glucose displayed a rise in ROS production and a decline in intracellular NO production. Two sources appeared to mediate HG-induced ROS production-NADPH oxidase and uncoupled eNOS, since blocking each one abolished the HG-induced rise in ROS production. Our studies are in agreement with previous work showing that uncoupling eNOS and NADPH oxidase in the glomeruli of rats with experimental DN are the major sources of superoxide mediated by the loss of BH_4 availability (6).In agreement with the importance of reduction in BH4 availability, exogenous administration of BH_4 in ihMCs abolished ROS production and reversed the decline of NO under HG levels. This finding suggests that ihMCs kept in HG mediums exhibit low levels of BH_4, leading to uncoupling of eNOS and a subsequent rise in ROS and decline in NO levels. This is further supported by our finding that BH_4 levels decreased and the oxidation of BH_4 rose to BH_2 in ihMCs kept in the HG medium.

Our present work further showed that ihMCs kept in the HG medium exhibited reduced levels of BH_4 in comparison to cells kept in the NG medium, due to the diminished *de novo* synthesis pathway of BH_4 formation, since activity of GTPCH I was reduced in cells cultured in HG. Diminished GTPCH I activity has also been reported in rats with experimental DN, leading to reduced BH_4 formation via the *de novo* synthesis (39). In

addition, the observation that a GTCH I blocker abrogated GT's effect in ROS and NO production in ihMCs exposed to HG and treated with GT further supports the concept that GT acts by improving GTPCH I activity. Our work also indicated that the recycling pathway of BH_4 is probably preserved in ihMCs exposed to HG, since BH_2 supplementation decreased ROS and increased NO bioavailability by enhancing BH_4 levels via DHFR. These findings are in agreement with previous *in vivo* (11) and *in vitro* studies (11,38) showing that DHFR plays a key role in regulating the BH_4 : BH_2 ratio and eNOS coupling under conditions of low total BH_4 availability. For example, in endothelial cells (38,40) expressing eNOS with low BH_4 levels, DHFR inhibition or knockdown further diminished the BH_4 : BH_2 ratio and exacerbated eNOS uncoupling.

Reduced BH_4 availability in ihMCs could also be attributed to reduced BH_4 stability. Diminished BH_4 stability has been reported in endothelial dysfunction (41), as well as in rats with DN (6). HG levels, as seen in diabetic conditions, increase the formation of superoxide through one main source in the kidney, NADPH oxidase activation. NO produced by eNOS and superoxide combine to form peroxynitrite anions. Oxidation of BH_4 by ROS, such as peroxynitrite, results in BH_2 formation, which inactivates the eNOS cofactor function, suggesting that reduced BH_4 stability uncouples eNOS, leading to reduced NO bioavailability and a further rise in the formation of diabetic glomeruli superoxides.

In conclusion, the current work indicates that GT reverses diabetes-induced uncoupling eNOS as experienced in renal mesangial cells exposed to HG levels and SHR diabetic rats. GT seems to ameliorate uncoupling eNOS via a rise in BH_4 levels/reduction in BH_4 oxidation, which occurs as a result of the *de novo* synthesis of BH_4. A rise in BH_4 levels would account, then, for reduced eNOS uncoupling leading to the amelioration of oxidative stress and enhanced NO availability.

ACKNOWLEDGMENTS

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Table 1 - Physiological characteristics of studied animals

Groups	**Initial body weight (g)**	**Final body weight (g)**	**Systolic blood pressure (mmHg)**	**Glycemia (mmol/L)**
CT SHR	277.2±17	345.5±13	204.3±10	8.4±1
DM SHR	277.6±7	192.2±38*	203.5±8	29.5±2*
DM SHR GT	269.7±15	194.9±25*	198.9±10	30.5±5*

CT SHR: control SHR; DM SHR: diabetic SHR; DM SHR GT: diabetic SHR treated with green tea; *p<0.0001 vs control SHR group.

LEGENDS

Figure 1: (A) Nitrite (NO_2^-) and nitrate (NO_3^-), the stable NO end products, were quantified as a measurement of NO levels in renal cortical homogenates by Griess reaction. Results were corrected for the protein concentration and are expressed as µmol NOx-/mg protein. *p = 0.02 vs. SHR CT; †p = 0.05 vs. SHR DM. (B) Representative Western blots of the renal cortical eNOS and p-eNOS (Thr495) expression from SHR control rats, SHR diabetic rats, and SHR diabetic rats treated with green tea. (C) Densitometric analysis of the eNOS/β-actin ratio, (D) p-eNOS (Thr495)/β-actin ratio (*p = 0.002 vs SHR CT, †p = 0.005 vs SHR DM) and (E) p-eNOS (Ser1177)/β-actin ratio (*p = 0.03 vs SHR CT, †p = 0.05 vs SHR DM), in the three different SHR groups of rats. Bars represent means ± SD. (F) Representative Western blots of the renal cortical of eNOS dimer and monomer expression from SHR control rats, SHR diabetic rats, and SHR diabetic rats treated with green tea. (C) Densitometric analysis of the percentage of eNOS dimer/monomer ratio (*p = 0.02 vs SHR CT, †p = 0.05 vs SHR DM).

Figure 2. (A and B) Total biopterin (black bars) and BH_4 (grey bars) expression analysis by UPLC (A) in renal cortex (Results were corrected for the protein concentration and expressed as nM / mg protein. * p = 0.002 vs. SHR CT, †p < 0.0001 vs. SHR DM, ‡p < 0.0001 vs. SHR CT) and (B) in urine. Results were corrected for 24-h urine volume. *p < 0.0001 vs. SHR CT. (C and D) Representative graphs of percentage of BH_4 to BH_2 oxidation (C) in renal cortex, * p = 0.01 vs. SHR CT, † p = 0.03 vs. SHR DM and (D) in urine. *p = 0.004 vs. SHR CT, †p = 0.05 vs. SHR DM.

Figure 3: (A) Representative micrographs of DAR-4M AM indicating NO production. ihMCs were cultured for 24-h in normal glucose (5.5 mM), high glucose (30 mM), and high glucose with green tea (100 µg/ml). (B) Quantification of NO levels in ihMCs via DAF-2DA. Values are mean ± SD and expressed as the percentage of fluorescence. Values were corrected by the number of cells at the end of each treatment. *p = 0.03 vs. NG, †p =

0.02 vs. HG. (C) Representative Western blots of the ihMCs of eNOS expression from ihMCs cultured under NG, HG, and HG treated with green tea for 24h. (D) Densitometric analysis of eNOS/β-actin ratio. (E) Representative Western blots of the ihMCs of eNOS dimer and monomer expression from ihMCs cultured under NG, HG, and HG treated with green tea for 24h. (F) Densitometric analysis of the percentage of eNOS dimer/monomer ratio (*p = 0.04 vs NG, †p = 0.05 vs HG).

Figure 4. (A) Representative micrographs of ihMCs probe with H_2DCF-DA indicating ROS production. Cells were cultured in normal glucose, HG, HG plus green tea, HG plus DPI, and HG plus L-NAME. (B) Quantification of total intracellular ROS levels and an assessment of enzymatic sources of ROS production in ihMCs. HMC was cultured for 24-h in normal glucose (5.5 mM) and high glucose (30 mM) in the presence and absence of green tea. Mannitol was used as an osmotic control; L-NAME, an inhibitor of NOS, diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, and rotenone, an inhibitor of mitochondrial complex I, were also used at high glucose levels to define the enzymatic sources of ROS production. Values are mean ± SD and expressed as the percentage of fluorescence. Values were corrected for the number of cells at the end of each treatment. *p < 0.0001 vs. NG, †p = 0.04 vs. HG, ‡p = 0.03 vs. HG.

Figure 5. (A) Total biopterin (black bars) and BH_4 (grey bars) expression analysis by UPLC in ihMCs lysate. Results were corrected for the protein concentration and expressed as nM/mg protein. *p = 0.05 vs. NG, †p = 0.02 vs. HG, ‡ p = 0.04 vs. HG. (B) Representative graphs of percentage of BH_4 to BH_2 oxidation in ihMCs lysate. *p=0.01 vs. NG, †p = 0.01 vs. HG. (C) Quantification of total intracellular ROS levels by H_2DCF-DA via a fluorimeter after incubation of ihMCs for 24 h in NG, HG mediums in the presence also of BH_4 (1 µM, 10 µM and 100 µM). *p = 0.0001 vs NG, †p = 0.004 vs HG. (D) NO levels were also quantified after incubation with DAF-2DA via a fluorimeter. *p = 0.007 vs NG and †p = 0.001 vs HG. The bars represent mean ± SD. Values are expressed as the

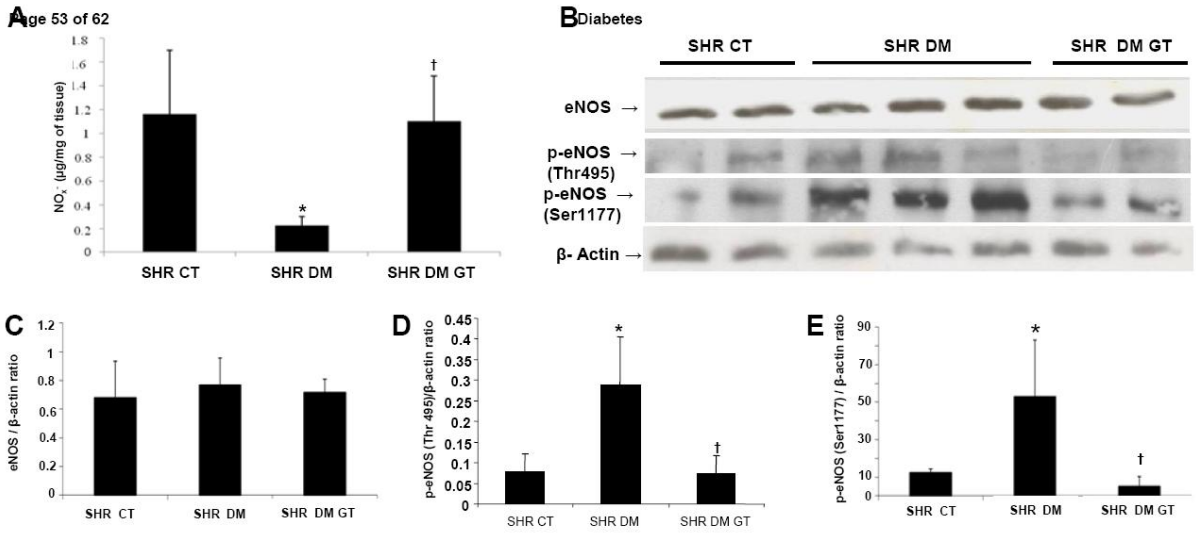
percentage of fluorescence units, and they were corrected by the number of cells at the end of each treatment.

Figure 6: (A) GTP-cyclohydrolase I (GTPCH I) activity was measured in HPLC by the concentration of neopterin in cell lysate. Results were corrected for the protein concentration and expressed as the percentage of pmol/ug protein. The levels of neopterin indicate GTPCH activity. The ihMCs were cultured for 24-h in normal glucose (5.5 mM), high glucose (30 mM), and high glucose with green tea (100 µg/ml). *p = 0.03 vs. NG, †p = 0.009 vs. HG. (B) Representative micrographs of H_2DCF-DA in ihMCs indicating ROS production. ihMC were kept for 24-h in NG, HG medium in the presence also of BH_2 (10 µM). (C) ROS measurement in ihMCs supplemented with BH_2 (1 µM, 10 µM, and 100 µM). *p = 0.0001 vs. NG, †p = 0.03 vs. HG. (D) Quantitative analysis of intracellular NO levels were also carried out after incubation with DAF-2DA. *p = 0.007 vs. NG, †p = 0.001 vs. HG. ROS measurement in ihMCs supplemented with BH_2 (1 µM, 10 µM, and 100 µM). *p = 0.0001 vs. NG, †p = 0.03 vs. HG. (E) ROS measurement in ihMCs pre-treated with HG and GT and supplemented with DAHP (100 µM, 500 µM, and 1 mM). *p = 0.0001 vs. NG, †p = 0.0001 vs. HG, ‡p = 0.0003 vs. HG. (F) Quantitative analysis of intracellular NO levels in ihMCs pre-treated with HG and GT and supplemented with DAHP. *p = 0.0004 vs. NG, †p = 0.0001 vs. HG, ‡p = 0.002 vs. NG. The bars represent mean ± SD and values are expressed as the percentage of fluorescence units. Values were corrected for the number of cells at the end of each treatment.

Figure 7: (A) Representative micrographs of H_2DCF-DA in ihMCs indicating ROS production. ihMCs were kept for 24-h in NG, HG mediums in the presence also of GT (100 µg/ml). (B) Quantification of total intracellular ROS levels by H_2DCF-DA via a fluorimeter was also carried out *p = 0.0001 vs. NG, †p = 0.0001 vs. HG. (C) ihMCs were cultured in HG and supplemented with GT (100 µg/ml) and BH_4, followed by measurement of

intracellular ROS by H_2DCF-DA. *$p < 0.0001$ vs. NG, $^{\dagger}p < 0.0001$ vs. HG. The bars represent mean ± SD and values are expressed as the percentage of fluorescence units.

