UNIVERSIDADE ESTADUAL PAULISTA

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

ANÁLISE HISTOLÓGICA, MORFOMÉTRICA, EXPRESSÃO DE GENES, PROTEÍNAS E WESTERN BLOT NA RETINA DE RATOS COM GLAUCOMA INDUZIDO E TRATADOS COM CITRATO DE SILDENAFIL

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Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Doutor.

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LISTA DE ABREVIATURAS E SIGLAS

CGR - Células ganglionares da retina

CS - Citrato de Sildenafil

PDE5 - Fosfodiesterase tipo 5

GMPc - Monofosfato cíclico de guanosina

NO - Nervo óptico

PIO - Pressão intraocular

ON - Óxido nítrico

PDE – Fosfodiesterase

EPR - Epitélio pigmentar da retina

SUMÁRIO

RESUMO

O glaucoma é a principal causa global de cegueira irreversível e o número de pessoas com glaucoma em todo o mundo aumentará para 111,8 milhões em 2040, mesmo com os tratamentos vigentes. Deste modo, enseja uma necessidade de desenvolver terapias neuroprotetoras que possam ser usadas para reduzir os efeitos perniciosos do glaucoma, como a morte de células ganglionares da retina (CGR). Avanços na compreensão da fisiopatologia do glaucoma é um fator chave na compreensão da patogênese da neuropatia glaucomatosa. Neste contexto a isquemia retiniana desempenha um papel central em várias doenças da retina. A patogênese da isquemia retiniana envolve alterações temporais da morfologia e morfometria da retina assim como mudanças na expressão gênica e protéica. O Citrato de Sildenafila (SC) mostrou efeito protetor nos modelos de isquemia/reperfusão (I/R) com efeitos neuroprotetores. Contudo, a administração oral de CS, em humanos, encontra inúmeros efeitos colaterais, tais como redução da pressão arterial, dores de cabeça, rubor e congestão nasal são concomitantes, assim como pacientes com moderada a grave doença cardiovascular ou aqueles submetidos a terapia a base de nitrato apresentam riscos secundários aumentados para efeitos cardiovasculares adversos. Deste modo, propomos que a administração tópica de CS pode ser uma alternativa à via oral e também ser igualmente neuroprotetor no glaucoma e podem minimizar os riscos indesejados no uso sistêmico desse fármaco, além de oferecer uma nova abordagem para a intervenção terapêutica na patogênese da neuropatia glaucomatosa. Caso nossos resultados sejam positivos, em um estudo tão abrangente, pode levar a um ensaio clínico deste fármaco seguro e promissor em pacientes com glaucoma.

Palavras-chave: Apoptose; Glaucoma; Células ganglionares da retina; Citrado de Sildenafil tópico.

ABSTRACT

Glaucoma is the leading global cause of irreversible blindness and the number of people with glaucoma worldwide will increase to 111.8 million by 2040, even with current treatments. Thus, there is a need to develop neuroprotective therapies that can be used to reduce the deleterious effects of glaucoma, such as retinal ganglion cell death (RBC). Advances in understanding the pathophysiology of glaucoma are a key factor in understanding the pathogenesis of glaucomatous neuropathy. In this context, retinal ischemia plays a central role in various diseases of the retina. The pathogenesis of retinal ischemia involves temporal changes in retinal morphology and morphometry as well as changes in gene and protein expression. Sildenafil Citrate (SC) showed protective effect in the ischemia / reperfusion (I / R) models with neuroprotective effects. However, oral administration of CS in humans finds numerous side effects such as reduced blood pressure, headaches, flushing and nasal congestion are concomitant, as well as patients with moderate to severe cardiovascular disease or those undergoing nitrate therapy have increased secondary risks for adverse cardiovascular effects. Thus, we propose that the eye drops administration of CS may be an alternative to the oral route and also be equally neuroprotective in glaucoma and may minimize the undesirable risks in the systemic use of this drug, as well as offer a new approach for the therapeutic intervention in the pathogenesis of neuropathy glaucomatous. If our results are positive, in such a comprehensive study, it may lead to a clinical trial of this safe and promising drug in patients with glaucoma.

Palavras-chave: Apoptosis; Glaucoma; Retinal Gnaglion Cells; Eye drops Citrate Sildenafil.

1. INTRODUÇÃO

Após a catarata, o glaucoma é a segunda causa de cegueira no mundo, sendo considerado um problema de saúde pública e a principal causa de cegueira irreversível. O glaucoma é uma neuropatia óptica crônica, progressiva, caracterizada por alterações típicas do disco óptico e da camada de fibras nervosas da retina, com repercussões características no campo visual. É acompanhado, na maioria das vezes, de pressões intraoculares acima de níveis considerados estatisticamente normais e o desfecho principal é a cegueira irreversível (SAKATA et al., 2007;GUPTA et al., 2009; THAM et al., 2014).

Em 2013, o número de pessoas com glaucoma no mundo foi estimado em 64,3 milhões, e deve aumentar para 76 milhões em 2020 e 111,8 milhões até 2040 (QUIGLEY; BROMAN, 2006; THAM et al., 2014). No Brasil, há escassez de informações quanto à prevalência desta doença. O Conselho Brasileiro de Oftalmologia estima que existam 985.000 pessoas com glaucoma com mais de 40 anos de idade

Uma característica fisiopatológica do glaucoma é a perda gradual de células ganglionares da retina (CGR) e seus axônios, fibras do nervo óptico (NO), tais como as condições de outras doenças neurodegenerativas, como o Alzheimer ou o Parkinson (HAYREH; PE'ER; ZIMMERMAN, 1999; NICKELLS, 2007; LIN et al., 2014).

O glaucoma cursa como uma doença "sem solução", evidenciado pelo número crescente de doentes, sublinhando a necessidade urgente de desenvolver novas estratégias para tratar esta doença (DUGGAL et al, 2005;. QUIGLEY, BROMAN, 2006; SCHMIER, HALPERN, JONES, 2007;. BALTMR et al, 2010).

A degeneração glaucomatosa do nervo óptico e da retina está ligada ao desenvolvimento da pressão intraocular elevada (PIO) (NICKELLS a, 2007). Apesar do aumento da PIO ser o principal fator de risco para o glaucoma, outras evidências sugerem que a isquemia local, levando a uma redução na perfusão, é também um evento chave na patogênese da neuropatia glaucomatosa (FLAMMER, MOZAFFARIEH, 2007; QUIGLEY, 2011). Níveis reduzidos de óxido nítrico (ON), um mediador vasodilatador, foram demonstrados em pacientes com glaucoma e em modelos animais da doença (bem como em outras doenças neurodegenerativas) estando implicados na diminuição do fluxo sanguíneo ocular que leva a morte neuronal e perda de visão

(DOĞANAY et al, 2002; REICHSTEIN et al., 2007). A investigação sobre a patogênese do glaucoma foi auxiliada pelo desenvolvimento de modelos animais onde PIO elevada provoca a morte da CGR por apoptose (REICHSTEIN et al., 2007) e, a redução da PIO nos olhos afetados pode atenuar o processo degenerativo (NICKELLS a, 2007).

Atualmente as principais modalidades terapêuticas disponíveis para o glaucoma estão focadas principalmente em reduzir a PIO, seja por fármacos ou procedimentos cirúrgicos, as mesmas apresentam sucesso questionável na preservação da visão e também com efeitos colaterais em pacientes com glaucoma. Portanto, há uma necessidade permanente de desenvolver novas abordagens terapêuticas que possa ser utilizada para neuroproteção, em conjunção com medicamentos hipotensores para preservar a visão (LEVIN, 2005; WEINREB, 2007).

O Citrato de Sildenafil (CS), um medicamento aprovado para o tratamento de disfunção erétil, é um inibidor de fosfodiesterase (PDE), uma enzima que degrada o GMPc, o mensageiro secundário do ON. Portanto, o tratamento com CS demonstrada ser neuroprotetor em vários modelos de doenças neurodegenerativas, uma vez que níveis elevados de GMPc prolonga o efeito de ON, com vasodilatação e melhora na circulação vascular (JOHNSTON, 2005; OREJANA et al, 2012.).

Contudo, a administração oral de CS encontra inúmeros efeitos colaterais em humanos, tais como perda da visão temporária, redução da pressão arterial, dores de cabeça, rubor e congestão nasal, além de aumento do risco de efeitos secundário adversos em indivíduos com cardiomiopatias (LEE, CHENG, 2004; HUANG et al, 2010). A administração tópica de CS pode ser considerada uma alternativa para a via oral, a fim de evitar estes efeitos secundários adversos, encurtar o tempo de latência e manter o efeito por períodos mais longos (YONESSI, 2005; ELNAGGAR; EL-MASSIK; ABDALLAH, 2011).

Baseado nos resultados preliminares do grupo de pesquisa (EZRA-ELIA et al., 2017; ZANONI et al., 2017) e visando minimizar os riscos na administração oral de CS, investigaremos a eficiência do tratamento com CS tópico, que pode ser igualmente neuroprotetor no glaucoma, e pode, assim, oferecer uma nova abordagem para a intervenção terapêutica na patogênese da neuropatia glaucomatosa.

2. REVISÃO DE LITERATURA

2.1 O olho

O olho foi descrito por Charles Darwin como perfeito e complexo. Existem várias estruturas e variações funcionais do "olho" entre organismos, no entanto, considera-se incorreto especular que um é superior à outro. Essa é a perfeição que o olho contempla; cada olho evoluiu precisamente para atender às necessidades das espécies. Deste modo, o mesmo pode ser comparado a uma câmera que reúne luz e transforma em imagens, porém mais complexo (LITZINGER; RIO-TSONIS, 2002; MASLAND, 2012; ADDO; BAMIRO; SIWALE, 2016).

Entre os órgãos dos sentidos do corpo, o olho é o mais utilizado, sendo considerado um dos órgãos sensoriais mais complexos e importantes do corpo, possui um mecanismo de busca e de focalização automática do objeto de interesse, um sistema de lentes que refratam a luz (uma fixa e outra regulável), pupila de diâmetro regulável, filme de revelação rápida das imagens, com um sistema de proteção e manutenção da transparência do olho (MCCAA, 1982; KOLB, 1995; MCALOOSE; MUNSON; NAYDAN, 2007; ADDO; BAMIRO; SIWALE, 2016).

A maioria das informações que coletamos sobre o nosso entorno são coisas que vemos com nossos olhos, o mesmo se encontra dentro de uma cavidade craniana em forma de cone, a órbita, que aloja o bulbo do olho e várias outras estruturas do tecido mole e os anexos oculares, como por exemplo, músculos e glândulas, que agem sobre o bulbo do olho funcional. Com relação ao formato do olho, apresenta forma majoritariamente esférica devido à pressão gerada pelo humor aquoso e à falta de elasticidade da esclera e da córnea (KOLB, 1995; LEITE; OLIVEIRA, 2013; ADDO; BAMIRO; SIWALE, 2016).