Figure 1


A
Page 53 of 62
NOx (µg/mg of tissue)
SHR CT
SHR DM
SHR DM GT
B
Diabetes
SHR CT
SHR DM
SHR DM GT
eNOS →
p-eNOS → (Thr495)
p-eNOS → (Ser1177)
β- Actin →
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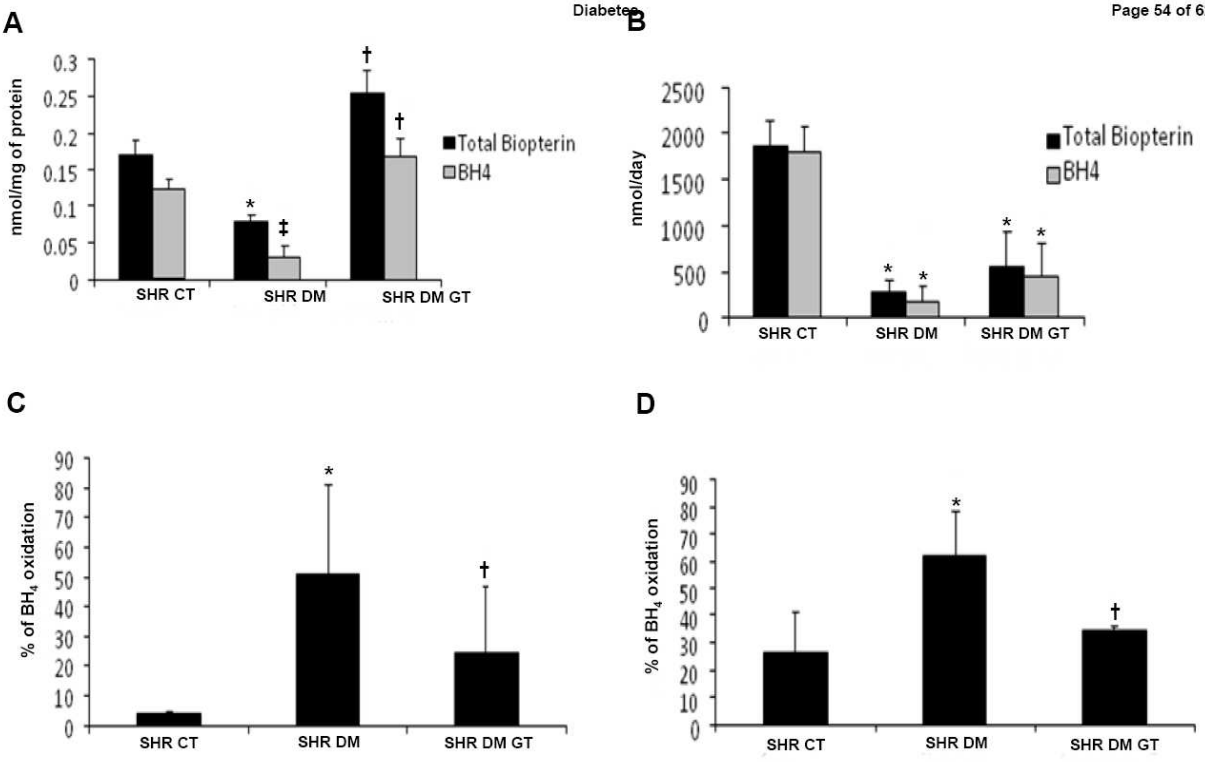
eNOS / β-actin ratio
D
p-eNOS (Thr 495)/β-actin ratio
E
p-eNOS (Ser1177) / β-actin ratio

Figure 2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0.25
0.2
0.15
0.1
0.05
0
Total Biopterin
BH4
SHR CT
SHR DM
SHR DM GT
B
nmol/day
2500
2000
1500
1000
500
0
Total Biopterin
BH4
SHR CT
SHR DM
SHR DM GT
C
% of BH4 oxidation
90
80
70
60
50
40
30
20
10
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SHR CT
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SHR DM GT
D
% of BH4 oxidation
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SHR CT
SHR DM
SHR DM GT

Figure 3

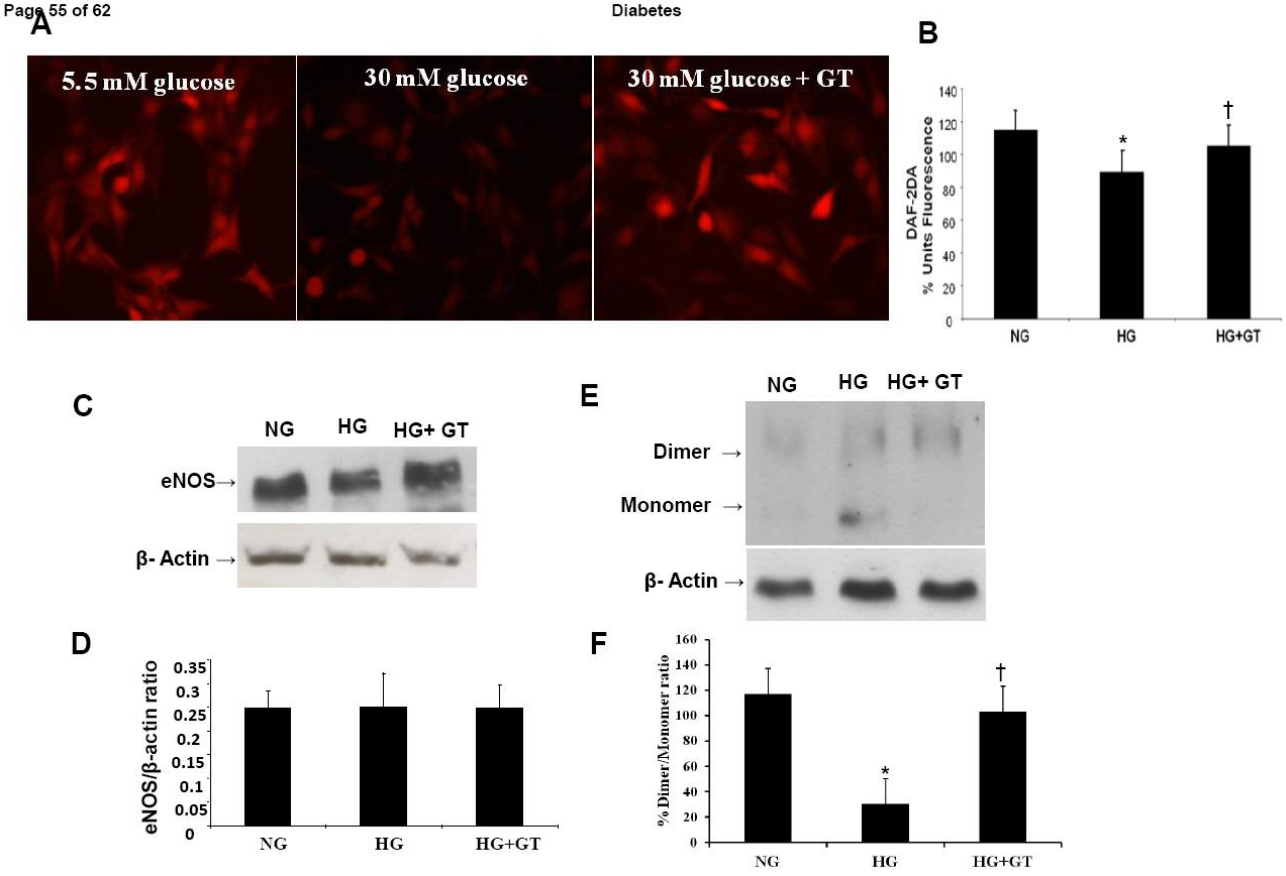

A
5.5 mM glucose
30 mM glucose
30 mM glucose + GT
B
DAF-2DA % Units Fluorescence
NG
HG
HG+GT
C
NG
HG
HG+ GT
eNOS→
β- Actin →
D
eNOS/β-actin ratio
NG
HG
HG+GT
E
NG
HG
HG+ GT
Dimer →
Monomer →
β- Actin →
F
% Dimer/Monomer ratio
NG
HG
HG+GT

Figure 4

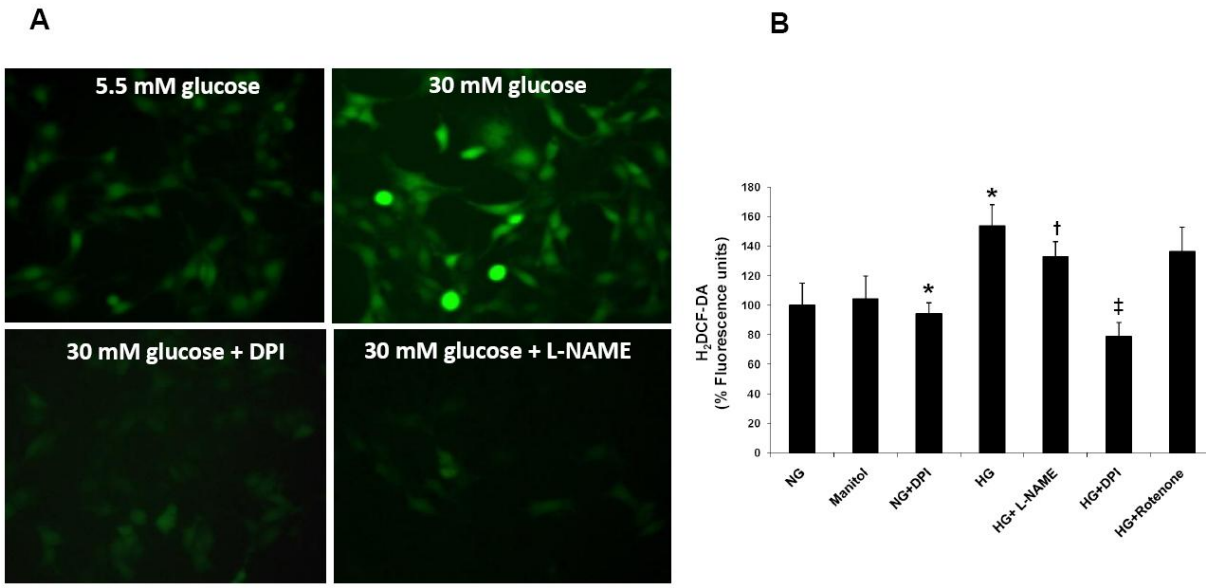

A
5.5 mM glucose
30 mM glucose
30 mM glucose + DPI
30 mM glucose + L-NAME
B
H2DCF-DA
(% Fluorescence units)
0
20
40
60
80
100
120
140
160
180
*
*
†
‡
NG
Manitol
NG+DPI
HG
HG+ L-NAME
HG+DPI
HG+Rotenone

Figure 5

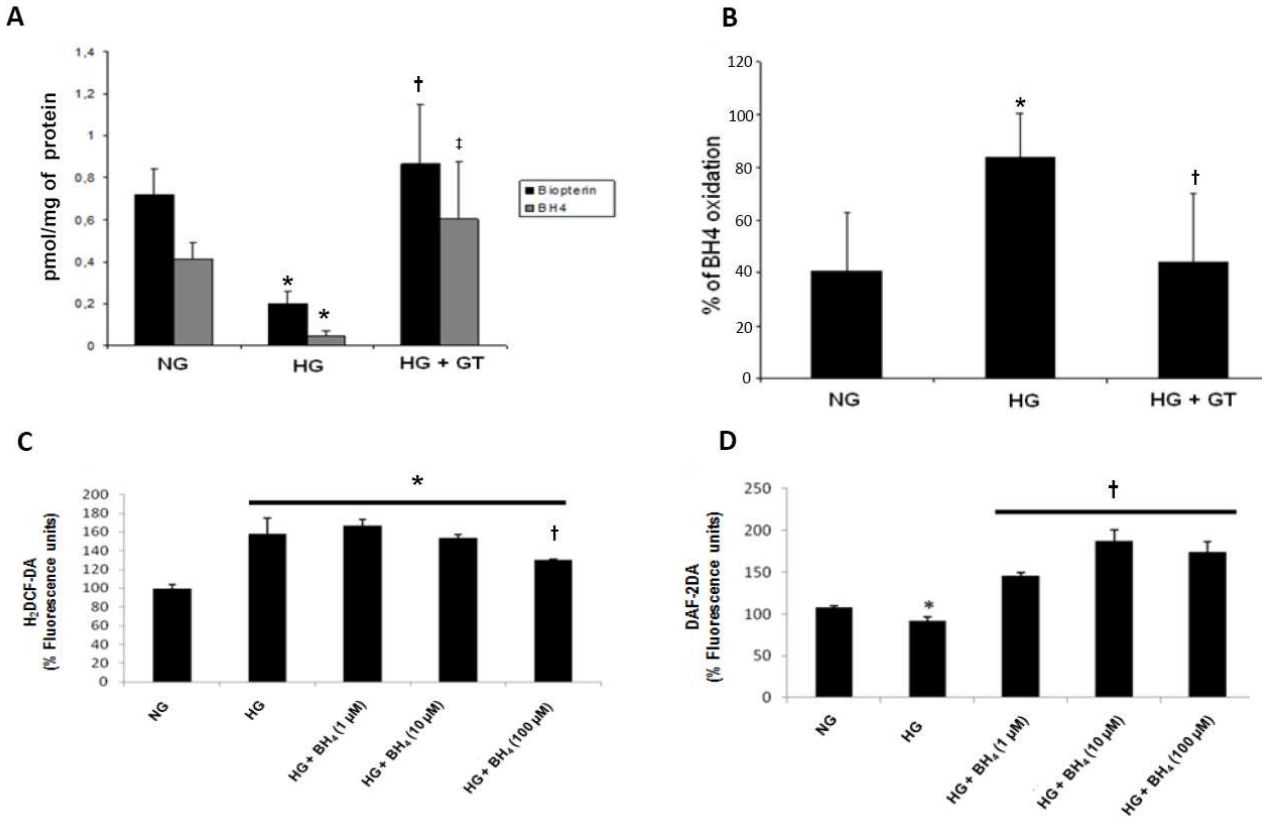

A
pmol/mg of protein
Biopterin
BH4
NG
HG
HG + GT
B
% of BH4 oxidation
NG
HG
HG + GT
C
H2DCF-DA
(% Fluorescence units)
NG
HG
HG + BH4 (1 µM)
HG + BH4 (10 µM)
HG + BH4 (100 µM)
D
DAF-2DA
(% Fluorescence units)
NG
HG
HG + BH4 (1 µM)
HG + BH4 (10 µM)
HG + BH4 (100 µM)

Figure 6

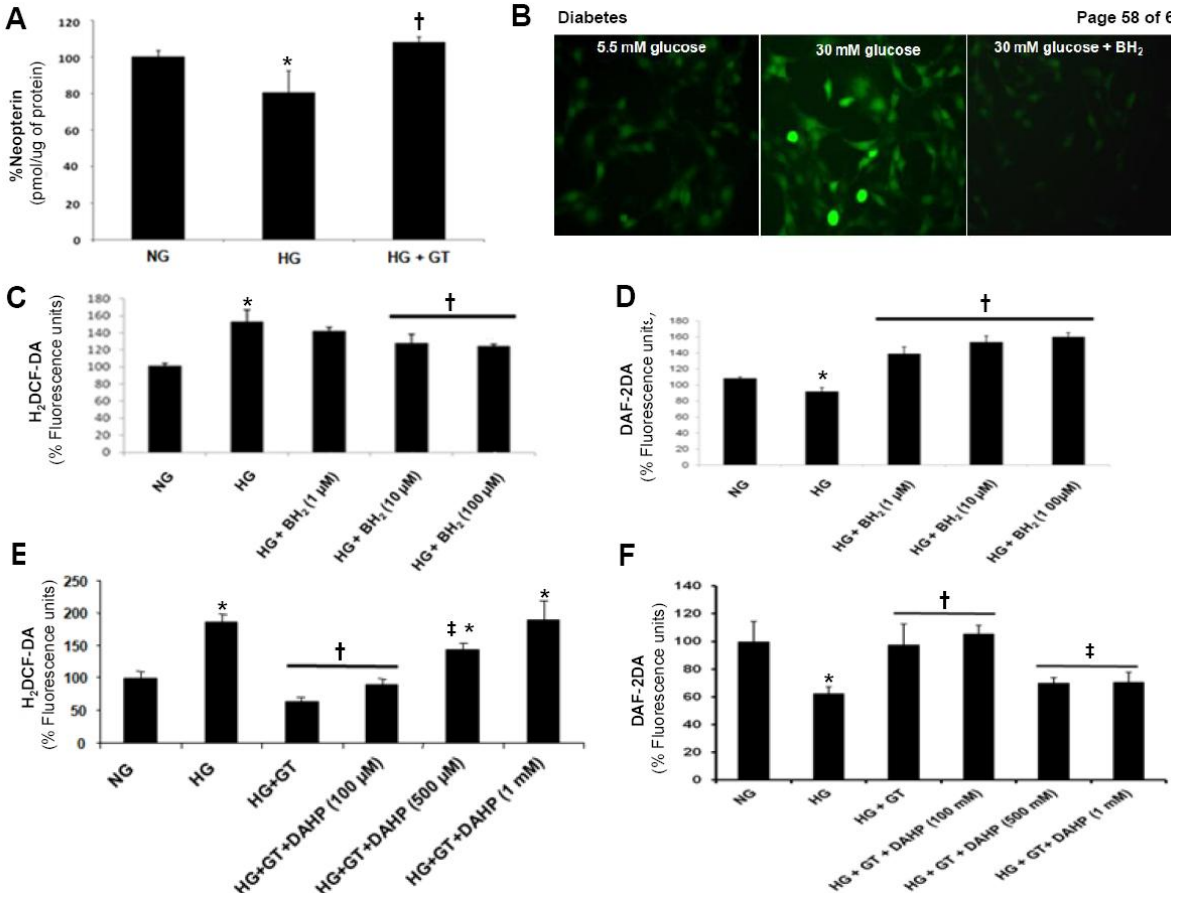
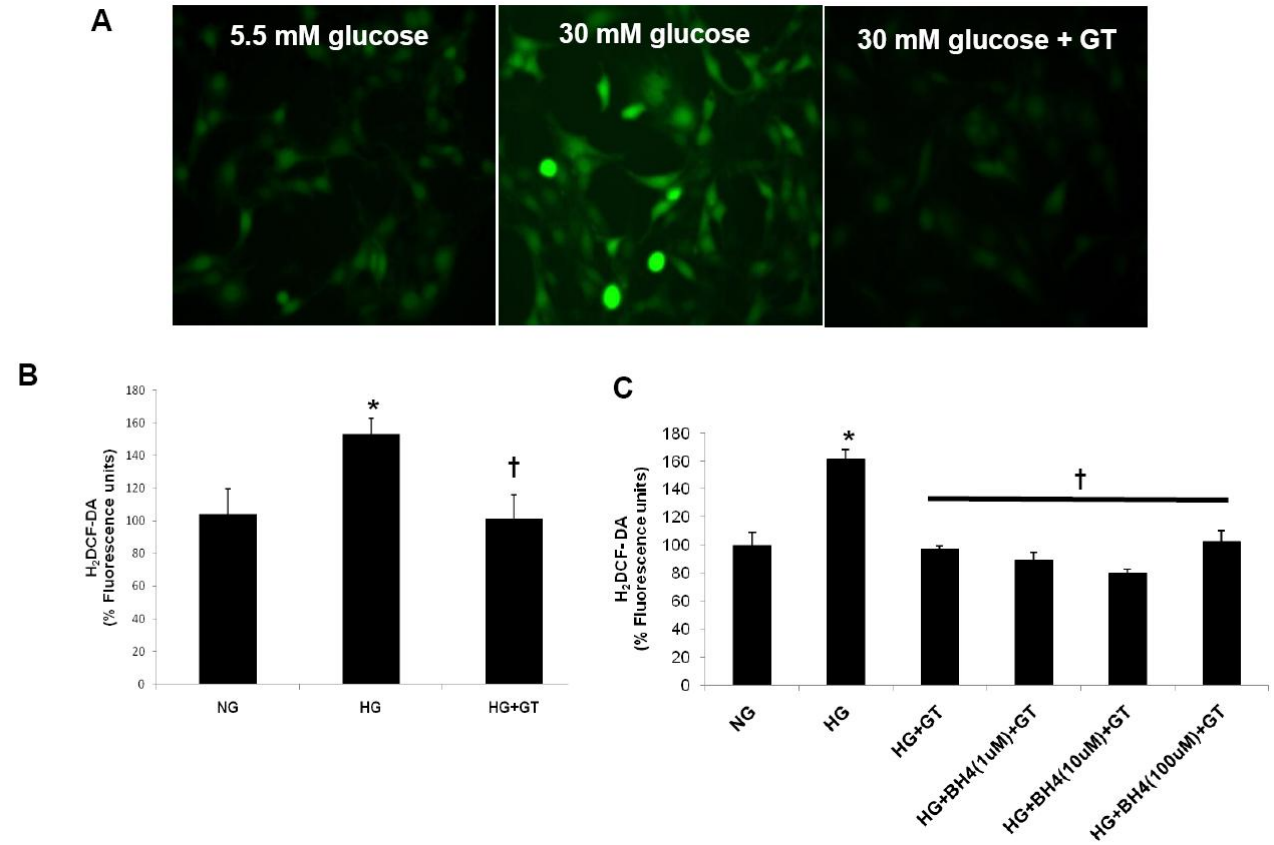

A
%Neopterin (pmol/ug of protein)
NG
HG
HG + GT
B
Diabetes
Page 58 of 6
5.5 mM glucose
30 mM glucose
30 mM glucose + BH2
C
H2DCF-DA (% Fluorescence units)
NG
HG
HG + BH2 (1 μM)
HG + BH2 (10 μM)
HG + BH2 (100 μM)
D
DAF-2DA (% Fluorescence units)
NG
HG
HG + BH2 (1 μM)
HG + BH2 (10 μM)
HG + BH2 (100μM)
E
H2DCF-DA (% Fluorescence units)
NG
HG
HG+GT
HG+GT+DAHP (100 μM)
HG+GT+DAHP (500 μM)
HG+GT+DAHP (1 mM)
F
DAF-2DA (% Fluorescence units)
NG
HG
HG + GT
HG + GT + DAHP (100 mM)
HG + GT + DAHP (500 mM)
HG + GT+ DAHP (1 mM)

Figure 7

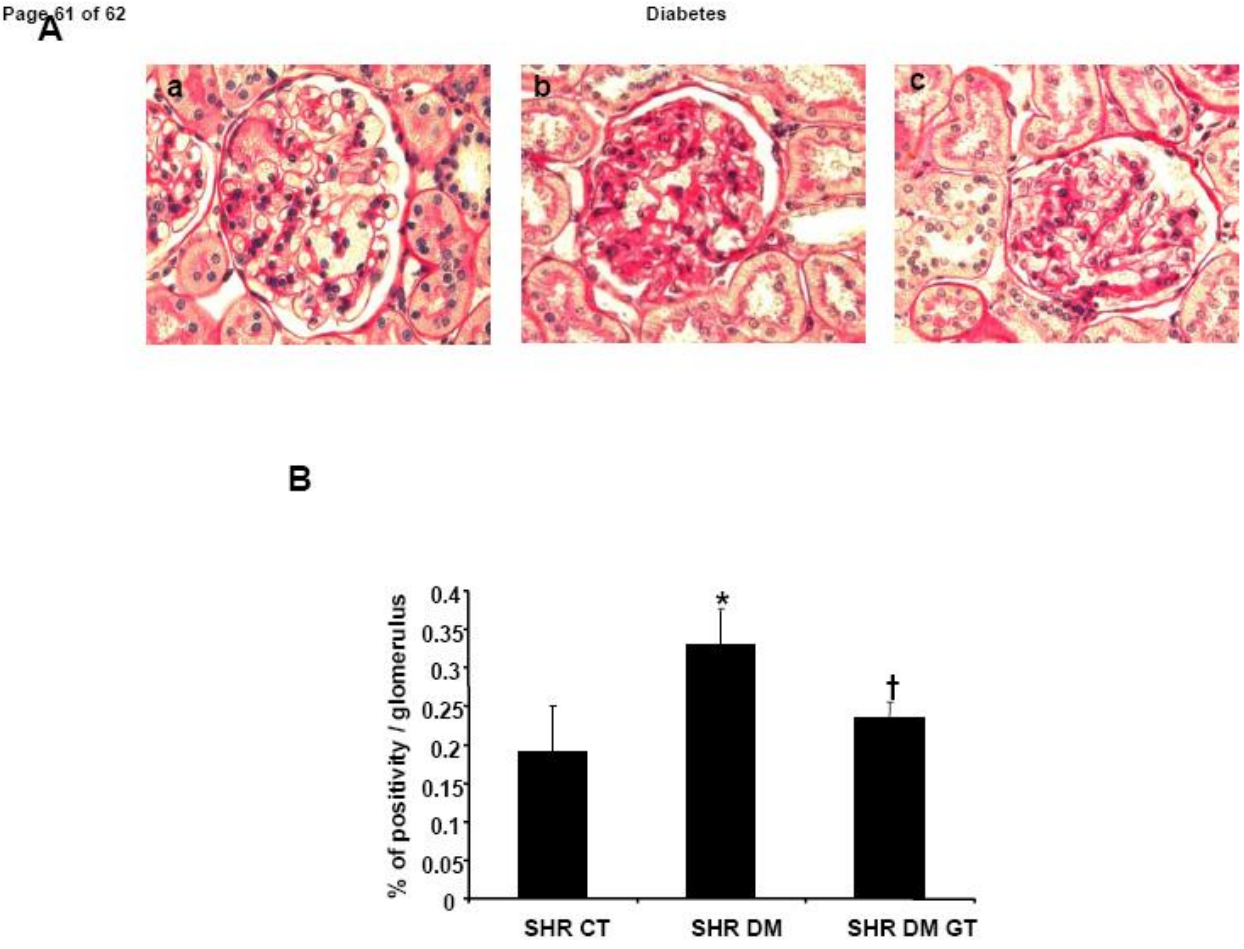

A
5.5 mM glucose
30 mM glucose
30 mM glucose + GT
B
H2DCF-DA (% Fluorescence units)
180
160
140
120
100
80
60
40
20
0
*
†
NG
HG
HG+GT
C
H2DCF- DA (% Fluorescence units)
180
160
140
120
100
80
60
40
20
0
*
†
NG
HG
HG+GT
HG+BH4(1uM)+GT
HG+BH4(10uM)+GT
HG+BH4(100uM)+GT

LEGENDS OF SUPPLEMENTAL FIGURES:

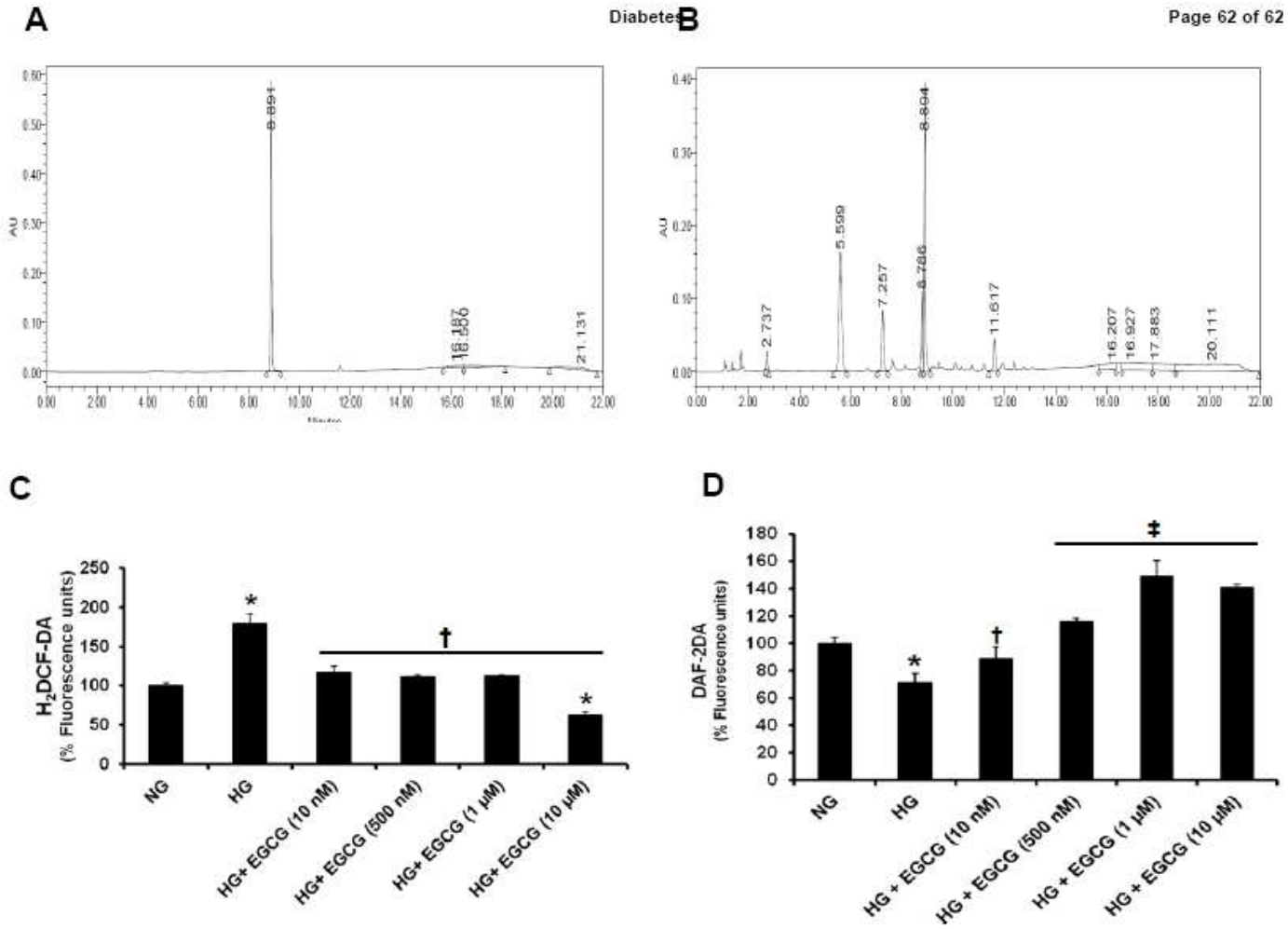
Figure S1: (A) Representative PAS stained glomerular histopathology in SHR CT (a), SHR DM (b) and SHR DM GT (c). (B) Quantification of matrix mesangial expansion (MME) was obtained using the software Leica Application Suite (LAS Image Analysis). The region of positive PAS staining was expressed as a function of total glomerular tuft area. Data are expressed as mean ± SEM. *p = 0.01 vs. NG, †p = 0.05 vs. HG.