O olho se desenvolve a partir do neuroectoderma do tubo neural, do mesoderma e do ectoderma superficial. O neuroectoderma forma a retina e o nervo óptico, ectoderma superficial origina a lente, as glândulas lacrimais, os epitélios do saco conjuntival e das pálpebras. Já o mesoderma origina a irís, corpo ciliar e coróide (DYCE; SACK; WENSING, 2010; HEAVNER; PEVNY, 2012; LEITE; OLIVEIRA, 2013).

O globo ocular é constituído por três túnicas, envolvendo três estruturas

transparentes, observadas em todos os vertebrados. A camada mais externa, conhecida como túnica fibrosa, é composta pela córnea e esclera. A camada média, conhecida como túnica vascular ou uvea, consiste a coróide, corpo ciliar e íris. O mais interno é a retina, que recebe sua circulação dos vasos da coróide, bem como dos vasos retinianos (MCCAA, 1982; LITZINGER; RIO-TSONIS, 2002; ADDO; BAMIRO; SIWALE, 2016). A principal irrigação do olho é efetuada pela artéria oftálmica externa, um ramo derivado da artéria maxilar, que passa ventral à órbita, irrigando estruturas mais rostrais à face. As ramificações das artérias oftálmicas podem ser divididas em três grupos: as que irrigam o bulbo do olho, as que irrigam os músculos oculares e as que deixam a órbita para irrigar estruturas adjacentes (DYCE; SACK; WENSING, 2010; HEAVNER; PEVNY, 2012; LEITE; OLIVEIRA, 2013).

A retina recebe seu suprimento sanguíneo de dois sistemas circulatórios: os vasos sanguíneos da retina e da coróide. A circulação da retina supre a retina interna, exceto pela zona foveal avascular. As camadas externas da retina avascular recebem seus nutrientes por difusão dos vasos coróides. O coriocapilar é fenestrado, o que permite o vazamento de moléculas para o epitélio pigmentar da retina (EPR). Sistemas especializados de transporte no EPR controlam o transporte de fluidos e nutrientes para os fotorreceptores (WILLOUGHBY et al., 2010; LEITE; OLIVEIRA, 2013; ADDO; BAMIRO; SIWALE, 2016).

Via de regra, a retina é o componente essencial da visão. As outras estruturas do olho, não menos importantes, são subsidiárias, regulam a quantidade de luz que entra ou fornecem nutrição, proteção, movimento e agem para focalizar imagens. A retina pode ser considerada como uma extensão periférica do sistema nervoso central, ao qual está ligado por fibras nervosas, o nervo óptico (MCCAA, 1982; GALLOWAY; AMOAKU, 1999; WILLOUGHBY et al., 2010; ADDO; BAMIRO; SIWALE, 2016).

Como no caso do cérebro e da medula espinhal, a retina está dentro de duas camadas de tecido que contribui para a proteção e nutrição. O lado de fora ddo globo ocular, correspondente à dura-máter, camada composta por tecido fibroso denso, similar a um envelope protetor, a túnica fibrosa. A parte posterior da túnica fibrosa, a esclera, é branca e opaca. Embora apresente função protetora, a porção anterior, a córnea, é clara e transparente, refrata e transmite a luz para a lente e a retina e protege o olho contra infecções e danos estruturais às partes mais profundas. A esclera forma um revestimento

de tecido conjuntivo que protege o olho de forças externas e mantém sua forma. Imediatamente interna à esclera, entre ela e a retina, encontra-se a úvea, uma túnica vascular análoga à pia-máter e aracnoide do sistema nervoso central (MCCAA, 1982; GALLOWAY; AMOAKU, 1999; WILLOUGHBY et al., 2010; LEITE; OLIVEIRA, 2013; ADDO; BAMIRO; SIWALE, 2016).

Primeiramente, a úvea fornece nutrientes para o olho. A porção posterior da úvea é a coróide, um tecido composto quase inteiramente de vasos sanguíneos. Uma segunda porção da úvea, o corpo ciliar, encontra-se imediatamente anterior à coróide e posterior à margem córneo-escleral, formando também fluido intra-ocular, o humor aquoso (MCCAA, 1982; LEITE; OLIVEIRA, 2013; ADDO; BAMIRO; SIWALE, 2016).

Além disso, o corpo ciliar contém músculos que fornecem um mecanismo de suporte e foco para a lente. A porção mais anterior do trato uveal, a íris, é desviada para o interior do olho. A íris atua como um diafragma, com uma abertura central arredondada, a pupila, que se dilata para permitir a passagem de mais luz para a retina com pouca luminosidade e se contrai em uma alta luminosidade. A íris também possui algum grau de função nutritiva, uma vez que atua para ajudar a regular o fluxo de fluido no olho (MCCAA, 1982; WILLOUGHBY et al., 2010; LEITE; OLIVEIRA, 2013; ADDO; BAMIRO; SIWALE, 2016).

A lente, responsável pelo foco do olho, está localizada imediatamente atrás da íris e é suportada pelo corpo ciliar por ligamentos suspensores, a zônula. O espaço entre a íris e a lente é chamada de câmara posterior. A câmara anterior é o espaço entre a íris e a córnea. Atrás da lente está o humor vítreo, transparente, que ocupa o espaço entre a lente e a retina (MCCAA, 1982; WILLOUGHBY et al., 2010; LEITE; OLIVEIRA, 2013; ADDO; BAMIRO; SIWALE, 2016).

A retina, também conhecida como túnica nervosa, é a área sensível à luz que consiste em milhões de células, as quais compreendem circuitos neurais complexos que convertem a atividade elétrica graduada nos fotorreceptores em potenciais de ação que viajam para o cérebro através de axônios no nervo óptico (II par de nervos cranianos), na forma de impulsos nervosos para o córtex visual (LITZINGER; RIO-TSONIS, 2002; WILLOUGHBY et al., 2010; MASLAND, 2012; ADDO; BAMIRO; SIWALE, 2016).

A retina se inicia onde o nervo óptico penetra na coróide, com o formato de um

cálice côncavo, revestindo a coróide e terminando na borda pupilar. Apenas dois terços, aproximadamente, da retina podem ser atingidos pela luz que penetra no olho através do espaço pupilar. Com isso, apenas essa porção da retina possui células receptoras (PURVES; WILLIAMS, 2001; WILLOUGHBY et al., 2010; LEITE; OLIVEIRA, 2013; ADDO; BAMIRO; SIWALE, 2016)

Embora tenha os mesmos tipos de elementos funcionais e neurotransmissores encontrados em outras partes do sistema nervoso central, a retina compreende apenas algumas classes de neurônios, e estes são organizados de uma maneira que tem sido menos difícil de desvendar do que os circuitos em outras áreas do cérebro (PURVES; WILLIAMS, 2001).

A retina pode ser dividida em dez camadas distintas. A camada mais externa da retina, o EPR, consiste em uma camada de células poligonal plana localizada entre a coróide e a camada fotorreceptora. Como o EPR adere mais proximamente à coróide do que ao tecido retiniano, podendo haver um espaço entre o EPR e a camada de fotorreceptores (o remanescente da vesícula óptica) (KOMÁROMY; SMITH; BROOKS, 1998; PURVES; WILLIAMS, 2001; MASLAND, 2012).

Já na retina neural existem dois tipos de fotoreceptores (elementos sensíveis à luz) - bastonetes e cones - responsáveis para receber e transformar fótons de luz em impulsos eletroquímicos. Ambos os tipos de fotorreceptores têm um segmento externo que é composto de discos membranosos que contêm fotopigmentos e fica adjacente à EPR, e um segmento interno que contém o núcleo da célula e dá origem a terminais sinápticos que entram em contato com células horizontais ou bipolares (KOMÁROMY; SMITH; BROOKS, 1998; PURVES; WILLIAMS, 2001; ADDO; BAMIRO; SIWALE, 2016).

A absorção de luz pelo fotopigmento inicia uma cascata de eventos que altera o potencial de membrana do receptor e, portanto, a quantidade de neurotransmissor liberada pelo fotorreceptor realiza sinapses nas células que eles entram em contato. As sinapses entre os terminais dos fotorreceptores e células bipolares (e células horizontais) ocorrem na camada plexiforme externa; mais especificamente, os corpos celulares de fotorreceptores compõem a camada nuclear externa, enquanto os corpos celulares de células bipolares ficam na camada nuclear interna (KOLB, 1995; PURVES; WILLIAMS, 2001; LITZINGER; RIO-TSONIS, 2002).

Os corpos celulares e os processos desses neurônios são empilhados em cinco camadas alternadas, com os corpos celulares localizados nas camadas nucleares internas, nucleares externas e células ganglionares, e os processos e contatos sinápticos localizados nas camadas plexiforme interna e plexiforme externa. Uma cadeia direta de três neurônios - célula fotorreceptora, célula bipolar e a célula ganglionar - é a principal rota de fluxo de informação dos fotorreceptores para o nervo óptico (PURVES; WILLIAMS, 2001; LITZINGER; RIO-TSONIS, 2002; MASLAND, 2012).

Os processos axonais curtos das células bipolares fazem contatos sinápticos, por sua vez, nos processos dendríticos das células ganglionares na camada plexiforme interna. Os axônios muito maiores das células ganglionares formam o nervo óptico e carregam informações sobre a estimulação retiniana para o restante do sistema nervoso central (KOLB, 1994; PURVES; WILLIAMS, 2001; WILLOUGHBY et al., 2010; MASLAND, 2012).

Os outros dois tipos de neurônios na retina, células horizontais e células amácrinas, têm seus corpos celulares na camada nuclear interna e são os principais responsáveis pelas interações laterais dentro da retina (KOLB, 1994; PURVES; WILLIAMS, 2001; WILLOUGHBY et al., 2010; MASLAND, 2012).

Essas interações laterais entre receptores, células horizontais e células bipolares na camada plexiforme externa são amplamente responsáveis pela sensibilidade do sistema visual ao contraste de luminância em uma ampla faixa de intensidades de luz (KOLB, 1994; KOMÁROMY; SMITH; BROOKS, 1998; PURVES; WILLIAMS, 2001; MASLAND, 2012). Os processos de células amácrinas, que se estendem lateralmente na camada plexiforme interna, são pós-sinápticos para os terminais das células bipolares e pré-sinápticos para os dendritos das células ganglionares Os processos das células horizontais se ramificam na camada plexiforme externa (KOLB, 1994; KOMÁROMY; SMITH; PURVES; WILLIAMS, 2001; BROOKS, 1998; MASLAND, 2012).