Figure S2: (A) Chromatography analyses of standard epigallo-cathechin gallate. (B) Chromatography analyses of green tea. (C) Quantification of total intracellular ROS levels by H2DCF-DA via a fluorimeter was carried out *p = 0.0001 vs. NG, †p = 0.0001 vs. HG. (D) Quantification of total intracellular NO levels by DAF-2DA via a fluorimeter. *p = 0.0002 vs. NG, †p = 0.009 vs. HG, ‡p = 0.0001 vs. HG. The bars represent mean ± SD and values are expressed as the percentage of fluorescence units.

SUPPLEMENTAL FIGURE 1 (Figure S1)



SUPPLEMENTAL FIGURE 2 (Figure S2)



CAPÍTULO 3

1. SUMÁRIO

No diabetes experimental em modelos *in vivo* e *in vitro* a eNOS é desacoplada diminuindo a produção de NO e se tornando uma importante fonte na produção de superóxido.

O desacoplamento da eNOS parece ser mediado pela redução dos níveis de BH_4.

O chá verde reverte o desacoplamento da eNOS e a redução dos níveis de BH_4 induzido pelo DM. Este último efeito ocorre através da ação do chá verde no restabelecimento a atividade do GTPCH I.

2. CONCLUSÃO

Em conclusão o chá verde aumenta a biodisponibilidade do NO pelo restabelecimento da produção de BH_4, acoplamento da eNOS e reduzindo o estresse oxidativo, alterações envolvidas na patogênese da nefropatia diabética.

CAPÍTULO 4

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CAPÍTULO 5

1. Apêndice (Artigo submetido)

Green tea-derived polyphenols protect against neurodegeneration in diabetic retinopathy

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Running title: GTPs neuroprotection in diabetic retina

Word count: 4199

Tables: 01

Figures: 07

ABSTRACT

Epigallocatechin gallate, the most active compound in green tea (GT) was demonstrated to be neuroprotective against brain ischemia. In this study, beneficial properties of GT polyphenols (GTPs) were demonstrated in diabetic retina and in retinal cells under diabetic milieu conditions. Spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats were induced diabetes and assigned to receive or not oral administration of GTPs for 12 weeks. The protective effects of GTPs were also evaluated in Müller and in ARPE-19 cells. In diabetic rats, there was increase in glial fibrillary acidic protein (GFAP) and oxidative retinal markers and decrease in occludin levels. Diabetic SHR rats also demonstrated blood-retinal barrier breakdown and impaired electroretinogram. Under high glucose, Müller cells presented increase expression of GFAP, decrease in glutamate transporter and receptor with augment in glutamine synthetase levels and reactive oxygen species (ROS) production with reduced glutathione content. ARPE-19 cells presented increase ROS production with decrease claudin-1 and glutamate transporter expressions. Treatment with GTPs fully restored all the above-mentioned alterations. The antioxidant potential of GTPs exerted retinal protection via glutamate cycle. These findings reveal novel mechanism by which GTPs protect the retina against neurodegeneration in disorders such as diabetic retinopathy.

Diabetic retinopathy (DR) is classical chronic microvascular complication of the retina caused by the deleterious metabolic effects of hyperglycemia, exhibiting an extensive and early neurodegeneration (1). Neuroretinal degeneration will initiate several metabolic and signaling pathways which will participate in the microvasculopathy process as well as in the disturbance of the blood-retinal barrier (BRB) (a key phenomenon in the pathogenesis of DR) (2).

Müller cell is the predominant glial cell in the retina, interacting with most neurons in a symbiotic relationship (3). They constitute the anatomical and functional link between retinal neurons and also play a critical role in the maintenance of the BRB (4), and the blood flow (5). They modulate the neuronal excitability and transmission, via release of gliotransmitters and other neuroactive substances (6), neurotransmitter recycling and release of precursors of neurotransmitters to the neurons. Glutamate is the major retinal excitatory neurotransmitter for the photoreceptor-bipolar-ganglion cell circuit and is toxic when in high concentrations causing neurodegeneration (7). Müller cells are intimately involved in regulating extracellular glutamate levels in the central nervous system (CNS) because possess high affinity glutamate uptake systems, converting glutamate to glutamine, a non-neurotoxic substance (8). Clearance of glutamate from the extracellular space is accomplished primarily by the action of glutamate transporters (9). GLAST, the high-affinity l-glutamate/l-aspartate transporter located in Müller cells is the only glial-type glutamate transporter in the retina. In studies with GLAST-deficient mice, the electroretinogram is deeply depressed and retinal damage is exacerbated. These evidences demonstrate that GLAST is required for normal signal transmission between photoreceptors and bipolar cells and that plays a neuroprotective role during ischemia in the retina (10).

GLAST is essential not only to keep the extracellular glutamate concentration below a neurotoxic level but also to maintain glutathione levels by transporting glutamate, which is substrate for glutathione synthesis into Müller cells, a major antioxidant defense in the retina. This is the primary route for uptake of cysteine (11), the rate-limiting substrate for the synthesis of glutathione (12). As retinal concentration of glutathione is decreased in GLAST-deficient mice, glutamate neurotoxicity and oxidative stress are involved in retinal degenerative disease (13).

Retinal pigmented epithelium (RPE) is a specialized epithelium lying in the interface between the neural retina and the choriocapillaris and is essential constituent of outer BRB. It plays an important role in the proper functioning and maintenance of the neural retina controlling the flow of solutes and fluid preventing accumulation of extracellular fluid in the subretinal space of the retina (14). The human retinal epithelial cell ARPE-19 is a spontaneously immortalized cell line that has been commonly used as a model for the outer BRB because it has been demonstrated to have structural and functional properties characteristic of the *in vivo* RPE cells. Of interest, a high-affinity glutamate transporter EAAC1, an excitatory amino acid transporter expressed by neurons in the CNS (15), has been found in RPE cells. Recent studies have demonstrated EAAC1$^{-/-}$ mouse exhibits a chronic mild neuron-specific oxidative stress (16). It is believed that RPE cells through its EAAC1 receptor, take part in regulation of the glutamate concentration in the subretinal space and also act as primary route for uptake of cysteine. However, a possible role of EAAC1 receptor in ARPE-19 cells under high glucose conditions has not been addressed.

Green tea (GT; *Camellia sinensis*) has been widely studied for its alleged beneficial properties in the treatment or prevention of human diseases. GT is a rich source of

polyphenols, particularly flavonoids. (-)-Epigallocatechin gallate (EGCG), the most active compound in GT, are reported to delay or prevent certain forms of cancer, arthritis, cardiovascular and other disorders (17) and also display a strong antioxidant activity (17-19). In addition, EGCG is a neuroprotective agent against brain ischemia (20). Recently has been described one mechanism of neuroprotection of EGCG through reestablishment of NMDA receptor-ROS mediated in an experimental model of Alzheimer disease (21). To our knowledge, the effects of GT polyphenols (GTPs) on experimental diabetic retinopathy have not been adequately investigated.

In the present study we sought to evaluate whether oral administration of GTPs can protect diabetic retina from the toxic effects of hyperglycemia and to access the underlying mechanisms through *in vitro* studies. We showed that GTPs abrogated the retinal alterations presented in diabetic rats and the retinal neuroprotective effect was through the reestablishment of glutamate cycle in Müller and ARPE-19 cells.

METHODS

Animal study

Spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats were provided by Taconic (Germantown, NY); care, use and treatment of all animals were in strict agreement with the guidelines of the Statement for the Use of Animals in Ophthalmic and Vision Research (ARVO), and approved by the local Committee for Ethics in Animal Research (CEEA/IB/Unicamp).

Diabetes was induced in 12-week-old male SHR and WKY rats with a single intravenous injection of streptozotocin (STZ, 50 mg/kg; Sigma, St. Louis, MO, USA) or vehicle as control. After induction, the diabetic rats were randomly assigned to receive or not oral treatment of GTPs (4,5g/kg body weight/day, Midori Industry of the Tea) instead of drinking water. The characterization of GT was done by high-performance liquid chromatography (HPLC) (22). During the study, the diabetic rats received two units of insulin (human insulin HI-0310; Lilly) three times per week subcutaneously. Twelve weeks after the induction of DM, the rats were submitted to electroretinography and then euthanized, and the retinas collected.

Immunohistochemistry for glial fibrillary acidic protein (GFAP), occludin, nitrotyrosine (NT) and neuronal nitric oxide synthase (phospho-nNOS Ser847) in retinal tissues

Paraffin embedded sections were incubated with goat polyclonal anti-GFAP (Santa Cruz Biotechnologies, Santa Cruz, CA), rabbit polyclonal anti-occludin (Invitrogen Corporation, Camarillo, CA), rabbit polyclonal anti-NT (Upstate Cell Signaling Solutions, Lake Placid, NY), or rabbit polyclonal to phospho-nNOS (Ser847) (Abcam Inc, Cambridge, MA) overnight at 4ºC. Subsequently the slides were incubated with appropriated secondary antibodies. The analyses were determined using Leica Application Suite (LAS Image Analysis).

Western blotting analysis for nNOS, endothelial nitric oxide synthase (eNOS) and Cu/Zn superoxide dismutase enzyme (Cu/Zn-SOD) in retinal tissue

The retinas were lysed in a RIPA buffer supplemented with a protease inhibitor cocktail. Membranes were incubated with primary antibodies for anti-phospho-nNOS (Ser847) (Abcam), anti-nNOS (Cell Signaling, USA), anti-phospho-eNOS (Ser1177) (Cell Signaling), eNOS/NOS type III (BD Transduction Laboratories, San Diego, CA) and Cu/Zn-SOD (Upstate). Subsequently the membranes were incubated with appropriated secondary antibodies. ß-actin was used as internal protein loading control.

Cyclic Guanosine Monophosphate (cGMP) enzyme-linked immunosorbent assay (ELISA)

The samples were prepared for the ELISA in accordance with a commercial cGMP ELISA assays (Cayman Chemical Company, Ann Harbor, MI). Protein concentrations were measured by the Bradford method (23).

BRB permeability in whole-mounted retinas

The BRB was performed by Evans blue method as previously reported (24). The retina was dissected and spread on clean glass slides and mounted in medium (Vectashield; Vector Laboratories, Burlingame, CA) vitreous-side up under coverslips. Retinal flat mounts were analyzed by confocal laser scanning microscope (CLSM, LSM510; Zeiss) using appropriate emission filters, to evaluate the BRB breakdown sites. Digital images were captured using specific software (LSM; Zeiss) and compared using a semi quantitative scale.

Full-Flash Electroretinogram (ERG) Recording

SHR animals had their retinal function measured at the end of the study using the UTAS-E3000 system (UTAS-E3000, LKGTechnologies Inc., Gaithersburg, MD) as previously described with some modifications (25). The pupils were dilated with tropicamide (Mydriacyl 0.5%; Allergan) and data collection occurred after general anesthesia with a mixture of ketamine and xylazine (75 and 7.5mg/kg, respectively) under dim red illumination (λmax = 650 nm); measurements were taken after overnight dark adaptation (>12 hours). ERG waveforms were recorded using a positive corneal custom made contact lens gold electrode, negative and reference stainless steel inserted under the skin of the head and in the tail, respectively. A dark-adapted 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.

In vitro studies

Primary rat retinal Müller cells

Enrichment of Müller cells was performed according to the previously described method (26). Cells were treated for 72 hours with 5.5mM $_D$-glucose (NG), 25mM $_D$-glucose (HG), HG plus 100 µg/ml of GT leaves (HG+GTPs) and NG + 19.5 mM of mannitol, as an osmotic control.

Transformed rat Retinal Müller cells line (rMC-1)

rMC-1, kindly donated by Dr. V.J. Sarthy (Northwestern University, Evanston, IL) were cultured with the same conditions as in primary Müller cells for 24, 48 and 72 hours. When

cells were 70% confluent, the proliferation rate was slowed down by reducing the concentration of FBS to 2%.

ARPE-19 cell line culture

ARPE-19 cells were obtained from Federal University of Rio de Janeiro (RJCB Collection). The experiments were conducted under NG and HG media, HG plus GTPs (100µg/ml), HG plus (-)Epigallocatechin Gallate in different concentrations (HG+EGCG at 10, 25 and 50 µM) and NG plus 19.5 mM of mannitol for 24 hours.

Cells were cultured in DMEM and Ham's F12 (DMEM:F12) supplemented with 10% FBS and 1% penicillin/streptomycin. When cells were 70% confluent, the proliferation rate was slowed down by reducing the concentration of FBS to 1%.

Before perform the *in vitro* studies, Thiazolyl Blue Tetrazolium Bromide (MTT) assay (27) was conducted in order to use only safe concentration for the retinal cells, i.e. below of 10% of cell toxicity.

Western blot

The protein cell lysate from primary Müller cells was obtained from scraped cells lysed in a RIPA buffer containing protease inhibitor; membranes were incubated with anti-GLAST (Alpha Diagnostic International, San Antonio, TX, USA), anti-N-methyl-D-aspartate receptor (NMDAR1-Millipore, Temecula, USA) and anti-glutamine synthetase (GS) (Millipore) with the appropriated secondary antibodies.

For ARPE-19 cells, protein was extracted with RIPA buffer in 1 mM PMSF, 2 mM Na_3VO_4, 100 mM NaF containing protease inhibitor; the membranes were hybridized with anti-claudin-1 (Invitrogen) and anti-EAAC1 (Alpha Diagnostic International) with the appropriated secondary antibodies.

Immunofluorescence assays

Primary rat Müller cells and ARPE-19 cells were seeded in cover slip of glass (12 mm of diameter) (Deckglaser, Knittel Glaser, Germany). After the treatments, cells were fixed in 2% paraformaldehyde or 100% methanol, washed and blocked. Primary Müller cells were stained with anti-GFAP antibody (Santa Cruz) and ARPE-19 were stained for claudin-1 (Invitrogen) followed by appropriated secondary antibody; then rinsed and cover-slipped with Vectashield containing 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Vector) or propidium iodide (Sigma) for nuclei staining. The sections were examined with a confocal laser scanning microscope (Zeiss), using appropriate emission filters. Digital images were captured using specific software (Zeiss).

Determination of intracellular ROS production

Intracellular ROS levels were measured as previously described (22), using the fluorogenic substrate DCF-DA, in which the fluorescent probe, 2,7-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma) is converted by intracellular esterases to DCFDA. Briefly, cells were grown on 96-well black plate. The cells were incubated for 30 min with 10 µM DCFDA (Invitrogen) in Hank's buffer. Relative fluorescence was measured using a

fluorescence plate reader (SynergyMx, Biotek, USA) at excitation and emission wavelengths of 485 and 528 nm, respectively.

Qualitative assessment of ROS was carried out in the ARPE-19 cells after 24 hours of treatment. Then, the cells were incubated for 30 min with 10 μM DCFDA in Hank's buffer, washed with PBS and imaged using a fluorescence microscope (Olympus IX-71).

Determination of reduced glutathione (GSH) levels

GSH levels in rMC-1 culture were measured using method described previously (22). The GSH concentration expressed as μM GSH/ μg protein. GSH was used for preparation of a standard curve.

Real-Time PCR in Müller cells

The Real-Time PCR was performed as previously (28). Total RNA from Müller cells was isolated with Trizol™ reagent (Invitrogen). RNA concentration and purity were determined by spectrophotometry (GeneQuant pro, GE Healthcare, Buckinghamshire, UK). Primers for the Glast (Glut1) and Nr1 (Grin1) gene were obtained from Applied Biosystems (Rn00570130_m1 and Rn01436034_m1). The glyceraldehyde 3-phosphate dehydrogenase (Gapdh) gene was used as controls (Applied Biosystems).

Statistical Analysis

The results were expressed as the means ± SD. The groups were compared by one-way analysis of variance (ANOVA), followed by the Fisher protected least-significant difference test. For ERG analyses, area under the curve (AUC) was used to compare the

ERG responses from each animal and compared by ANOVA, followed by the Fisher protected least-significant difference test. Statistical differences were considered significant at $P<0.05$

RESULTS

Animal study

GTPs treatment did not alter body weight or blood glucose levels

The final body weight was lower and blood glucose levels were higher in diabetic rats compared with nondiabetic groups ($p=0.002$ and $p<0.0001$, respectively) and the systolic blood pressure was higher in SHR than in WKY rats ($p<0.0001$) (Table 1). The GTPs treatment did not alter any of the physiological parameters.

The early markers of DR were ameliorated with oral administration of GTPs

In this study, we used GFAP and occludin expressions as indexes of glial reactivity and BRB breakdown, respectively, accepted as early retinal changes in diabetes. The presence of diabetes markedly exacerbated GFAP staining in both rat strains throughout the retinal tissue ($p=0.001$ and $p=0.0003$, for WKY and SHR, respectively) (figure 1A) and clearly decreased expression occludin compared with control rats ($p=0.03$ and $p=0.01$, for WKY and SHR, respectively) (figure 1B). The treatment with oral GTPs prevented these alterations observed in diabetic rats, remaining similar to control groups, suggesting protective effects of GTPS in the early phases of DR.

Oxidative stress was prevented in diabetic rats treated with GTPs

The nitration of tyrosine, an effect of peroxynitrite on proteins, was accessed by NT expression. The NT staining become strengthen throughout the retinal layers in diabetic compared with control animals ($p=0.002$ and $p=0.04$, for WKY and SHR, respectively). GTPs completely re-established the retinal nitrosative status to nondiabetic rats levels ($p=0.01$ and $p=0.04$, for WKY and SHR, respectively) (figure 2A). There was a tendency to decrease the expression of Cu/Zn-SOD, an important antioxidant defense, in diabetic SHR rats but did not reach significant levels. Only in SHR animals treated with GTPs, there was a marked elevated levels compared with non-treated groups ($p=0.0006$) (figure 2B).

The constitutive nNOS is impaired in diabetic SHR rats

Synaptic activity regulates production of Nitric oxide (NO), catalyzed by nNOS through pathways involving the phosphorylation. Phosphorylation of nNOS at Ser847 (phospho-nNOS Ser847) by the calcium-calmodulin protein kinase II inhibits its activity (29). Herein, we observed that in WKY rats there was no alteration in the expression of nNOS (total and phosphorylated forms) among the studied groups ($p>0.05$). By contrast, diabetic SHR rats presented a clear increase in the expression of phospho-nNOS compared with control animals ($p<0.0001$) and the treatment with GTPs re-established this expression to normal levels ($p=0.01$) (figure 3A). The phospo-nNOS staining exhibits a scattered pattern located mainly in the inner plexiform layer and is more intense in DM-SHR rats (figure 3B). These findings suggest that the changes in phospho-nNOS were dependent on the presence of hypertension. Probably, the exacerbation of retinopathy by hypertension involves inactivation of nNOS. But further studies are necessary in order to better clarify these results.

The cGMP levels in diabetic retina were re-established by administration of GTPs

Levels of NO in biological systems are controversial but evidence suggests that NO levels in the range 0.2–2.0 nM, activates soluble guanylate cyclase to produce cGMP and upregulate Akt signaling (30) thus preventing cell death (31). To access a possible link between nNOS and diabetic neurotoxicity, we quantified the levels of cGMP in retinal tissue by ELISA assay. In DM-SHR rats we observed a reduction in the cGMP levels compared with CT-SHR rats ($p=0.003$). The treatment with GTPs prevented this decrement ($p<0.05$) (figure 3C).

DM induced activation of constitutive eNOS and this change was prevented by treatment with GTPs

The NOS system in the retina tissue was also assessed by constitutive eNOS for understanding the contribution of eNOS for the BRB breakdown. It was already described that the activation of eNOS along with inflammatory markers represents important contributor to the BRB breakdown (32). NO produced by eNOS is essential for increased vascular permeability induced by vascular endothelial growth factor (VEGF) (33). In DM-SHR rats, there was an increase of phospho-eNOS Ser1177 (active form, $p=0.02$) and this expression was prevented by GTPs ($p=0.05$). The total form of eNOS remained unaltered among the groups (figure 4A).

The inner BRB breakdown, a key phenomenon in the pathogenesis of RD was ameliorated by oral GTPs

The inner BRB was estimated in SHR rats by Evans blue method in flat mounted retinas. We observed multiple and extensive vascular leakage in retina from diabetic rats compared with control; the treatment with GTPs significantly ameliorated this effect (figure 4B).

Retinal function revealed an important protective effect of GTPs in treated diabetic rats

It was observed a significant decrease in amplitude with an increased implicit time of *b*-wave and markedly reduced oscillatory potentials in DM-SHR rats compared with CT-SHR ($p>0.05$). The treatment with oral GTPs alleviated the retinal responses indicating an improvement of inner retinal function ($p=0.03$)

In vitro studies

Primary Müller cells

GFAP was used either as a marker of Müller cell as an index of glial reaction. In presence of HG medium, there was a marked increase of GFAP expression and treatment with GTPs abolished this increment (figure 5A).

The glutamate/glutamine cycle inside the Müller cells was accessed by expressions of transporter GLAST, receptor NMDAR1 and the glutamate metabolizing enzyme, GS. After 72 hours in HG, GLAST and NMDAR1 protein levels were significantly decreased when compared with NG medium ($p<0.05$) accompanied by a compensatory increase in GS levels ($p=0.01$); the GTPs treatment prevented these alterations (figure 5B-D). Similarly, mRNA for NMDAR1 evaluated through quantitative real-time PCR showed decreased levels in HG medium ($p=0.002$); the treatment with GTPs increased this level but did not achieve statistical significance.

Rat MC-1 (rMC-1) cells

To address the relationship between oxidative stress and neurodegeneration, we evaluated the oxidative balance in Müller cells and glutamate pathway. First, we evaluated the time course effect of HG medium in ROS production. In 48 and 72 hours of treatment the increments of ROS production were not significant (supplemental figure 1). Therefore, the experiments were conducted at 24 hours of treatment.

The rMC-1 cell exposed to HG medium presented increased ROS production in comparison with NG ($p=0.01$) and the presence of GTPs at 10 and 100 µg/ml prevented this increment ($p=0.001$ and $p<0.0001$ vs HG, respectively) (figure 6A). At HG conditions, GSH level, depiction of glutathione defense system present in these cells, showed a decrease in its levels compared with NG ($p=0.001$); GTPs treatments (10 and 100µg/ml) recovered this parameter to NG levels ($p=0.006$) (figure 6B). To verify whether the impairment of GSH system is due to compromised uptake of cysteine in HG conditions, we treated the cells with N-acetylcysteine (NAC, 1mM), a direct intracellular cysteine donor. As expected the supplementation of cysteine fully re-established the GSH ($p=0.1$ vs NG); by contrast, the treatment with tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, 1 mM), a superoxide dismutase mimetic did not affect the GSH levels ($p=0.5$ vs HG). These findings indicate that in HG conditions, rMC-1 cells present a marked inadequacy in oxidative defense probably due to deficiency in cysteine. Aligned with the oxidative stress parameters, the gene expressions of Glast and Nr1 depicted a significant decrease which were re-established with GTPs (10 and 100ug/ml for Glast and 100ug/ml for Nr1, $p<0.05$ and $p=0.03$ respectively) (figure 6C,D). Collectively, the impairment of glutamate transporter GLAST

and receptor NMDAR1 with a compensatory upregulation of metabolizing enzyme GS in HG condition, restrained the cystein uptake diminishing the GSH levels and increasing ROS production; the treatment with GTPs re-establishing both glutamate receptor and transporter, protecting these glial cells against HG induced oxidative stress.

ARPE-19 CELLS

Since ARPE-19 cells express glutamate transporter (EAAC1) and represent an *in vitro* model of outer BRB, we evaluated whether ARPE-19 cells exposed to HG in presence of GTPs showed restoration of the expression of EAAC1 and the integrity of tight junction and total ROS production. Similarly observed in Müller cells, there was an increased production of total intracellular ROS in presence of HG ($p<0.0001$) and the concomitant treatment with GTPs or EGCG counteracted this effect ($p<0.0001$) (figure 7A). Concomitant, the protein expression of EAAC1 decreased and the presence of GTPs markedly increased its levels ($p=0.03$) (figure 7B). Finally, the integrity of intercellular junction, estimated by claudin-1 expression, demonstrated an expressive decrease in HG medium ($p=0.02$) and GTPs treatment restituted to normal conditions ($p=0.03$) (figure 7C-D). Combined, these observations indicate that GTPs treatment in HG conditions protected ARPE-19 cells from oxidative stress, ameliorating the glutamate receptor EAAC1 thus restoring the intercellular junction structure.

DISCUSSION

The present study aimed to evaluate the potential protective effect of GTPs as oral antioxidant in experimental diabetic retina and to access the underlying mechanisms. In

animal study, GTPs were able to ameliorate the structural lesions present in DR and also to maintain the retinal function evaluated by electroretinography similar to control groups. In *in vitro* studies, GTPs played a pivotal role in glutamine/glutamate cycle in Müller cells re-establishing the glutamate transporter/NMDAR1 status, restoring the cysteine route thus preventing the oxidative stress related excitotoxicity present in HG conditions. In ARPE-19 cells, the GTPs treatment re-established the intercellular junction and prevented the decrease in EAAC1 transporter expression. From the present study, our results provide novel evidence that GTPs, possible EGCG, act as a potent neuroprotector in diabetic retina and also in retinal cells.

The importance of NO in neurodegeneration relies on the evidence that in diabetic retina there is a correlation between the localization of NO production and the nNOS immunoreactivity in plexiform layers, nNOS expression is decreased and its subcellular localization is altered (34). Expression of nNOS-immunoreactive amacrine cells was also demonstrated to be in close proximity to the retinal vasculature and the levels was reduced after one week of diabetes and remained decreased in long term diabetic rats; this depletion of nNOS-containing neurons may contribute to alterations in the autoregulation of blood flow which occurs in DM (35). One regulator of nNOS is the NMDAR, consisting of NR1 and NR2 subunits which is targeted at excitatory synapses (36). NO derived from NOS system activates soluble guanylyl cyclase and increases cyclic GMP production resulting in retinal transduction (37). Therefore, the levels of cGMP could be taken as an index of neuroprotection, since neurons expressing nNOS are dependent on their NO for survival via cGMP-dependent activation of the Akt survival pathway (38). In this study, the increase in

phospho-nNOS acompained by decrease in cGMP levels in diabetic animals were reverted by oral administration of GTPs. To our knowledge this is the first report demonstrating that oral GTPs improves NO/cyclic GMP pathway in models of diabetic retinal disease. As a result, there is a full retinal function protection in experimental model of DR. Aligned with our findings, Zhang and collaborators demonstrated in a model of retinal ischemia that orally administered EGCG attenuated the injuries to the retina caused by ischemia/reperfusion, suggesting a neuroprotection in glaucoma disease in a rat model (39).

To investigate the role of oxidative stress in glutamate/glutamine cycle imbalance, we evaluated the ROS production and the reduced glutathione levels in primary Müller cells and cell line under HG conditions and treated with GTPs. The presence of GTPs at 10-100µg/ml reduced ROS formation with increasing in reduced glutathione levels, preventing the oxidative stress induced by HG conditions; Glast and Nr1 mRNA levels were re-establishing. This piece of information indicates that in HG, glutamate transporter/receptor impairment leads to oxidative stress and in presence of GTPs, the glutamate uptake is restored thus preventing oxidative damage. Concordant with our findings, Zeng and collaborators verified that in high glucose-treated Müller cells there was a decreased GLAST expression and treatment with taurine, a retinal amino acid, avoided degradation under diabetic conditions via its antioxidant mechanism (40). Studies by Siu and collaborators with mass spectrometry has shown that in retinal homogenates, the treatment with glutamate induced lipid peroxidation and the presence of catechin, a polyphenol presents in GTPs, significantly reversed the changes in thioredoxin peroxidase, 5-hydroxytryptamine receptor, peroxiredoxin 6 and pyruvate thus protecting the retinal tissue from glutamate-induced lipoperoxidation (41). The antioxidative efficacy of catechin was

described to be approximately 11-fold compared with trolox against the glutamate-induced lipoperoxidation (41).

In ARPE-19 cells (an immortalized RPE cell line), our results suggest that the upregulation of claudin-1 by GTPs may be involved in the outer BRB sealing. The functional consequences and clinical applicability of these observations need further studies. More recently, the role of outer BRB breakdown in the pathogenesis of diabetic retinopathy is gaining importance. Data from Simo's group have demonstrated that the complexity of this barrier and that occludin, ZO-1 and claudin-1 might have different attributes in outer BRB barrier (42).