Várias subclasses de células amácrinas que fazem contribuições distintas para a função visual. Uma classe de células amácrinas, por exemplo, desempenha um papel importante na transformação das respostas persistentes de células bipolares para a luz nas breves respostas transitórias exibidas por alguns tipos de células ganglionares (KOMÁROMY; SMITH; BROOKS, 1998; PURVES; WILLIAMS, 2001). Outro tipo

serve como um passo obrigatório no caminho que transmite informações de fotorreceptores de bastão para células ganglionares da retina. A variedade de subtipos de células amácrinas ilustra a regra mais geral de que, embora existam apenas cinco tipos básicos de células retinianas, pode haver diversidade considerável dentro de um determinado tipo de célula. Essa diversidade é a base para caminhos que transmitem diferentes tipos de informações aos alvos centrais de maneira paralela (KOLB, 1994; GALLOWAY; AMOAKU, 1999; LITZINGER; RIO-TSONIS, 2002; PURVES; WILLIAMS, 2001; MASLAND, 2012).

2.2 Glaucoma

O glaucoma não é uma doença, mas um grupo de doenças oculares com características anatômicas, como ângulo aberto (onde o ângulo da câmara anterior do olho permanece aberto) e ângulo fechado (ângulo da câmara anterior do olho permanece fechado). Se o olho não apresentar doença preexistente, o glaucoma é considerado primário. Pacientes que têm glaucoma em um olho com doença preexistente são diagnosticados como glaucoma secundário. Há vários tipos diferentes de glaucoma, e eles foram classicamente divididos nas categorias de primárias ou secundárias de ângulo aberto e de primárias ou secundárias de ângulo fechado (LEE, D. A.; HIGGINBOTHAM, 2005; WEINREB; AUNG; MEDEIROS, 2014). Há também a hipertensão ocular (HO) que é definida como uma PIO aumentada (acima de 21 mmHg) na ausência de perda de campo visual ou de dano glaucomatoso no nervo óptico. Assim como o glaucoma de pressão normal é a forma em que há dano no nervo óptico ou no campo visual na ausência da PIO elevada e de anormalidades oculares ou sistêmicas que possam aumentar a PIO (KILLER; PIRCHER, 2018).

Geralmente, aceita-se que a degeneração glaucomatosa do nervo óptico e da retina esteja ligada ao desenvolvimento da pressão intraocular elevada (PIO) (NICKELLS, 2007a). Sabe-se que o aumento da PIO é um dos principais fatores de risco para o glaucoma, contudo, evidências sugerem que a isquemia local, levando a uma redução na perfusão é também um evento chave na patogênese da neuropatia glaucomatosa, além de vários fatores de risco já identificados, tais como: idade maior que 40 anos, escavação do nervo óptico aumentada, etnia (negra para o de ângulo aberto e amarela para o de angulo fechado), história familiar, ametropia (miopia para o de

ângulo aberto e hipermetropia para o de angulo fechado) e pressão de perfusão ocular diminuída (FLAMMER; MOZAFFARIEH, 2007; QUIGLEY, 2011). Níveis reduzidos de óxido nítrico (ON), um mediador vasodilatador, também foram demonstrados em pacientes com glaucoma e em modelos animais da doença (bem como em outras doenças neurodegenerativas) estão implicados na diminuição do fluxo sanguíneo ocular, levando a morte neuronal e perda de visão (DOGANAY et al., 2002; REICHSTEIN et al., 2007).

O desaparecimento da CGR representa o caminho final comum da perda de visão no glaucoma. Vários estudos demonstram que a CGR morre pelo mecanismo de apoptose em modelos animais de glaucoma (p.ex: modelos de isquemia-reperfusão, transecção do nervo ópitico) e também no glaucoma humano. Apoptose é um mecanismo básico de morte celular observado em várias condições neurodegenerativas. Constitui um programa de "suicídio" codificado geneticamente, ativado quando as células não são mais necessárias ou foram seriamente danificadas e é tipificado pela rápida fagocitose sem inflamação. Estas células demonstram alterações morfológicas características na microscopia: condensação da cromatina nuclear, compactação de organelas citoplasmáticas, vacuolização citoplasmática, vacuolização de membrana, lise celular e a formação de corpos apoptóticos (LAM et al., 1999; D'ONOFRIO; KOEBERLE, 2013; SCHMID et al., 2014; EZRA-ELIA et al., 2017; ZANONI et al., 2017).

2.3 Apoptose

Apoptose é uma modalidade de morte celular programada que pode ser identificada por diferentes características morfológicas e o envolvimento de proteínas específicas que regulam a mesma (REGNER, DA ROCHA, 2007; LUCHS, PANTALEÃO, 2010). A morte celular é também parte do desenvolvimento normal e do ciclo de maturação, também é um componente de muitos padrões de resposta de tecidos vivos para agentes xenobióticos (por exemplo, micro-organismos e produtos químicos) e as modulações endógenas, tais como a inflamação e fornecimento vascular irregular (CLAVIEN et al., 2000).

Apoptose é uma resposta celular fundamental que tem um papel crucial durante o desenvolvimento e na regulação da homeostase dos tecidos, eliminando as células não desejadas (DEGTEREV, YUAN, 2008). O termo "apoptose", definido como um tipo de

morte celular controlada que pode ser induzida por uma variedade de agentes farmacológicos e fisiológicos, foi descrita pela primeira vez por Kerr, Wyllie, e Currie em 1972 para descrever uma forma morfologicamente distinta da morte celular com base nos seguintes critérios morfológicos principais: encolhimento celular, condensação e marginação da cromatina nuclear, a fragmentação do DNA, vacuolização citoplasmática, vacuolização de membrana, lise celular e a formação de corpos apoptóticos.

As vias da apoptose foram descrita em Caenorhabitis elegans no início da década de 1990 e a subsequente análise genética da apoptose dos mamíferos apresentou-se mais complexa. Esses achados sugerem uma especialização funcional, assim como a regulação compensatória de sinalização e execução apoptótica poderia ser características importantes na apoptose dos mamíferos (DEGTEREV; YUAN, 2008).

Em determinados casos o tipo de estímulo determina se as células morrem por apoptose ou necrose. Em doses baixas, uma variedade de estímulos nocivos, tais como calor, radiação, hipóxia e quimioterápicos citotóxicos podem induzir apoptose, mas estes mesmos estímulos podem resultar em necrose em doses mais elevadas. Finalmente, a apoptose é um processo ordenado e dependente de energia que envolve a ativação de um grupo de proteases e cisteínas denomidadas "caspases" e uma cascata complexa de eventos que ligam os estímulos de iniciação para o desaparecimento final da célula (ZEISS, 2003; ELMORE, 2007).

Usando histologia convencional, nem sempre é fácil distinguir apoptose de necrose. Ambas podem ocorrer simultaneamente dependendo de fatores tais como a intensidade e duração do estímulo, extensão da depleção de ATP e a disponibilidade de caspases. A necrose é um processo descontrolado e passivo que normalmente afeta grandes áreas de células enquanto a apoptose é controlado, dependente de energia e pode afetar células individuais ou grupos de células. (ZEISS, 2003; ELMORE, 2007).

De modo geral, o mecanismo primário de lesão das CGR em glaucoma não é bem compreendido, mas há evidências experimentais de que a lesão neuronal na doença ocorra em grande parte por apoptose. É amplamente aceito que fatores neurotróficos promovem a sobrevivência neuronal inibindo vias apoptóticas padrão, uma vez que durante o desenvolvimento do sistema nervoso, neurônios jovens necessitam de fatores tróficos para a sua sobrevivência, diferenciação e o estabelecimento de conexões

sinápticas (DEGTEREV, YUAN, 2008; ALMASIEH et al, 2012). Os fatores neurotróficos são produzidos em quantidades limitadas, portanto, apenas os neurônios expostos a níveis ótimos dessas moléculas sobrevivem, enquanto os demais são eliminados por apoptose (LEWIN et al, 1998; ALMASIEH et ai, 2012).

A indução de apoptose pode ocorrer por estímulo externo ou interno. Há duas principais vias gerais de indução de apoptose: receptor ou via extrínseca e mitocondrial ou via intrínseca (GRIVICICH, REGNER, DA ROCHA, 2007). A via extrínseca promove a ativação de Caspases 2, 8, 9 e 10, que cliva o gene pró-apoptótico da família Bcl-2, por ligação do receptor Fas e Tnf-r (PAROLIN, RAZÃO, 2001). A via intrínseca é mediada por estímulos internos (intracelular), bem como lesão de DNA ou perturbação do ciclo celular ou em vias metabólicas (LUCHS, PANTALEAO, 2010).

Uma vez ativada as vias extrínsecas e intrinsecas, a maioria das caspases têm a capacidade de catalizar a ativação de vários outros membros desta família, resultando na amplificação da cascata proteolítica (PAROLIN, RAZÃO, 2001). As caspases são classificadas em dois grupos: as caspases iniciadoras (Caspase-2, 8, 9 e 10) e a caspases executoras (Caspase-3, 6 e 7). As formas funcionais das caspases iniciadoras promovem diretamente ou indiretamente a ativação de caspases executoras (PAROLIN, RAZÃO, 2001; GRIVICICH, REGNER, DA ROCHA, 2007; LUCHS, PANTALEÃO, 2010).

Na via extrínseca, um receptor de morte ativa uma proteína de morte intracelular TNF\Fas-associado que, por sua vez ativa pro-caspase-8 para formar um complexo de sinalização. A Caspase-8 é clivada e ativada através da autoproteólise levando a subsequente ativação de Caspase-3 e Caspase-6. A expressão de ambos os iniciadores e as caspases efetoras foi investigada em CGR após uma lesão do nervo óptico agudo ou crônico (HANNINEN et al., 2002; LUCHS, PANTALEAO, 2010; LEVKOVITCH-VERBIN et al., 2010; ALMASIEH et al., 2012).

Sinais apoptóticos extrínsecos incluem uma variedade de receptores de morte: Tnf-α, Fas-l e indutores de apoptose relacionado com Tnf (TRAIL) que se ligam aos seus respectivos receptores para induzir a morte celular. Os resultados da ativação desses receptores, recruta o iniciador procaspase-8 que conduz à ativação da Caspase-8, seguido por ativação da Caspase-3 e consequentemente a morte celular (ALMASIEH et al., 2012).

Na via intrínseca, o citocromo C é liberado a partir da mitocôndria e, juntamente com Apaf-1 e pro-caspase-9 forma o apoptossomo, o que facilita a ativação de Caspase-9 e promove clivagem de Caspase-3. O citocromo C, é libertado a partir da mitocôndria danificada, promovendo a formação de um heptamérico 'apoptossomo' megacomplexo de APAF1 e Caspase-9 (um membro da CED-3 como uma família de protease Cys). Isto leva à alteração conformacional e a ativação de Caspase-9. Ativado Caspase-9, por sua vez cliva e ativa caspases, incluindo Caspase-3, Caspase-6 e Caspase-7 que realiza a fase de execução da apoptose (DEGTEREV, YUAN, 2008; ALMASIEH et al., 2012).

Bax é uma proteína normalmente presente no citoplasma das células, mas depois da ativação do sinal de morte celular, o mesmo irá translocar e inserir na membrana mitocondrial externa. Vários estudos sugerem que monômeros de Bax podem formar uma estrutura de múltiplas subunidades de poro suficientemente grande para permitir a fuga de moléculas, como o citocromo C. Camundongos knock-out para o gene Bax funcional, apresentam várias populações de neurônios supranumerários, incluindo as células ganglionares da retina, indicando a importância da Bax na regulação da morte celular programada durante o desenvolvimento neuronal (WEI et al., 2001; KIRKLANDRA et al., 2002).

A elevação do glutamato endógeno e a ativação dos receptores de glutamato têm sido apontados como principais fatores para uma variedade de desordens neurológicas agudas e crônicas, incluindo acidente vascular cerebral, exocitose, várias formas de demência e neurodegeneração A hipótese central de lesão citotóxica é que o excesso de glutamato se liga à superfície celular nos receptores de glutamato ionotrópicos (Nmdar), principalmente de N-metil-D-aspartato (Nmda), desencadeando a ativação maciça e influxo de Ca, ocorrendo uma sinalização pró-apoptóticos nos neurônios. (KALIA et al,. 2008; NING et al., 2013).

Na retina, o excesso de glutamato tem sido proposto como a base comum doenças neurodegenerativas, tais como a oclusão da artéria da retina e glaucoma. Grande número de estudos demonstram que as CGR são extremamente sensíveis a Nmda aplicado exogenamente, uma vez que provoca a morte rápida destes neurônios, e que os inibidores de Nmdar e/ou suas vias são neuroprotetores em modelos

experimentais de isquemia da retina e glaucoma (SEKI et al., 2010; ALMASIEH et al., 2012; NING et al., 2013).

2.4 Neuroproteção e Sildenafil

Embora a terapia atual do glaucoma estaeja focada, principalmente, em reduzir a PIO, diversos grupos de pesquisa têm se dedicado ao desenvolvimento de drogas que oferecem proteção neural. Várias substâncias têm sido sugeridas como candidatos para a terapia neuroprotetora baseado em mecanismos de inibição de degeneração e apoptose das CGR, a fim de promover a sua sobrevivência (SANDALON et al., 2013). O óxido nítrico (ON) é uma molécula gasosa lábil liberada a partir de células endoteliais. Ela induz a vasodilatação, com aumento do fluxo sanguíneo e diminui a resistência vascular. A sua inibição conduz a uma redução da perfusão.

Não é surpreendente, portanto, que a disfunção do endotélio vascular, que resulta na diminuição dos níveis de ON, é encontrada em pacientes com glaucoma com consequente aumento da resistência vascular da retina. A deficiência na produção de ON é, por conseguinte, um participante na patogênese de glaucoma, enquanto o aumento de sua síntese e liberação pode impedir a progressão das manifestações nocivas (DOGANAY et al., 2002; JOHNSTON, 2005; TODA, NAKANISHI, 2007; OREJANA et al., 2012.).

O Sildenafil é um fármaco vasoativo que tem sido desenvolvido para o tratamento da disfunção erétil. Ele aumenta GMPc intracelular através da inibição da enzima PDE, bem como pelo aumento da sinalização mediada ON/GMPc. Há evidências experimentais de que o aumento de GMPc intracelular podem evitar a indução de estresse oxidativo e peroxidação lipídica (ABDOLLAHI et al., 2003; MILANI et al., 2005).

O efeito neuroprotetor do CS foi demonstrado em vários modelos de doenças neurodegenerativas, prolongando o efeito do ON, com consequente vasodilatação e melhora da circulação (JOHNSTON, 2005; OREJANA et al., 2012). O tratamento com SC resultou no aumento da sobrevivência neuronal, e até mesmo a neurogênese, em modelos de lesão cerebral e medula espinal (OZDEGIRMENCI et al., 2011; SERARSLAN et al., 2010).

Evidências sugerem que o CS apresentou efeito protetor em modelos isquemia/reperfusão (I/R) em rins de ratos (MEDEIROS et al., 2010), cardioproteção em modelos animais (WANG et al., 2015), reduziu a apoptose das células cardíacas induzida por diabete (EBRAHIMI et al., 2009), atenuou a apoptose do miocárdio em um modelo crônico de cardiotoxicidade com doxorrubicina (FISHER et al., 2005) e efeitos neuroprotetores no modelo de I/R da retina (EZRA-ELIA et al., 2017; ZANONI et al., 2017).

No olho, o CS pode afetar o fluxo sanguíneo ocular e o volume da coroide devido aos seus efeitos sobre os músculos lisos vasculares. Ele também pode ter efeitos vasodilatadores sobre sinusóides da coroide e vasos da retina semelhantes à vasodilatação de sinusóides do corpo cavernoso (MARMOR; KESSLER, 1999; EGAN; POMERANZ, 2000).

Estudos demonstraram que o CS não provoca danos ao DNA, apoptose, neurotoxicidade e inflamação. Quando a concentração de ON é maior do que 1 mM, os efeitos predominantes incluem desamidação, oxidação ou nitração de DNA através da interação de ON com superóxido ou radicais de oxigênio. Quando a concentração é inferior a 1 mM, suas ações são diretas, sem interagir com radicais superóxido ou oxigênio, podendo regular as atividades fisiológicas por diferentes vias de sinalização (LEE, CHENG, 2004).

A administração oral de CS encontra diversos obstáculos. O fármaco é vulnerável ao metabolismo sistêmico e efeitos colaterais podem ocorrer, tais como redução da pressão arterial, dores de cabeça, rubor e congestão nasal são concomitantes com a administração oral. Pacientes com moderada a grave doença cardiovascular ou aqueles submetidos a terapia de nitrato estão em risco aumentado para potencialmente graves efeitos secundários cardiovasculares adversos com a terapia CS. O pico relativamente tardio (30-45 minutos) e curta duração de ação têm sido demonstradas por CS, com uma meia vida de 4 horas. Entretanto, as doses utilizadas são necessárias para sustentar níveis de droga no plasma (LEE, CHENG, 2004; HUANG et al, 2010).

A administração tópica de CS através de uma área local do tecido pode ser considerada como uma alternativa para a via oral, a fim de evitar estes efeitos secundários adversos, para encurtar o tempo de latência, e para manter o efeito por

períodos mais longos (YONESSI, 2005; ELNAGGAR; EL-MASSIK; ABDALLAH, 2011).

2.5 Animais como modelos experimentais

Modelos animais têm sido um dos pilares da pesquisa básica e aplicada, permitindo a rápida progressão da descoberta científica, assim como melhor compreensão das vias envolvidas na doença, sem causar danos ao ser humano (MINHAS G, 2012). Modelos animais tiveram um lugar central na pesquisa médica para melhor compreender a etiologia, patologia e evolução da isquemia retiniana e também para ajudar na avaliação, desenvolvimento e melhoria das estratégias terapêuticas para o tratamento de doenças humanas e animais, bem como em ensaios pré-clínicos. (JOHNSON; TOMAREV, 2010; MINHAS G, 2012; NIWA et al., 2016).

Em geral, os modelos animais de glaucoma são classificados em duas categorias: modelos de ocorrência natural e modelos induzidos. Uma variedade de modelos de glaucoma de ocorrência natural foi descrita em diferentes espécies de animais, incluindo cachorro (beagle), coelho albino da Nova Zelândia e camundongos DBA/2J (ISHIKAWA et al., 2015; REICHSTEIN et al., 2007). Contudo, os modelos de glaucoma que ocorrem naturalmente são ruins para controlar o início e o curso patológico da doença. Os modelos de glaucoma induzidos foram desenvolvidos com o objetivo de criar condições adequadas para experimentos controlados (ISHIKAWA et al., 2015).

Por conseguinte, foram desenvolvidos alguns modelos em mamíferos *in vivo* e *ex vivo* para melhorar a precisão e a repetibilidade de condições experimentais e para examinar mecanismos patológicos. Assim como surgiram modelos glaucomatosos de camundongos transgênicos e geneticamente modificados (MINHAS G, 2012; D'ONOFRIO; KOEBERLE, 2013; ISHIKAWA et al., 2015; NIWA et al., 2016). Modelos in vitro sempre foram usados para obter informações sobre eventos bioquímicos e moleculares causados por isquemia; mas modelos animais são essenciais para entender a fisiopatologia da isquemia retiniana (JOHNSON; TOMAREV, 2010; MINHAS G, 2012).

Os modelos de isquemia da retina em ratos são frequentemente utilizados, visando estudar o equilíbrio entre oferta e demanda de energia, alterações vasculares e

neuronais, assim como a distribuição do suprimento sanguíneo da retina e da coroideia é bastante semelhante à dos seres humanos (MINHAS G, 2012). Dos modelos animais de glaucoma disponíveis, aqueles baseados em roedores são altamente atraentes por inúmeras razões, incluindo seu potencial para manipulação experimental (incluindo, mas não se limitando a genética), vida útil curta, baixo custo, estrutura ocular e fisiologia relativamente comparáveis aos humanos (JOHNSON; TOMAREV, 2010; MINHAS G, 2012; ISHIKAWA et al., 2015).

Existe uma grande variedade de modelos para o glaucoma, pois como a PIO elevada está fortemente associada ao início e progressão do glaucoma e os únicos tratamentos clinicamente aprovados para o glaucoma envolvem terapia farmacológica ou cirúrgica para reduzir a PIO, modelos de glaucoma que envolvem dano ao nervo óptico mediado pela hipertensão ocular são mais comuns (JOHNSON; TOMAREV, 2010; MUNEMASA; KITAOKA, 2013).

2.5.1 Mensurando a pressão intraocular

Uma ampla variedade de modelos de glaucoma demonstra que a extensão e duração da PIO elevada está correlacionada positivamente com perda de CGR, dano ao nervo óptico e díficit na função visual (EZRA-ELIA et al., 2017; GROZDANIC et al., 2003; JOHNSON; TOMAREV, 2010; ZANONI et al., 2017). Para interpretar os dados obtidos em modelos de hipertensão ocular, é importante poder determinar e com precisão medir a PIO em animais vivos ao longo do tempo. O método mais direto disponível para medir a PIO em roedores é pela manometria de fluidos um transdutor de pressão após a canulação da câmara anterior com uma microagulha (JOHN et al., 1997). Enquanto esta técnica padrão-ouro é altamente preciso, é um método invasivo que possui certas limitações. Cura da córnea no local da canulação deve ocorrer entre medições sucessivas. Além disso, a PIO deve ser medida sob anestesia geral, que demonstrou rapidez e reduzir substancialmente a PIO em alguns casos (JOHN et al., 1997; JOHNSON; TOMAREV, 2010).