In conclusion, the results from the current study are novel and describe the neuroprotective effects of GTPs in animal model of diabetic retinopathy and *in vitro* studies mimicking diabetic conditions. GTPs prevented the early markers of DR, oxidative stress imbalance and restored neuronal NOS isoform, thus preserving the retinal function in diabetic hypertensive rats. Also, GTPs ameliorate the oxidative imbalance and glutamate pathway in retinal Müller and ARPE-19 cells. In addition, these findings enhance our understanding of how oxidative stress impairs the glutamate/glutamine cycle in different retinal cells.

AUTHOR CONTRIBUTIONS

K.C.S.[1] and M.A.B.R. acquisition of data, wrote manuscript, reviewed/edited manuscript. D.E.H and K.C.S. [2] study design, reviewed manuscript. A.M.F contributed to discussion and reviewed manuscript. P.A.O.R performed experiments. J.M.L.F. and J.B.L.F. study design, reviewed the data, wrote manuscript, reviewed/edited manuscript.

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J.M.L F, J.B.L.F, K.C.S. are guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1 - Physiological characteristics of studied animals

Groups	Initial body weight (g)	Final body weight (g)	Systolic blood pressure (mmHg)	Glycemia (mmol/L)
CT-WKY	322.3±22	498.7±48	143.7±5	8.9±0.4
DM-WKY	331.2±16	384.6±66[#]	139.8±3	28.5±5[π]
DM-WKY GTPs	325.6±16	367.2±42[#]	137.5±8	27.4±7[π]
CT-SHR	277.2±17	345.5±13	204.3±10[¶]	8.4±1
DM-SHR	277.6±7	192.2±38[*]	203.5±8[¶]	29.5±2[*]
DM-SHR GTPs	269.7±15	194.9±25[*]	198.9±10[¶]	30.5±5[*]

CT-WKY: control WKY; DM-WKY: diabetic WKY; DM-WKY GTPs: diabetic WKY treated with GTPs; [#]p=0.002 vs control WKY group, [π]p<0.0001 vs control WKY group. CT-SHR: control SHR; DM-SHR: diabetic SHR; DM-SHR GTPs: diabetic SHR treated with GTPs; [*]p<0.0001 vs control SHR group, [¶]p<0.0001 vs WKY group.

LEGENDS

Figure 1: *GTPs regulate the early markers of diabetic retinopathy, including GFAP and occludin levels.*

(A) Representative photomicrograph of immunohistochemistry of glial reactivity by glial fibrillary acidic protein (GFAP) in rat retinas from WKY and SHR. In non-diabetic rat retina, there was a light retinal glial reaction mainly in astrocytes. After 3 months of diabetes, we can observer a marked staining throughout the retina; the treatment with oral administration of GTPs reduced this diabetes-mediated response. Magnification X100. Bars represent mean ± SD of percentage of GFAP positive retinal cells per mm^2 of retina. *p=0.02 and *p=0.0003 for WKY and SHR, respectively.

(B) Representative photomicrograph of immunolocalization of occludin in WKY and SHR animals. In control groups, the occludin is present in ganglion cell layer and also in inner nuclear layer around the vessels. In diabetic animals, there is a reduction in its expression and the treatment with GTPs arrested this effect. Magnification X100. Bars represent mean ± SD of percentage of positivity of occludin/ mm^2 of retina. *p= 0.02 and *p= 0.01 for WKY and SHR, respectively.

GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; CT WKY: control WKY; DM WKY: diabetic WKY; DM WKY GT: diabetic WKY treated with GTPs; CT SHR: control SHR; DM SHR: diabetic SHR; DM SHR GT: diabetic SHR treated with GTPs.

Figure 2: *Retinal oxidative stress was ameliorated by oral GTPs*

(A) Representative photomicrograph of immunolocalization of nitrotyrosine in WKY and SHR animals. Nitrotyrosine is a stable product formed from the reaction of peroxynitrate on tyrosine residues of protein and is accepted as an index of nitrosative damage. In normal retina, the signal of nitrotyrosine is faint and present in all retinal layers; by contrary, in diabetic retina, the staining turned out stronger and is expressed throughout the retinal tissue. This abnormality is abrogated by GTPs administration. Bars represent mean ± SD of percentage of nitrotyrosine positive retinal cells/mm^2 of retina. *p=0.04. (B) Western blot analysis of Cu/Zn superoxide dismutase enzyme (Cu/Zn SOD) in total retinal lysates of the SHR rats. The superoxide dismutase (SOD) family is a major antioxidant system, and deficiency of Cu/Zn-superoxide dismutase (SOD1) leads to features of ROS-mediated retinal degeneration. In this present study, there was no significant decrement of Cu/Zn SOD levels after 3 months of diabetic but the administration of GTPs significantly increased its levels by 2-folds. Equal loading and transfer were ascertained by reprobing the membranes for ß-actin. The bars represent mean ± SD of band densities expressed in arbitrary densitometric units from at least three independent experiments. *p=0.0006.

Figure 3: *Oral administration of GTPs re-establish the constitutive neuronal nitric oxide synthase/cyclic GMP pathway in diabetic SHR rats*.

(A) The constitutive neuronal NOS and its phosphorilated form at Ser847 (inactivated form) were accessed by Western blot analyses in total retinal lysates of the studied groups. In WKY rats, after 12 weeks of DM induction there was not change in phospho-nNOS:

total nNOS: ß-actin ratio. By contrast, in diabetic SHR there was an increased in this ratio, meaning decrease of nNOS activation in retinal tissue; the GTPs restored this abnormality. Equal loading and transfer were ascertained by reprobing the membranes for ß-actin. The bars represent mean ± SD of band densities expressed in arbitrary densitometric units from at least three independent experiments. *p=0.001. (B) Representative photomicrograph of immunolocalization of phospho-nNOS in SHR animals. In control rats, there was positivity mainly in IPL; there is a marked increase in its expression in presence of DM and the treatment with GTPs reduced this increment. Magnification ×100. (C) Cyclic guanosine monophosphate (cGMP) levels analyzed by enzyme-linked immunoassay (ELISA) kit in retinas from SHR rats. The values represent mean ± SD of cGMP levels in pmol/mg protein of retina tissue. *p<0.05.

Figure 4: *GTPs prevented the eNOS activation in diabetic SHR rats leading to a re-establishment of BRB breakdown and retinal function*.

(A) Western blot analysis of phospho-eNOS and total eNOS in total retinal lysates of SHR groups. Equal loading and transfer were ascertained by reprobing the membranes for ß-actin. The bars represent mean ± SD of band densities expressed in arbitrary densitometric units from at least three independent experiments. *p<0.05. (B) Representative images of vessel leakages in whole-mounted retinas. Evans blue binds to the albumin in the blood stream, thus allowing detection of leakage sites in retinal tissue. Evans blue was injected through the tail vein 10 minutes before animal been euthanized. On control flat mounted retina, the capillary bed is intact and nearly no leakage was observed. After 12-weeks of DM, Evans blue dye leaked in several sites into retinal tissue demonstrating a clear inner

BRB breakdown. The oral administration of GTPs reduced the leakage to normal levels. (scale bar: 200µm for all groups. In DM-SHR, higher magnification for better visualization; scale bar :100µm).

To quantify the leakage, a semi quantitative scale was established, based on the extension and sites of retinal leakage.

Level 0: absence of fluorescein leakage in the entire retina;

Level 1: presence of fluorescein leakage in only one quadrant;

Level 1^{+}: more than two sites of leakage in only one quadrant;

Level 2: presence of fluorescein leakage in two quadrants;

Level 2^{+}: more than two sites of leakage in one of two quadrants;

Level 2^{++}: more than two sites of leakage in both of two quadrants;

Level 3: presence of fluorescein leakage in three quadrants;

Level 3^{+}: more than two sites of leakage in one of three quadrants;

Level 3^{++}: more than two sites of leakage in two of three quadrants;

Level 3^{+++}: more than two sites of leakage in three quadrants;

Level 4: presence of fluorescein leakage in all four quadrants

(C) The figure 4C shows representative waveforms of *a*- and *b*-waves of the studied rats. The *a*-wave is the first negative deflection which originates and represents the activity of the photoreceptors. The inner retinal function response, *b*-wave, is a positive deflection generated, in part by the Müller and mainly bipolar cells potentials. Representative full flash ERG waveform for a CT-SHR (red), DM-SHR (blue) and DM-SHR GTPs (green). The bars represent the mean amplitude and latency in AUC of the *a*- and *b*-waves components and total latency of the ERG from the SHR animals. *$p<0.05$ and $^{\#}p<0.03$.

Figure 5: *GTPs lead to neuroprotection in primary rat Müller cells*.

(A) Immunofluorescence for GFAP in primary Müller cells cultured for 72h. Scale bar: 20 µm. (B) Representative Western blots for GLAST in total cell lysates. *p<0.03. (C) Representative Western blots for NMDAR1 in total cell lysates. *p<0.04. (D) Nr1 gene expression profile normalized with Gapdh in primary Müler cell mRNA. *p=0.002. (E) Representative Western blots for Glutamine synthetase in total cell lysates. *p<0.01. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for ß-actin. The bars represent mean ± SD of band densities expressed in percentage of variation from at least three independent experiments.

NG: normal $_{D}$-glucose; HG: high $_{D}$-glucose, HG+GT: high $_{D}$-glucose + 100µg/ml of green tea; MAN: mannitol.

Figure 6: *GTPs lead to neuroprotection in rMC-1 by oxidative balance.*

(A) Quantification of total intracellular ROS levels in rMC-1 cultured for 24 h in normal $_{D}$-glucose (NG, 5mM) and high $_{D}$-glucose (HG, 25mM) in the presence and absence of green tea leaves (HG + GT: 1, 10 and 100 µg/ml). Mannitol (MAN) was used as an osmotic control. Values are means ± SD and expressed as percentage of fluorescence units. Values were corrected by the number of cells at the end of each treatment. *p<0.01 vs NG; $^{\#}$p<0.01 vs HG; ¶p<0.04 vs HG+GT 1µg/ml; §p<0.0007 vs HG+GT 10µg/ml. (B) Concentration of reduced glutathione (GSH) from rMC-1 cells cultured for 24 h (µM glutathione/µg of protein). Cells in HG treated with GT (10-100 µg/ml) or NAC (1mM) prevented the decrease in GSH levels. Bars represent mean±SD. *p<0.001. (C) Glast gene expression profile normalized with Gapdh in rMC-1. *p=0.02 vs NG; $^{\#}$p=0.02 vs HG; ¶p<0.05 vs HG.

(D) Nr1 gene expression profile normalized with Gapdh in rMC-1. *p<0.04 vs NG; #p=0.03 vs HG.

Figure 7: *GTPs in ARPE-19 cells prevented outer BRB dysfunction, ROS production and glutamate transport decreased levels*.

(A) Representative images of H_2DCF-DA via fluorescent microscopy in ARPE-19 cells. The fluorescent intensity of H_2DCF-DA indicates ROS production. The bars represents the quantification of total intracellular ROS levels in ARPE-19 cultured for 24 h. Values are means ± SD and expressed as percentage of fluorescence units. Values were corrected by the number of cells at the end of each treatment. *p<0.0001. (B) Representative Western blots for claudin-1 in ARPE-19 cells. *p<0.02. (C) Immunofluorescence analysis in ARPE-19 cells for claudin-1. Scale bar: 20 µm. (D) Representative Western blots for EAAC1 in ARPE-19 cells. *p<0.03. Equal loading and transfer were ascertained by reprobing the membranes for ß-actin. The bars represent mean ± SD of band densities expressed in percentage of variation from at least three independent experiments. NG: normal $_D$-glucose; HG: high $_D$-glucose, HG+GT: high $_D$-glucose + 100µg/ml of green tea; HG+EGCG (10, 25 and 50µM): high $_D$-glucose plus (-)-Epigallocatechin gallate; MAN: mannitol.

FIGURE 1

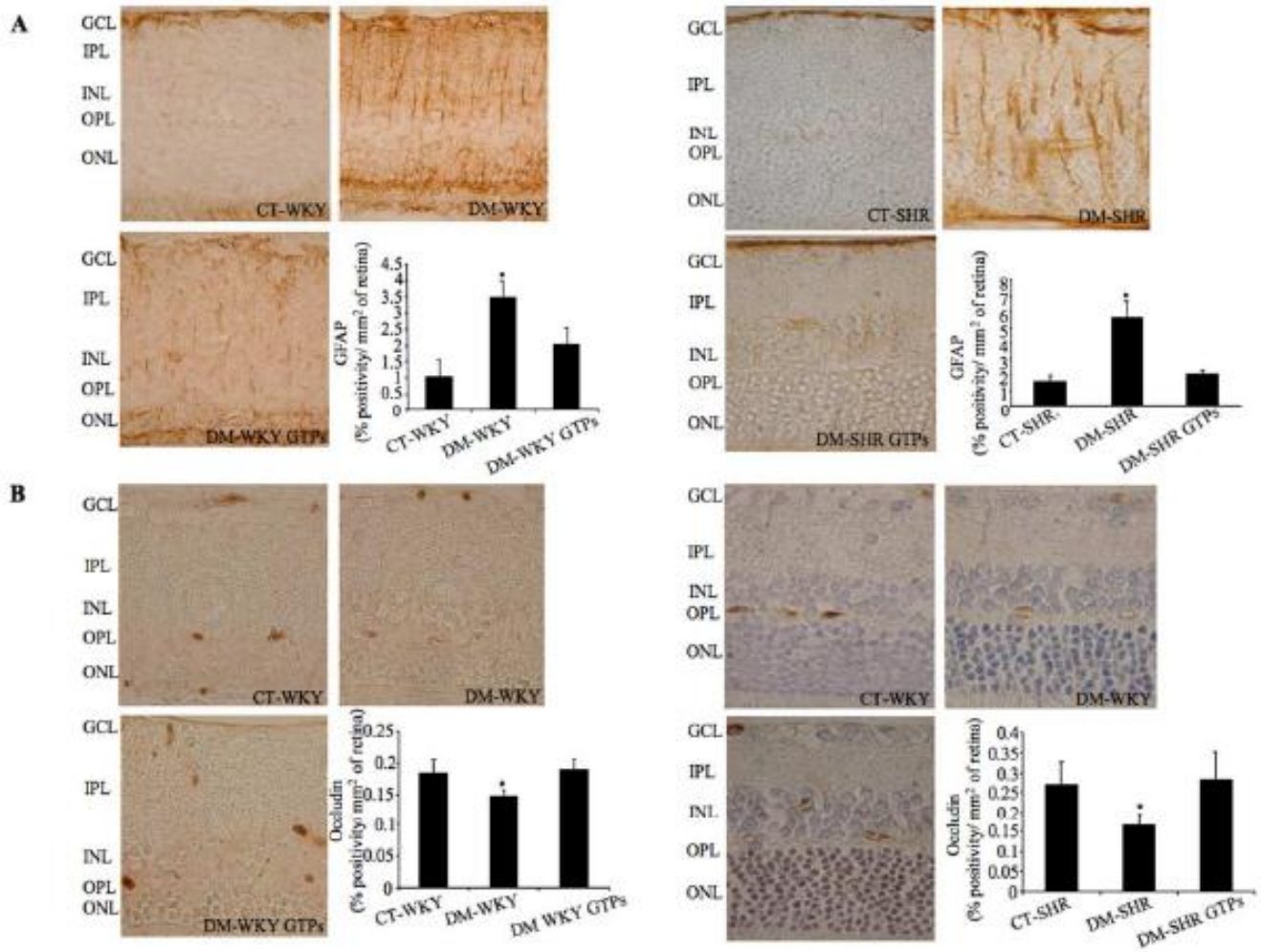

A
GCL
IPL
INL
OPL
ONL
CT-WKY
DM-WKY
DM-WKY GTPs
GFAP
(% positivity/ mm² of retina)
CT-WKY
DM-WKY
DM-WKY GTPs
CT-SHR
DM-SHR
DM-SHR GTPs
CT-SHR
DM-SHR
DM-SHR GTPs
B
Occludin
(% positivity/ mm² of retina)
DM WKY GTPs

FIGURE 2

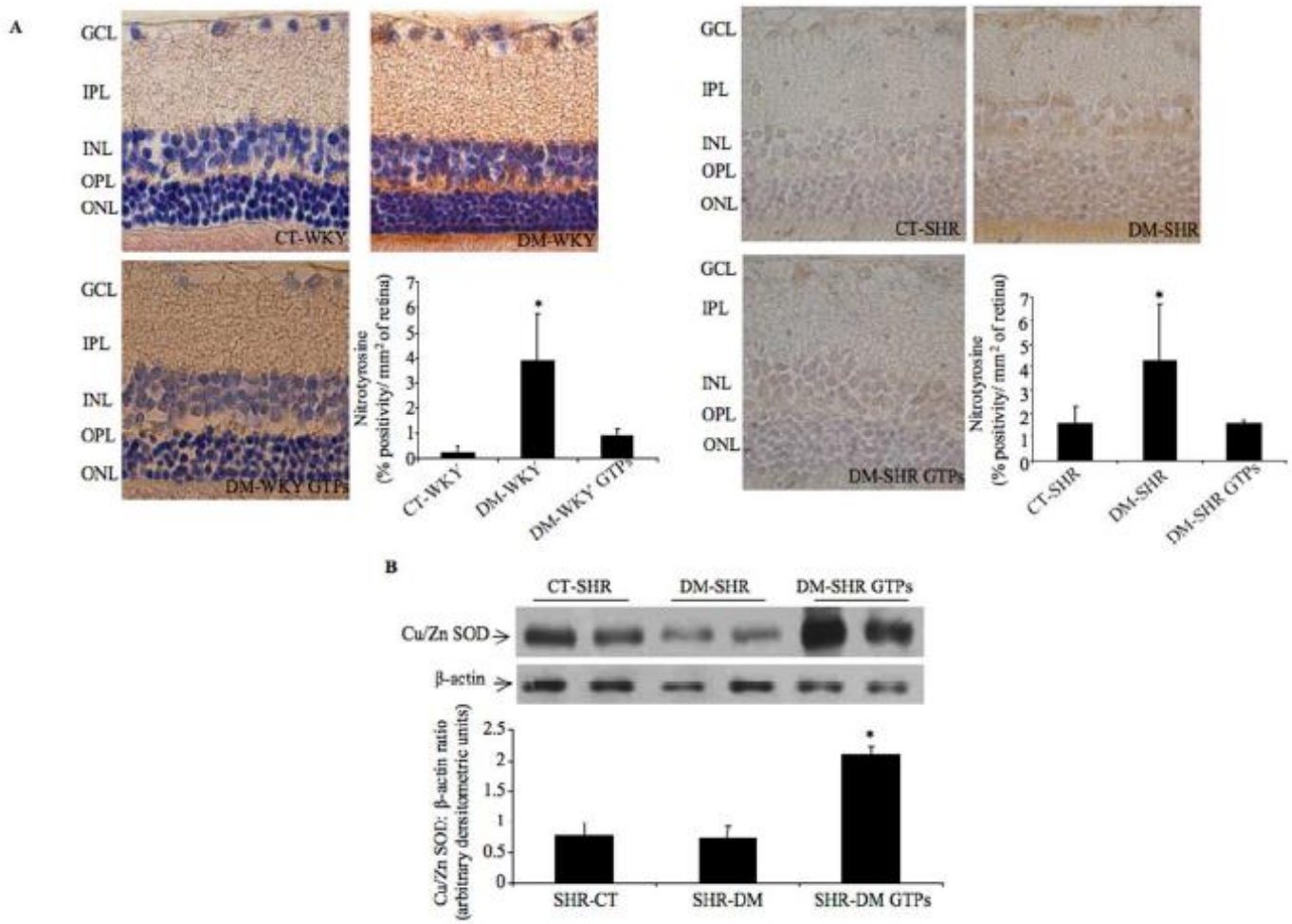

A
GCL
IPL
INL
OPL
ONL
CT-WKY
DM-WKY
DM-WKY GTPs
CT-SHR
DM-SHR
DM-SHR GTPs
Nitrotyrosine (% positivity/ mm² of retina)
CT-WKY
DM-WKY
DM-WKY GTPs
CT-SHR
DM-SHR
DM-SHR GTPs
B
CT-SHR
DM-SHR
DM-SHR GTPs
Cu/Zn SOD
β-actin
Cu/Zn SOD: β-actin ratio (arbitrary densitometric units)
SHR-CT
SHR-DM
SHR-DM GTPs

FIGURE 3

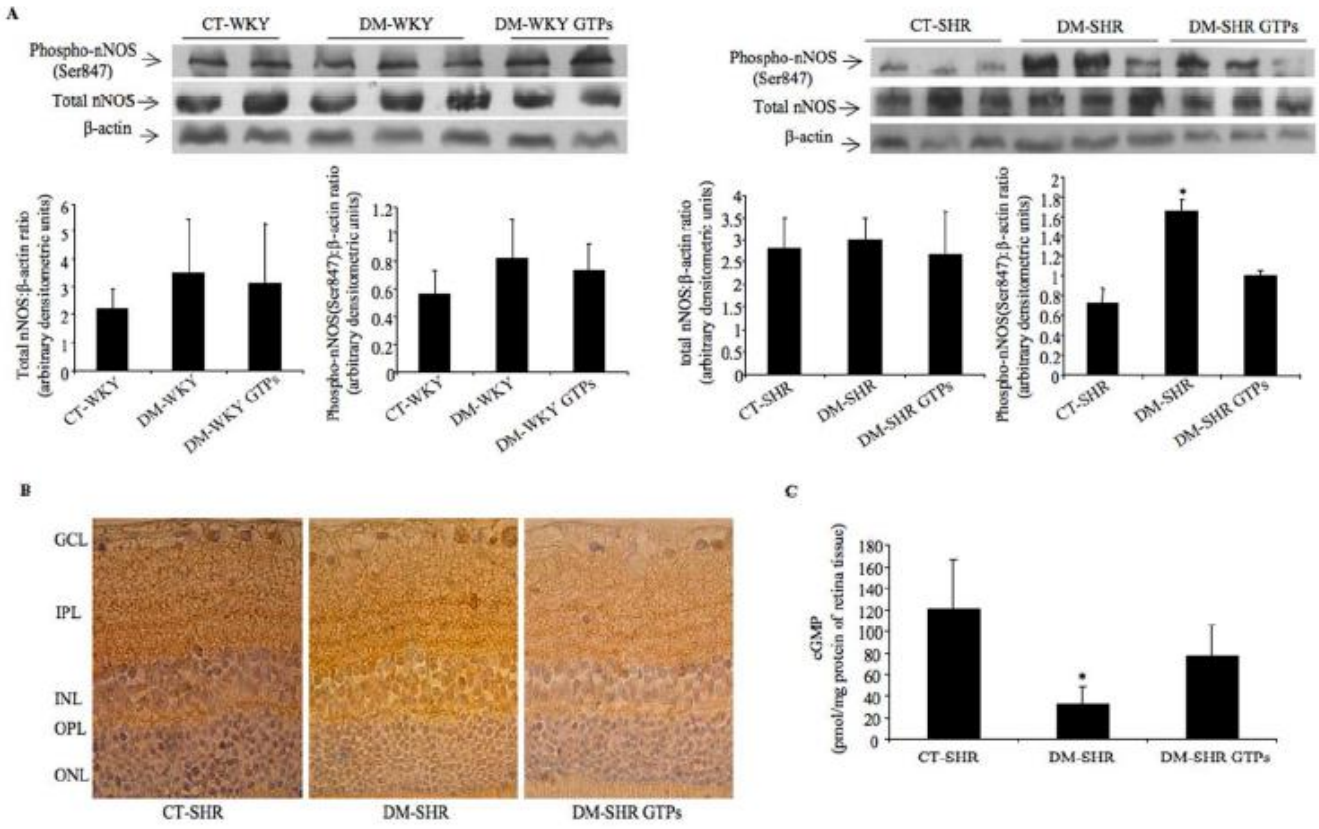
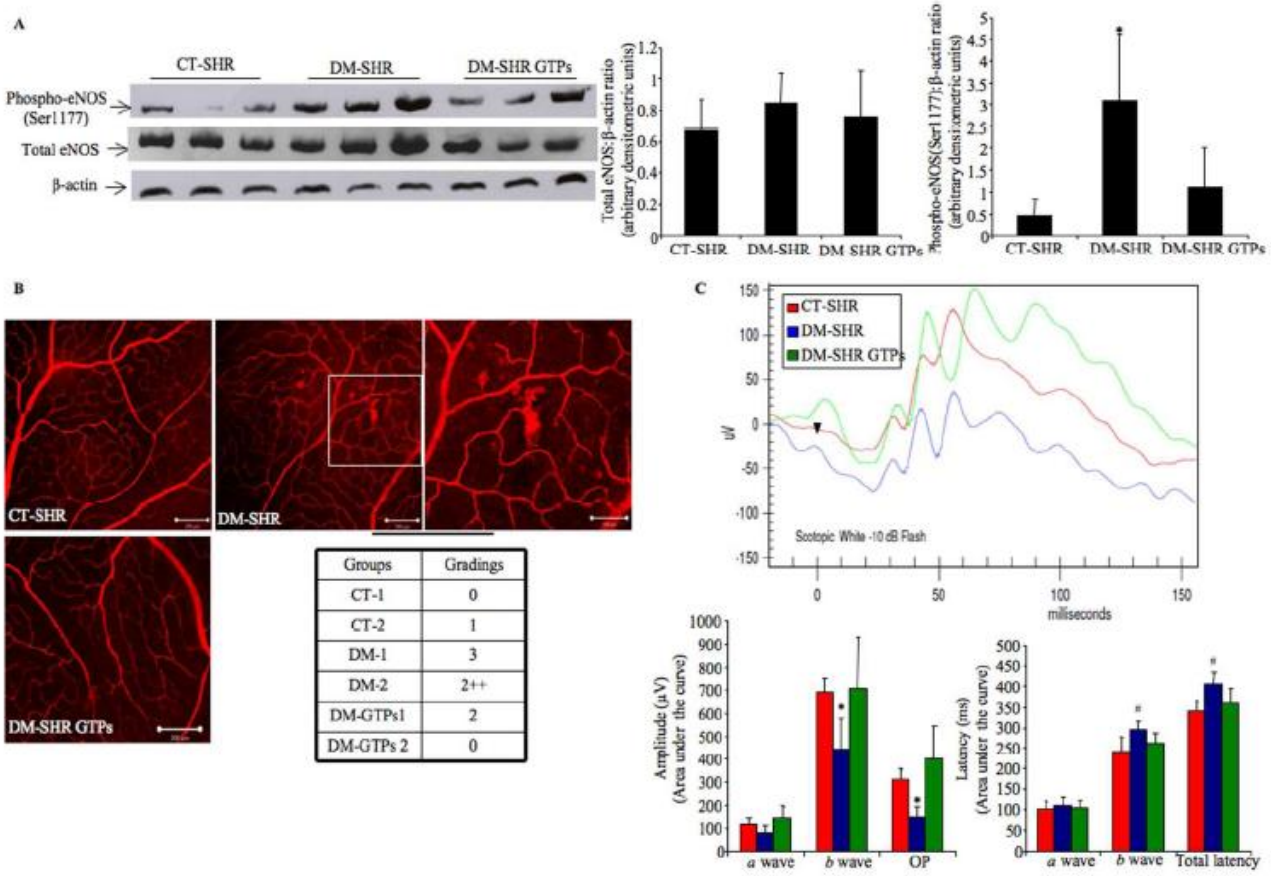

A
CT-WKY
DM-WKY
DM-WKY GTPs
Phospho-nNOS (Ser847)
Total nNOS
β-actin
Total nNOS:β-actin ratio (arbitrary densitometric units)
Phospho-nNOS(Ser847):β-actin ratio (arbitrary densitometric units)
CT-SHR
DM-SHR
DM-SHR GTPs
total nNOS:β-actin ratio (arbitrary densitometric units)
Phospho-nNOS(Ser847):β-actin ratio (arbitrary densitometric units)
B
GCL
IPL
INL
OPL
ONL
C
cGMP (pmol/mg protein of retina tissue)