O TonoPen foi utilizado com algum sucesso em roedores, mas a ponta relativamente grande que foi originalmente projetada para uso humano parece produzir resultados menos confiáveis em ratos (MOORE; MILNE; MORRISON, 1993). Mais recentemente, um tonômetro de indução de impacto, o TonoLab, foi desenvolvido e tem

mostrado maior eficácia na mensuração da PIO em roedores conscientes. Em comparações diretas entre a manometria TonoPen ou TonoLab e microagulhas, o TonoLab parece produzir medições mais precisas (SAEKI et al., 2008; ZANONI et al., 2017)

2.5.2 Elevando a pressão intra-ocular

A indução de isquemia aguda da retina por um período entre 30 à 120 minutos seguida de reperfusão causa a morte dentro de uma variedade de tipos de células dentro da retina, p.ex. apoptose nas CGRs, o dano ocorre ao longo das várias camadas da retina. Como tal, pode-se argumentar que este modelo representa degeneração da retina global em vez de glaucoma per se. Não obstante, tem sido utilizado para investigar a morte e disfunção de CGR e tem sido particularmente importante na investigação do potencial papel da insuficiência vascular no glaucoma (NIWA et al., 2016; PEACHEY; GREEN; RIPPS, 1993; ZANONI et al., 2017).

As lesões na retina pode pode ser induzida através de uma variedade de métodos (p.ex. transação do nervo óptico, injeção ocular de agentes excitotóxicos e isquemia/reperfusão). Dentre os diversos modelos de indução e isquemia, a canulação da câmara anterior de camundongos e ratos com uma microagulha é um modelo de fácil reproduação e permite controlar com precisão a pressão intra-ocular. Ao ajustar a PIO acima da pressão de perfusão ocular (geralmente a cerca de 110 mmHg), o fluxo sanguíneo através da vasculatura retiniana e uveal é suprimido (JOHNSON; TOMAREV, 2010; MINHAS G, 2012; NIWA et al., 2016). A PIO pode ser normalizada pela redução da pressão do sistema de perfusão após o término do período de exposição isquêmica. Embora este método envolva hipertensão ocular aguda extrema, o efeito neurodegenerativo é pensado para ser mediado principalmente através do insulto isquêmico e ativando a via de apoptose, embora, pela indução PIO, seja possível que outros danos nas CGR e retina também pode desempenhar um papel na lesão, semelhantes aos observados no glaucoma (JOHNSON; TOMAREV, 2010; D'ONOFRIO; KOEBERLE, 2013; MORGAN, 2012; NIWA et al., 2016).

O método mais utilizado para estudar os mecanismos envolvidos na isquemia retiniana demonstraram que o modelo de isquemia retiniana induzida pela pressão intra ocular alta (PIO) foi estabelecido como um importante modelo PEACHEY et al (1993).

O modelo de PIO cria isquemia ao elevar e manter a pressão intraocular acima da pressão arterial sistêmica (JOHNSON; TOMAREV, 2010; ISHIKAWA et al., 2015; ZANONI et al., 2017). Este aumento da pressão intra ocular bloqueia a circulação sanguínea da retina e, portanto, leva à isquemia (JOHNSON; TOMAREV, 2010; ISHIKAWA et al., 2015; MINHAS G, 2012). Este modelo é utilizado para estudar mudanças na expressão de proteínas, excitotoxicidade e alteração nas propriedades celulares em vários modelos. Os modelos animais de PIO também foram usados para estudar as mudanças nas reatividades de anticorpos séricos após isquemia (NICKELLS, 2007; WURM et al., 2011; SOTO; HOWELL, 2014; NIWA et al., 2016).

Modelos animais de glaucoma são essenciais para interromper ou elucidar o curso natural da doença e o desenvolvimento de intervenções terapêuticas para reverter a progressão da condição. Embora nenhum modelo experimental único seja ideal, cada um dos sistemas existentes tem sido usado com sucesso para descobrir aspectos importantes da patologia do glaucoma e pode ser usado para desenvolver novas terapias para a doença no futuro (NICKELLS, 2007a; JOHNSON; TOMAREV, 2010; D'ONOFRIO; KOEBERLE, 2013; NIWA et al., 2016).

O desenvolvimento contínuo desses modelos e aprofundando a exploração de suas alterações fisiopatológicas contribuirá para avanços na compreensão e tratamento do glaucoma em pacientes humanos no futuro.

CAPÍTULO 2 – Trabalho Científico

Trabalho a ser enviado para a revista: Experimental Eye Research.

Guia para publicação: https://www.sciencedirect.com/journal/experimental-eye-research

Topic administration of Sildenafil citrate attenuates morphological changes in the retinal ischemia/reperfusion injury in rats.

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Purpose: Retinal ischemia/reperfusion (I/R) injury is an important cause of visual impairment. However, questions remain on the overall I/R mechanisms responsible for progressive damage to the retina. In this study, we investigate the effects of eye drops Sildenafil Citrate (SC) in retinal ischemia/reperfusion (I/R) model in rats and characterized the pathogenesis by analyzing temporal changes of retinal morphology and morphometry

Methods: A total of eighty-four male rats (*Rattus norvegicus*), over 6 months old, average weight between 350g to 450g, were used in this study. The animals were divided into seven groups: a control group (CG) and six groups submitted to retinal ischemia / reperfusion (I/R) model. Each experimental group consisted of 12 animals treated with Sildenafil Citrate eye drops (2%) and 12 placebo animals treated for 7, 14 or 21 days, and a control group. Paracentesis of the anterior chamber was made with a 30G needle, coupled to saline (0.9%) and maintained for 60 minutes. Intraocular pressure was measured by rebound tonometer (Tonovet®). The eyes were collected for histopathology and morphometry

Results: Acute I/R injury significantly decreased retinal thickness and its respective layers in PG groups, compared to CG, as well as RGC cell number, together with significant morphologic changes. The use of eye drops SC protected the retina, that showed less lesions in morphology, compared to placebo group, as well as morphometric parameters closer to normal animals.

Conclusion: Our results demonstrated that eye drops sildenafil can exert a neuroprotective role against retinal I/R injury. The maintenance of retinal morphology was evident in the retinal ganglion cell layer of treated animals, shown in histology and morphometry.

Key Words: Glaucoma, Sildenafil citrate, Ischemia-reperfusion, Retinal ganglion cell, Morphometry, Morphology.

INTRODUCTION

Retinal ischemia, in its various forms, is a common cause of visual impairment and blindness in the industrialized world. Diseases associated with ischemia include retinal vascular occlusion, diabetic retinopathy, glaucoma, and ocular trauma. It is caused by a reduction of the retinal blood supply that decreases the delivery of oxygen and other nutrients to various retinal layers (OSBORNE et al., 2004; BERGERT; FUNK, 2008; KIM et al., 2013; SCHMID et al., 2014) All of these diseases have been shown to lead to thinning of the nerve fiber layer, as a result of ganglion cell injury or death. In experimental studies, ganglion cells loss have been described in result to retinal ischemia (OSBORNE et al., 2004).

In animal models, retinal I/R can be induced through different techniques, and, one of the most frequently used models to study pathologic processes, molecular mechanisms and to explore possible potential therapeutic strategies for retinal ischemia has been a model of acute elevation of intraocular pressure (IOP) followed by reperfusion (SCHMID et al., 2014; JUNG et al., 2016; JOACHIM et al., 2017; EZRA-ELIA et al., 2017). This model, where the pressure in the eye is temporarily increased through the infusion of liquid into the anterior chamber via cannulation, shows compression of the passing vasculature through the optic disc supplying the retina (LAM; ABLER; TSO, 1999; JUNK et al., 2002; ZHENG et al., 2007; JUNG et al., 2016).

Extensive studies in this model demonstrate that there are loss of neuronal cells, especially in the inner retinal layers, retinal ganglion cells (RGCs) (SELLÉS-NAVARRO et al., 1996; HAYREH et al., 2004; SCHMID et al., 2014). Previous studies indicate that these cells are most sensitive to ischemia. This leads to a reduced thickness of the retina (ZHAO et al., 2013; JOACHIM et al., 2017). Longer periods of ischemia also affect the outer retinal layers, including photoreceptors (JUNK et al., 2002; ZHENG et al., 2007; JUNG et al., 2016; LIU et al., 2016; UEDA et al., 2016; JOACHIM et al., 2017). Therefore, neuroprotection is always the aim to reduce or prevent RGC and retinal damage with pharmaceutical intervention or molecular genetics techniques (EZRA-ELIA et al., 2017; KUEHN; FINGERT; KWON, 2005; LIU et al., 2016; ZANONI et al., 2017).

Sildenafil citrate (SC) (Viagra®), a potent inhibitor of the vascular-associated enzyme phosphodiesterase type 5 (PDE5), is used to treat erectile dysfunction (ABBOTT et al., 2004; THARAKAN; MANYAM, 2005). It induces vasodilation by enhancing the smooth muscle relaxing effect of nitric oxide. Nitric compounds produce an increase in optic nerve head circulation and retinal venous vasodilation (GRUNWALD et al., 2001; ELTONY; ABDELHAMEED, 2017). Initially, the primary clinical target for SC treatment was erectile dysfunction, but during the last years, it has been shown to be useful for the prevention and treatment of high altitude sickness and pulmonary arterial hypertension (WIROSTKO et al., 2012).

Evidences suggest that SC has a protective effect in other models, such as rat kidney I/R model (MEDEIROS et al., 2010; ORUC et al., 2010), cardio protection in animal models and humans (WANG et al., 2015), reducing apoptosis of cardiac cells induced by diabetes (EBRAHIMI et al., 2009), and attenuating myocardial apoptosis in a chronic model of cardiotoxicity with doxorubicin (FISHER et al., 2005) and affects neuroprotection in retinal I/R model (EZRA-ELIA et al., 2017; ZANONI et al., 2017).

However, oral administration of CS has side effects in humans, such as reduced blood pressure, temporary vision loss, headache, flushing and nasal congestion, as well as an increased risk for adverse cardiovascular effects in patients with moderate to severe cardiovascular disease or those undergoing nitrate therapy (LEE; CHENG, 2004). Eye drops administration of CS may be considered an alternative to the oral route, avoiding the adverse side effects, decreasing the latency time and maintaining the effect at the site for a longer period (YONESSI, 2005; ELNAGGAR et al., 2011).

Previous study from our group showed a protective effect of systemic Sildenafil treatment for I/R lesions in the rat retina (EZRA-ELIA et al., 2017; ZANONI et al., 2017). Due to the adverse effects of systemic SC treatment described in the literature, we aimed to investigate the effects of SC eye drops in I/R retinal lesion in rats, as well as the role of the treatment in the morphological changes.

METHODS

Animals

A total of forty-four male rats (*Rattus norvegicus*), over six months old, average weighing between 350g to 450g, were used in this study. The animals were clinically and ophthalmically evaluated by routine methods and allocated in appropriate cages, clean, sanitized, ventilated environment and, with access to food and water "*ad libitum*" and were kept under 12-hour cycles of light and controlled temperature.

Ethical approval statement

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures performed in the present study involving animals were in accordance with the ethical standards of the Ethics Committee on Animal Use (CEUA), Sao Paulo State University (UNESP), Botucatu, Medical School (Protocol No. 1199-2016).

Groups and experimental protocols

The animals were divided into seven groups: a control group (CG) consisting of six animals without any intervention, and other six groups with induced acute retinal I/R injury, treated with sildenafil (SG) for 7 days (n = 12), 14 days (n = 12) and 21 days (n = 12) or placebo (PG) for 7 days (n = 6), 14 days (n = 6) and 21 days (n = 6) after I/R injury.

Topical proparacaine hydrochloride (0.5%), and a combination of intramuscular ketamine (75 mg/kg) and xylazine (10 mg/kg) were used for anesthesia.

A 30-gauge needle connected to saline reservoir was inserted into the anterior chamber through the cornea of right eyes, coupled to a bag of 0.9% saline, positioned at a height of 150 cm above the eye (according to Zanoni et al., 2017 and Schmid et al. 2014). Intraocular pressure was raised to 45 mmHg for 60 min, and IOP was measured using a rebound tonometer (TonoVet Icare, Vantaa, Finland). Afterwards, the animals were treated with dipyrone (200 mg/kg) and morphine (1 mg/kg) by hypodermic injection, as analgesics. Sildenafil Citrate eye drops (2%) was manufactured by Eye

Pharma Company (São Paulo – Brazil) (pH 4.7; vehicle - buffer with sodium chloride, monosodium phosphate, disodium phosphate, benzalkonium chloride and water for injectables), and was instilled as eye drops BID (between 8:00 a.m. - 8:30 a.m. and, between 8:00 p.m. - 8:30 p.m. in the right eyes) on the day of I/R injury induction and for every day until euthanasia, while for PG we used the same vehicle solution as SC, but without SC, and with equal criteria.

All animals (SC and PG) were euthanized by intramuscular injection of a combination of ketamine (75 mg/kg) and xylazine (10 mg/kg) and a subsequent intracardiac injection of sodium pentobarbital (120 mg/kg) 1 h after the last eye drop instillation. The CG animals were euthanized using the same method as SC and PG after attesting healthy eyes by clinical and ophthalmological evaluation.

Collection, storage and preparation of samples

After euthanasia, half of the animals (n=6) in each group had their right eyeball excised with the optic never. The eyes were fixed in Davidson's solution for 48 hours and, the macroscopic cut were made in a parasagittal section of the eye (positioned the cutting blade adjacent to the optic nerve and perpendicular to the posterior ciliary artery). These eyes were than washed in running water, maintained in 70% ethanol up until routinely processed for paraffin embedded. The entire eye was embedded in paraffin and the paraffin block was cut transversely in 3μm thick consecutive sections, in an automatic microtome (Leica - RM2255, Heidelberg, Germany), until it reached the center of the optic nerve, including the retina. For each eye three slides were made, and HE stained for morphometry and histopathology evaluation.

The other half of the SC group had their right eye excised. Vitreous humor sample was collected in a sterile syringe and placed in an Eppendorf tube, maintained in -80 °C until Elisa test.

Measurement of Sildenafil Citrate Concentration by ELISA

Sildenafil of the treatment group was measured from the vitreours humor (VH) using an ELISA kit (MaxSignaL® Sildenafil/Vardanafil ELISA Kit, Bioo Scientific, Austin, USA) according to the manufacturer's instructions. Briefly, was established a dilution factor using VH obtained from one animal per group for adequation of samples

into dilution curve to the manufacturer's instructions. So, the samples were diluted at 1:240 in PBS, and reagents were added according to the manufacturer's instructions. The values were read from a six-way parameter fit standard curve. Averaged concentrations of duplicates were then multiplied by the dilution factor. The manufacturer reports that assay antibodies have 100% cross-reactivity with SC. The results were expressed in ng/ml and the coefficient of variation was measured.

Morphometry and histology

The morphometric analysis was performed using an image analysis program QWin v3.013 (Leica, Heidelberg, Germany) and a conventional optical microscope (Leica - DMR, Heidelberg, Germany) equipped with a digital camera (Leica - DFC500, Heidelberg, Germany). Five images (fields) were obtained from each slide with a 20X objective, covering the full extent of the retina, from ora serrata to ora serrata, including the central portion of the optic nerve. In each image, we evaluated the total thickness of the retina and each of its layers [the retinal ganglion cell (RGC), inner plexiform (IPL), inner nuclear (INL), outer plexiform (OPL) and the outer nuclear (ONL)] and counted the cells from the retinal ganglion cell layer. The mean thickness of each layer and the mean number of cells in the ganglionar layer, in each treatment group, were compared to the control group (according to ZANONI et al., 2017). The histopathological evaluation was performed according to JOHNSON et al. (2000) and SCHMID et al. (2014), including vascular, inflammatory (edema and inflammatory infiltrate), cellular (necrosis/apoptosis) and tissue changes (atrophy). The analysis was descriptive, according to the treatment group.

Statistics

For the histological changes, descriptive analyses were employed. For morphometry, the thickness of the total retina and its layers, and for the total number of cells in the retinal ganglion cell layer, the results were evaluated for normality by Kolmogorov–Smirnov test. ANOVA and t-test were used to evaluate the morphometry, RT-qPCR and Elisa results. We used ANOVA test to evaluate the statistical difference between all groups (e.g. CG vs. SG 7 days vs. PG 7 days). T-test was used to evaluate

statistical difference between two variables (e.g. CG vs. SG 7 days, SG 7 days vs. PG 7 days and CG vs. PG 7 days). The results statistical significance was set at $p < 0.05$.

RESULTS

Sildenafil Citrate Concentrations

Concentrations of SC in humor vitreous are shown in Figure 1. We observed a progressive increase of SC concentration according to the treatment time, but with no statistical difference between groups. SC concentration was 236.7 ± 45.64 (7 days), 378.5 ± 124.4 (14 days) and 515.3 ± 133.8 ng/ml (21 days). The coefficient of variation from eye drop containing 2% of sildenafil were 17.4%.

Morphometry and Histopathology

Acute I/R injury significantly ($p < 0.05$) decreased retinal thickness and its respective layers in PG groups, compared to CG, as well as RGC cell number, together with significant morphologic changes (Figures 2-4 and Table 1-2).

All groups had statistically significant decrease in total retina thickness comparing to CG, where it was greater and progressive in the experimental PG, than in SG. The RGC layer in the PG was significantly ($p < 0.05$) decreased at 21 days after I/R injury compared to CG eyes by approximately 40%, which correlates with morphological changes of I/R-injured retinal layers. However, cell number in the RGC layer of SG decreased 13% after I/R injury (Table 2).

In the PG, the layers IPL, INL, OPL and ONL showed decreased thickness values for all time points, with statistically significant reduction compared to CG and, the analysis showed more preservation in all SG for all time points when compared with PG and CG, except in OPL SG 7 days (table 2). In SG, the total cell number of the RGC was preserved in the different treatment times, comparing to PG.

The number of animals with inflammatory response was higher in PG (all treatment moments), as well as vascular changes, especially in 21 days of treatment (Figures 2-4; table 2).

DISCUSSION

Neuroprotection involves prevention of neuronal death secondary to injury or disease and is also a popular subject in the treatment of ocular disease. Surprisingly, the neuroprotective and/or neurorestorative potential of sildenafil on retinal diseases have not been highly explored. Most of the available studies on the effect of sildenafil on the retina investigated the risks of sildenafil side effects on the function or structure of the retina (DÜNDAR et al., 2001;ABDULSAHIB et al., 2015; KUMARI et al., 2016; MOSCHOS; NITODA, 2016), but our group have been investigating the potential use of SC for eye treatment (EZRA-ELIA et al., 2017; ZANONI et al., 2017).

In the present study, our hypothesis was that SC does not cause damage in the retinal cells and is a neuroprotective therapy, being effective in the treatment of diseases characterized by ischemia, associated with increased loss of retinal cells. We demonstrate for the first time, that eye drops SC at 2% have histological and morphological results similar to oral administration (EZRA-ELIA et al., 2017; ZANONI et al., 2017), being a safe component for the retina. Furthermore, the present eye drops SC administration, were better than oral SC, with more preservation of RGC layer and retina at 21 days.

Our results, concerning retinal safety after the use of SC was also described by WIROSTKO et al. (2012), that demonstrated that chronic oral sildenafil treatment, did not lead to any significant signals on ocular examination, on assessment of visual function, or through visual disturbance questionnaires. These authors found that, with respect to changes in subjective visual function, the only effect was a low dose dependent, and in many cases reversible incidence of the transient phosphodiesterase type 6 mediated effects that have been reported from clinical experience with sildenafil single use in the treatment of erectile dysfunction. Moreover, treatment with the clinically approved dose for pulmonary arterial hypertension, 20 mg three times daily, resulted in a frequency of these effects that was indistinguishable from that with placebo treatment.

Retinal thickness and RGC count in PG had a significant and progressive decreased after I/R injury, both morphologic and morphometric analysis. Theses results were similar to other studies, demonstrating that there was loss of neuronal cells,

especially in the retinal layers, as well as in RGCs layer (KALESNYKAS et al., 2012; SCHMID et al., 2014; UEDA et al., 2016; JOACHIM et al., 2017). On the other hand, the treatment with eye drops SC compared to our previous study (ZANONI et al., 2017) had a better neuroprotection effect *in vivo* in I/R injury, reduced retinal cell injury as demonstrated by morphological and morphometry results in SG, with preservation in the number of RGC and thickness in all groups in comparison to the PG and control. In addition, the progressive retinal degeneration after I/R injury was also strongly correlated with significant cell loss in the RGC layers and other layers in the PG. Interestingly, we found a cell loss ratio in histologic analysis of40% at 21 days in the PG compared to CG, which correlates with morphological changes of I/R-injured retinal layers. However, cell number in the RGC layer of SG decreased 13% after I/R injury. KIM et al. (2013) found cell loss ratio in histologic data (~30%) at 28 days after I/R injury in rats. These data sugest that I/R injury caused cell loss of RGC and that SC had a protective effect on cell loss, since it was decreased 13% in the SG group at 21 days.

The RGC are the primary cellular population affected in glaucoma, and the numbers of surviving RGC and their axons are important determinants in the extent of visual impairment in the glaucomatous patients. One major goal in glaucoma research and clinical management of glaucoma is to prevent RGCs from degeneration. (CHEN; TANG, 2011; WURM et al., 2011; ZHAO et al., 2013). So, by the results shown in this research, there was maintenance in RGC population in the animals treated with eye drops SC.

CONCLUSION

Our data indicated that eye drops SC is a neuroprotector in induced retinal ischemia, also in the different stages of injury. However, neuroprotective mechanisms of sildenafil on the retina remain to be elucidated and further studies should be made to better understand the effects of this agent for the treatment of the retina, optic nerve, and visual axis associated with acute retinal I/R injury.

These histopathologic and morphometric observations demonstrated that eye drops SC treatment reduced the RGC loss and retinal injury after I/R and provides a protection of the retina.