FIGURE 4



Groups	Gradings
CT-1	0
CT-2	1
DM-1	3
DM-2	2++
DM-GTPs1	2
DM-GTPs 2	0

FIGURE 5

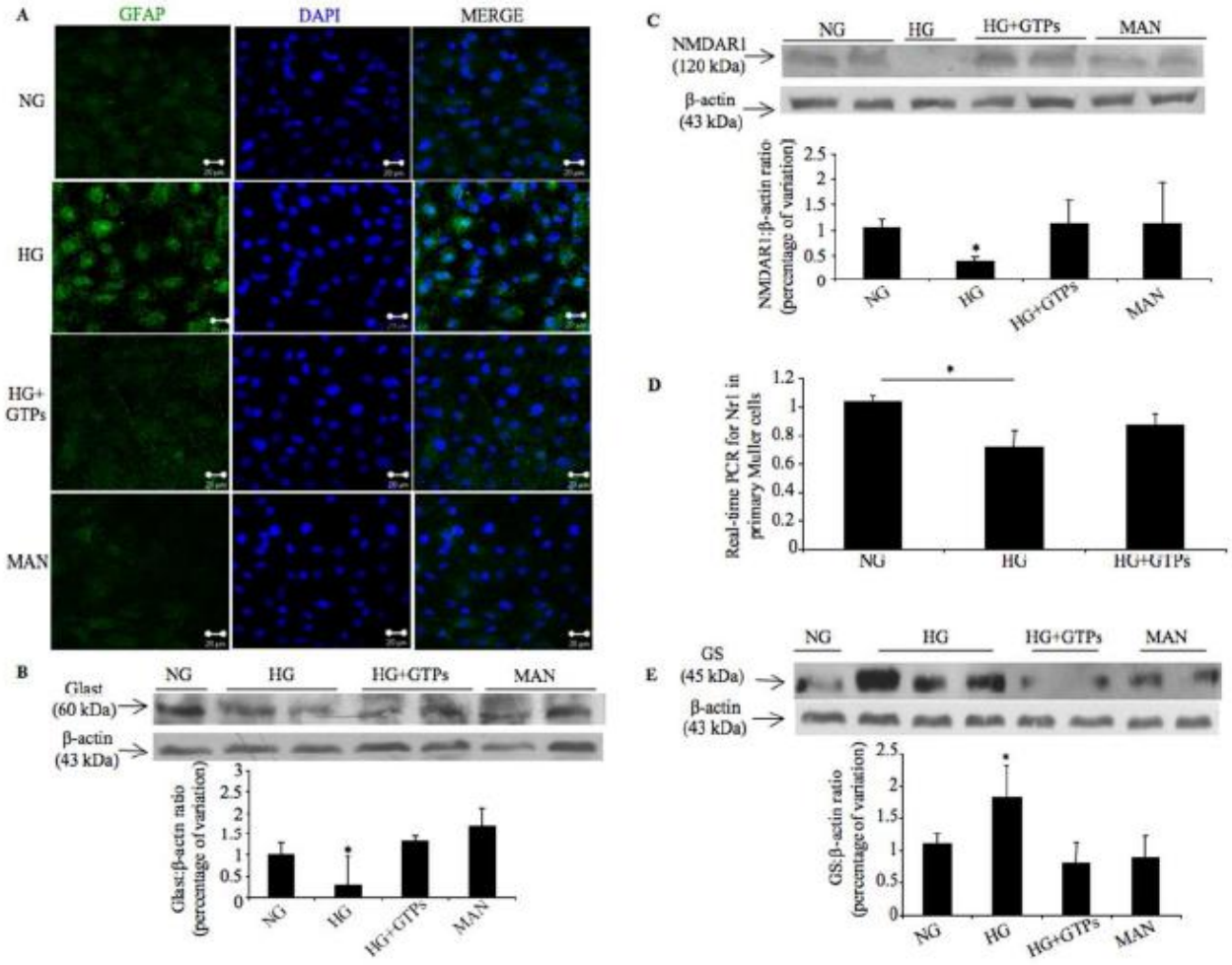

A
GFAP
DAPI
MERGE
NG
HG
HG+ GTPs
MAN
B
Glast (60 kDa)
β-actin (43 kDa)
NG
HG
HG+GTPs
MAN
Glast:β-actin ratio (percentage of variation)
C
NMDAR1 (120 kDa)
β-actin (43 kDa)
NMDAR1:β-actin ratio (percentage of variation)
D
Real-time PCR for Nr1 in primary Muller cells
E
GS (45 kDa)
β-actin (43 kDa)
GS:β-actin ratio (percentage of variation)

FIGURE 6

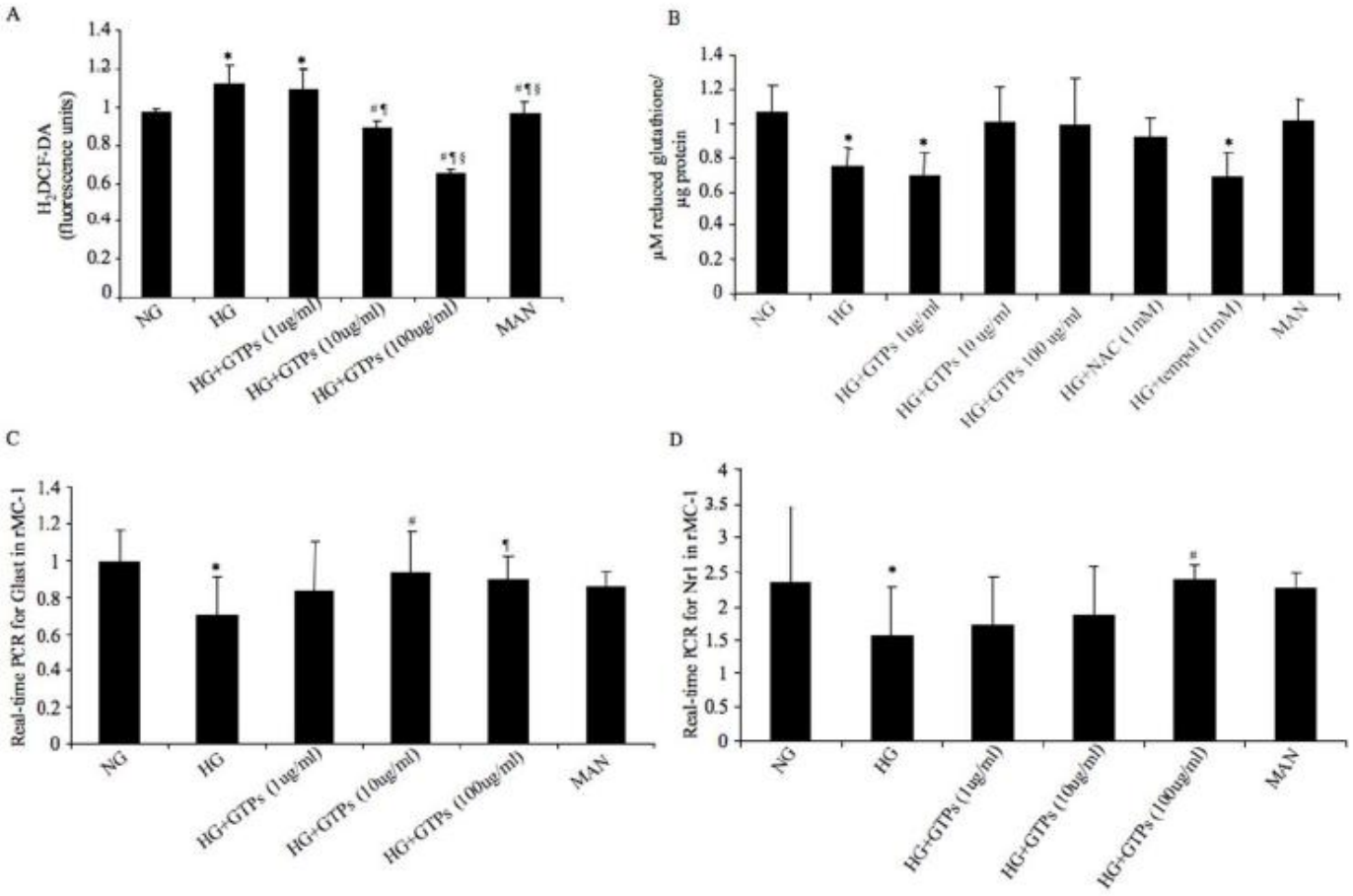
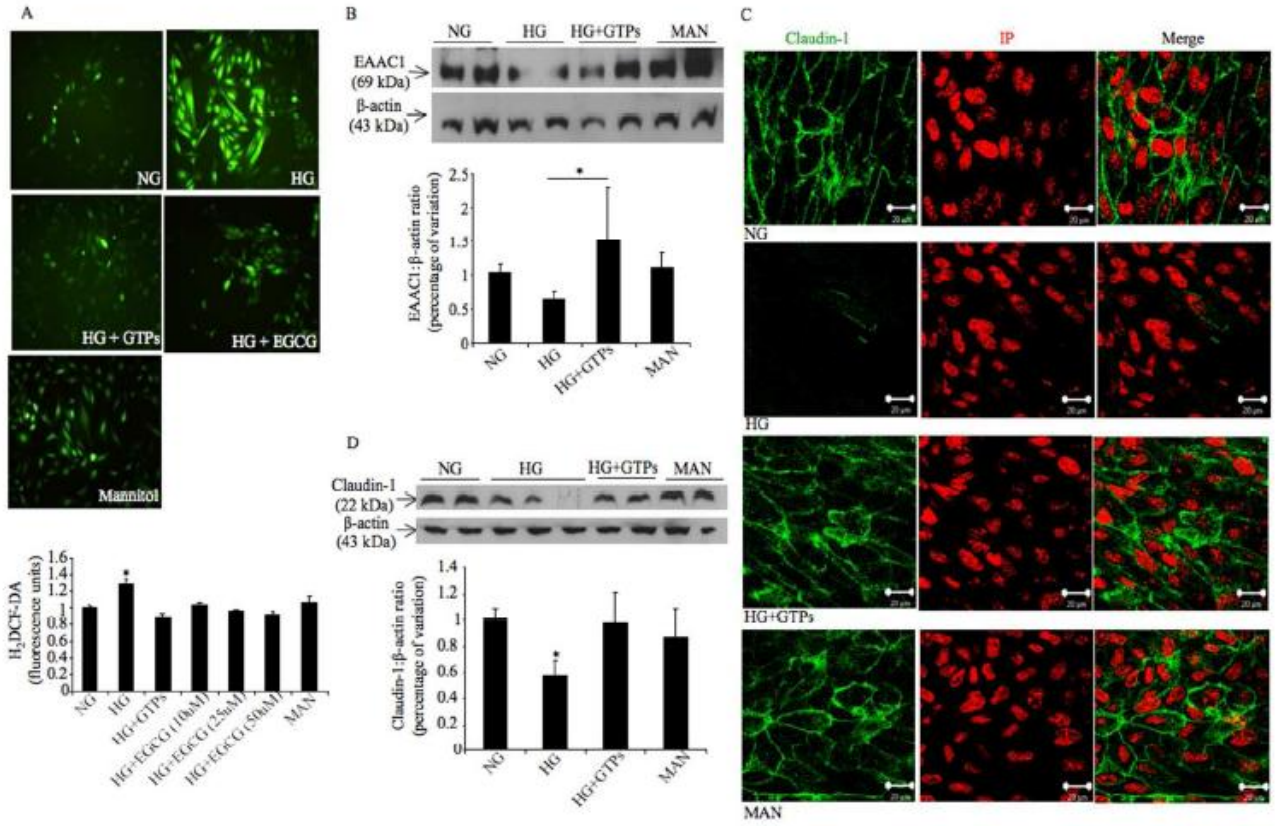

A
H_2DCF-DA (fluorescence units)
NG
HG
HG+GTPs (1ug/ml)
HG+GTPs (10ug/ml)
HG+GTPs (100ug/ml)
MAN
B
μM reduced glutathione/ μg protein
NG
HG
HG+GTPs 1ug/ml
HG+GTPs 10 ug/ml
HG+GTPs 100 ug/ml
HG+NAC (1mM)
HG+tempol (1mM)
MAN
C
Real-time PCR for Glast in rMC-1
NG
HG
HG+GTPs (1ug/ml)
HG+GTPs (10ug/ml)
HG+GTPs (100ug/ml)
MAN
D
Real-time PCR for Nrf1 in rMC-1
NG
HG
HG+GTPs (1ug/ml)
HG+GTPs (10ug/ml)
HG+GTPs (100ug/ml)
MAN

FIGURE 7


A
NG
HG
HG + GTPs
HG + EGCG
Mannitol
H2DCF-DA (fluorescence units)
NG
HG
HG+GTPs
HG+EGCG (10nM)
HG+EGCG (25nM)
HG+EGCG (50nM)
MAN
B
NG
HG
HG+GTPs
MAN
EAAC1 (69 kDa)
β-actin (43 kDa)
EAAC1:β-actin ratio (percentage of variation)
C
Claudin-1
IP
Merge
NG
HG
HG+GTPs
MAN
D
Claudin-1 (22 kDa)
β-actin (43 kDa)
Claudin-1:β-actin ratio (percentage of variation)

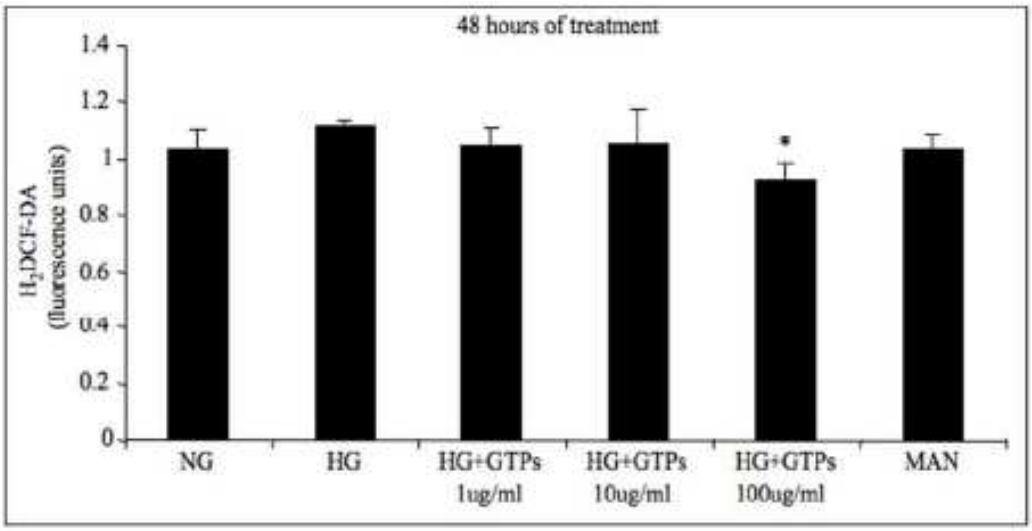
LEGEND OF SUPPLEMENTAL FIGURE 1

ROS production in rMC-1 in 48 and 72 hours of treatment.

(A) Quantification of total intracellular ROS levels in rMC-1 cultured for 48 h in normal D-glucose (NG, 5mM) and high D-glucose (HG, 25mM) in the presence and absence of green tea leaves (HG + GTPs: 1, 10 and 100 .g/ml). *p<0.03 vs HG, HG+GTPs 1 and 10.g/ml. (B) Quantification of total intracellular ROS levels in rMC-1 cultured for 72 h in NG and HG in the presence and absence of GTPs 100ug/ml. There was no difference between the treatments. Mannitol (MAN) was used as an osmotic control. Values are means ± SD and expressed as percentage of fluorescence units. Values were corrected by the number of cells at the end of each treatment.

SUPPLEMENTAL FIGURE 1

A



B