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Table1. Number of animals in each group, according to the histopathological changes in the retina, from rats with induced ischemic/reperfusion injury model, treated with eye drops sildenafil (SG) or not treated (PG).

	Histopathological changes															
	Vascular				**Inflamatory**				**Cellular**				**Tecidual**			
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
PG-7d	1/6	5/6	-	-	3/6	2/6	1/6	-	1/6	2/6	3/6	-	-	5/6	1/6	-
SG-7d	1/6	5/6	-	-	4/6	2/6	-	-	2/6	3/6	1/6	-	2/6	3/6	1/6	-
PG-14d	1/6	3/6	2/6	-	2/6	4/6	-	-	-	2/6	3/6	1/6	-	2/6	3/6	1/6
SG-14d	2/6	4/6	-	-	5/6	1/6	-	-	3/6	3/6	-	-	2/6	4/6	-	-
PG-21d	3/6	3/6	-	-	3/6	3/6	-	-	-	1/6	4/6	1/6	-	3/6	2/6	1/6
SG-21d	4/6	2/6	-	-	5/6	1/6	-	-	3/6	3/6	-	-	2/6	3/6	1/6	-

*PG, placebo group, SG, sildenafil group; 7, seven-day treatment; 14, fourteen-day treatment; 21, twenty-one-day treatment. **0, no change; 1, discrete; 2, moderate; 3, severe.

Table 2. Mean values (SD) of retinal layers thickness (μm) and the count of cells from the retinal ganglion layer (RGC layer) from normal rats (CG) and rats with induced retinal ischemia/reperfusion injury, treated (SG) or not (PG) with Sildenafil for 7, 14 and 21 days. Whole retinal thickness and sub-layer (IPL, INL, OPL and ONL) thickness and count RGC layer at each time point was measured and statistically analyzed. Data represent mean ± standard error of percentages at each time point from control and I/R eyes compared to the day 0-control eyes, which were set as 100%.

THICKNESS OF LAYERS (μm)

GROUPS	**TREATMENT**	**Whole Retinal Layer** (mean±sd) Eye Glaucoma		**Internal Plexiform** (mean±sd) Eye Glaucoma		**Internal Nuclear** (mean±sd) Eye Glaucoma		**Outer Plexiform** (mean±sd) Eye Glaucoma		**Outer Nuclear** (mean±sd) Eye Glaucoma		**Ganglion Cell (Score)** (mean±sd) Eye Glaucoma	
7 days	7 days - Placebo	148.5 ± 3.8[ab]	p<0.05	36.50 ± 1.5[ab]	p<0.05	23.05 ± 0.8[ab]	p<0.05	7.425 ± 0.1[a]	p<0.05	39.13 ± 0.5[ab]	p<0.05	236.7 ± 9.9[ab]	p<0.05
	7 days – Sildenafil	168.5 ± 3.5[ab]		41.40 ± 1.4[b]		26.15 ± 0.9[ab]		7.134 ± 0.1[a]		41.28 ± 0.3[ab]		292.0 ± 4.1[ab]	
14 days	14 days – Placebo	130.0 ± 2.3[ab]	p<0.05	30.70 ± 0.9[ab]	p<0.05	24.00 ± 0.6[ab]	p<0.05	7.161 ± 0.3[ab]	p<0.05	41.77 ± 0.7[ab]	p<0.05	197.8 ± 28.4[ab]	p<0.05
	14 days – Sildenafil	167.5 ± 1.7[ab]		36.37 ± 0.7[ab]		28.33 ± 0.5[ab]		8.135 ± 0.1[ab]		45.59 ± 0.91[b]		292.8 ± 13.4[b]	
21 days	21 days – Placebo	129.1 ± 3.6[ab]	p<0.05	32.77 ± 1.5[ab]	p<0.05	24.94 ± 0.8[ab]	p<0.05	6.909 ± 0.2[ab]	p<0.05	36.42 ± 0.3[ab]	p<0.05	202.8 ± 10.2[ab]	p<0.05
	21 days – Sildenafil	166.1 ± 2.3[ab]		38.54 ± 1.1[ab]		30.44 ± 0.6[ab]		8.339 ± 0.1[ab]		45.72 ± 0.1[b]		289.2 ± 8.0[ab]	
	Control	181.8 ± 3.4		43.99 ± 1.2		33.25 ± 0.5		8.906 ± 0.2		46.90 ± 0.9		332.2 ± 11.9	

[a] There was a significant difference in the groups compared to the control. [b] There was a significant difference in the SG to the PG. P value represents statistical significance of differences between groups by ANOVA test.

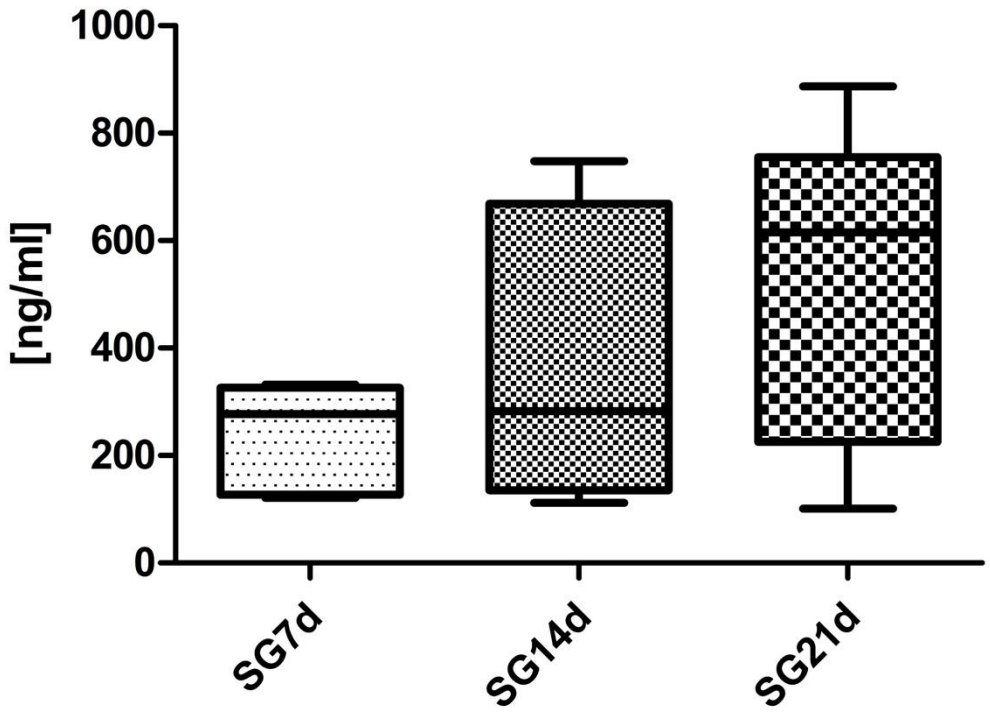

Figure 1. Mean Sildenafil concentration (ng/mL) in the humour vitreous of rats with ischemic reperfusion retinal lesion, after 7 days, 14 days and 21 days treatment with eye drops administration of 2% Sildenafil Citrate.

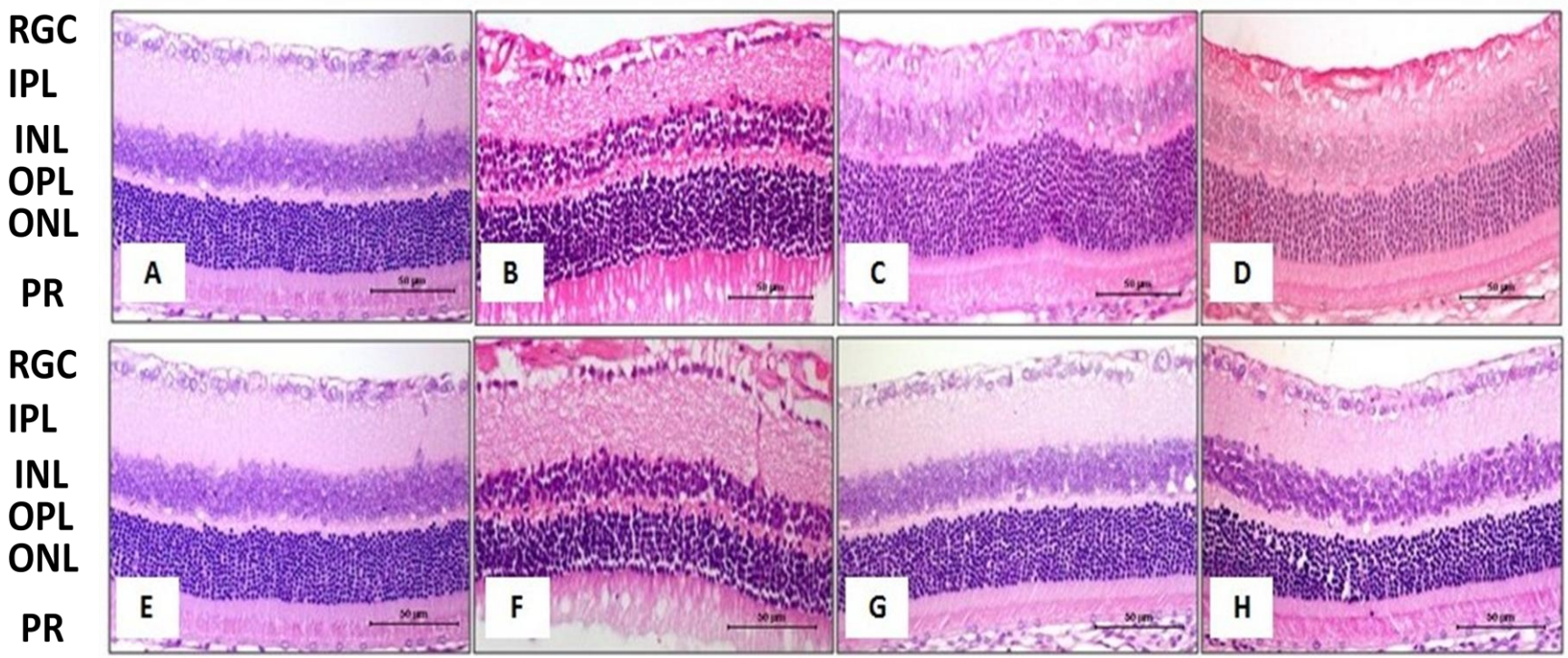

Figure 2. Retinas from rats with induced ischemia–reperfusion; morphological changes. (A and E) control group. (B) PG 7 days, retinal with discrete retinal atrophy, discrete loss of the both plexiform, nuclear and retinal ganglion cell layers. (C) PG 14 days, moderate retinal decrease thickness with the loss of RGC layer and moderate atrophy of the inner plexiform layer. (D) PG 21 days, significantly decrease of the retinal thickness with the market loss of RGC layer. (F) SG 7 days, maintance of retinal thickness and lower loss of the cells from the RGC layer. (G) SG 14 days, retinal with discrete retinal atrophy, discrete loss of the both plexiform, nuclear and RGC count. (H) SG21 days, discrete retinal atrophy with the discrete loss of RGC count and discrete atrophy of inner plexiform layer and the inner nuclear layer. HE, 40 X, scale 50 bar. RGC: Retinal ganglion cell; IPL: Inne plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; PR: Photoreceptor layer.

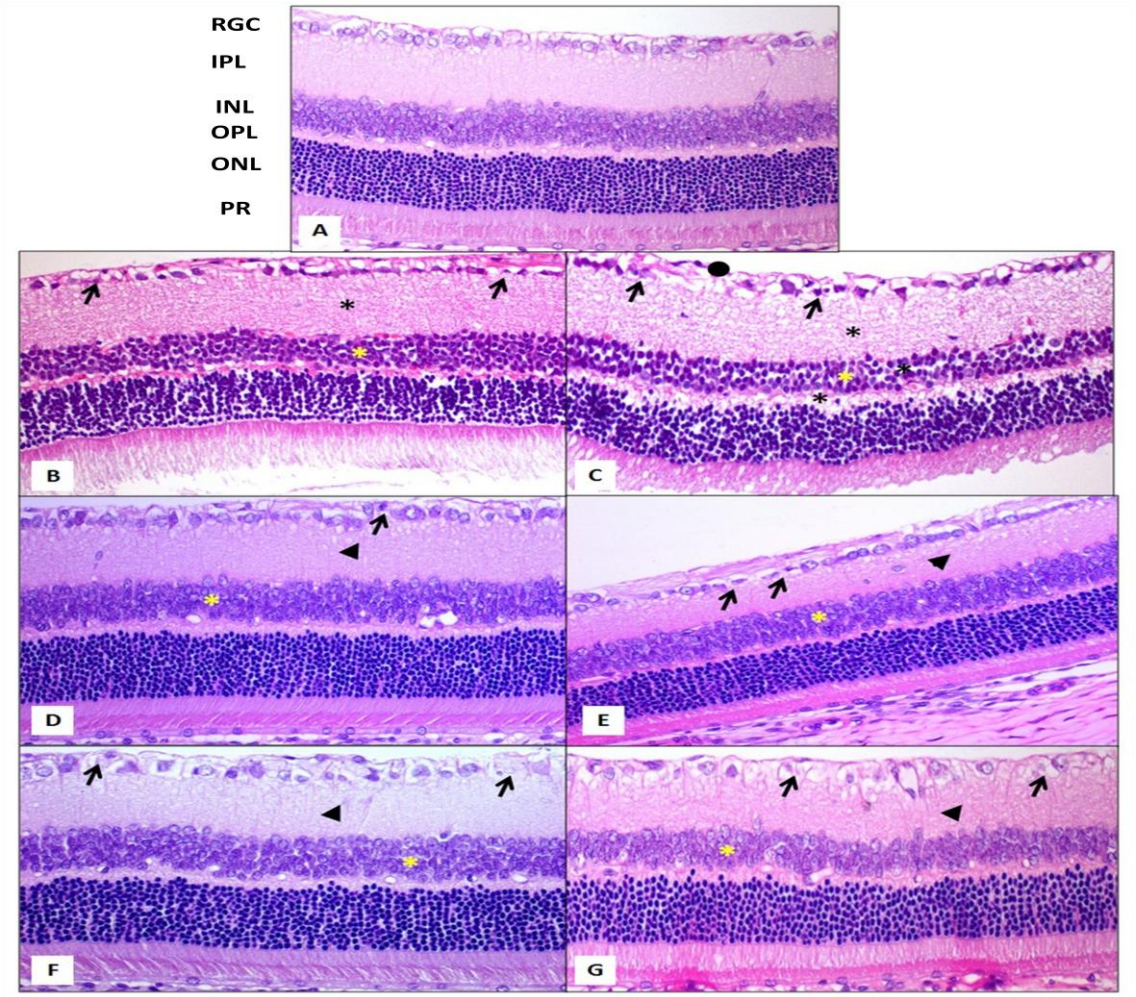

Figure 3. Morphological changes in the retina from rats with induced ischemia–reperfusion; (a) control group. (b) SG 7 days, retinal with few apoptotic bodies; pyknosis, karyolysis and chromatolysis (arrow), discrete extracellular edema (black asterisk) and low decrease in plexiform layer thickness (yellow asterisk). (c) PG 7 days, retinal with few apoptotic bodies ;nuclear pyknosis, karyolysis and chromatolysis (arrow), moderate extracellular edema (black asterisk) in IPL, INL, OPL, discrete leukocytes infiltrate (black circle) and discrete decrease in INL thickness (yellow asterisk). (d) SG 14 days, PG 7 days, retinal with few apoptotic body; nuclear pyknosis, karyolysis and chromatolysis (arrow), discrete decrease in IPL thickness (arrowhead) and discrete decreasein INL thickness (yellow asterisk). (e) PG 14 days, retinal with moderate number of apoptotic bodies; nuclear pyknosis, karyolysis and chromatolysis (arrow), moderate decrease in IPL thickness (arrowhead) and moderate decrease INL (yellow asterisk). (f) SG 21 days, retinal with decrease RGC number and moderate number of apoptotic bodies; nuclear pyknosis, karyolysis and chromatolysis (arrow), moderate decrease in IPL thickness (arrowhead) and discrete decrease in INL thickness (yellow asterisk). (g) PG 21 days, significantly decrease in RGC count and apoptotic bodies; nuclear pyknosis, karyolysis and chromatolysis (arrow), marked decrease IPL (arrowhead) and marked decrease INL (yellow asterisk). HE, 40 X , scale 50 bar. . RGC: Retinal ganglion cell; IPL: Inne plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; PR: Photoreceptor layer.

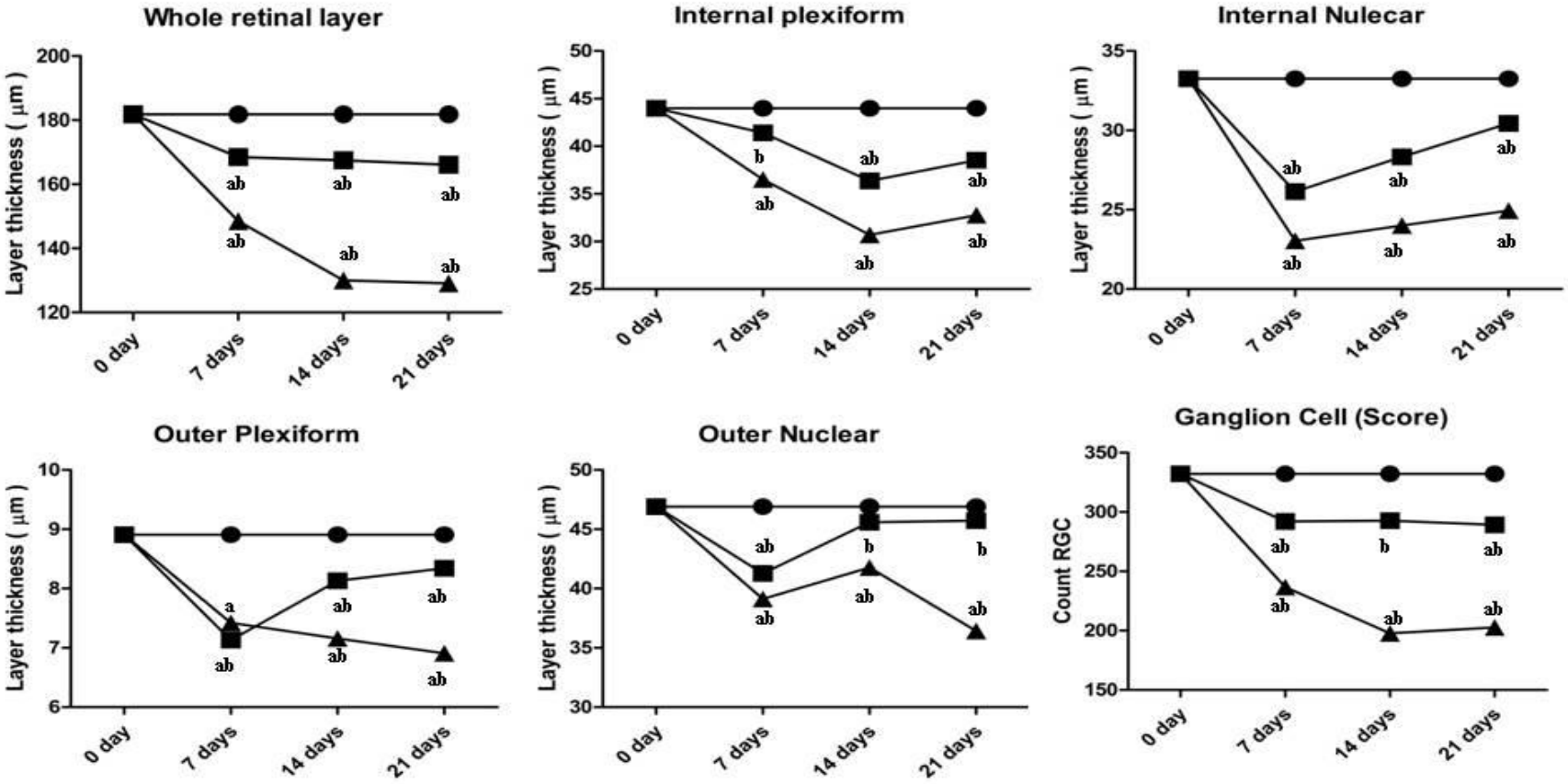

Figure 4. Graphic representation of retinal layers thickness (µm) and the count of cells from the retinal ganglion layer (RGC layer) from normal rats and rats with ischemia/reperfusion injury, treated (SG) or not (PG) with eye drops Sildenafil citrate for 7, 14 and 21 days. [a] There was a significant difference in the groups compared to the control. [b] There was a significant difference between SG and PG ($p<0.05$). ● Control; ■ Sildenafil Group; ▲ Placebo Group.

CAPÍTULO 3 – Trabalho Científico

Trabalho a ser enviado para a revista: Progress in Retinal and Eye Research.

Guia para publicação: https://www.elsevier.com/journals/progress-in-retinal-and-eye-research/1350-9462/guide-for-authors

Role of topical Sildenafil Citrate (2%) on ocular neuroprotection after retinal ischemia reperfusion model in rats

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Purpose: Retinal ischemia plays a central role in several retinal diseases. The pathogenesis of retinal ischemia involves changes in gene and protein expression. Sildenafil Citrate (SC) showed a protective effect in ischemia/reperfusion (I/R) models with neuroprotective effects. We investigated the effects of eye drops Sildenafil Citrate (SC) in retinal ischemia/reperfusion (I/R) model in rats and determined whether neuroprotection was related to apoptosis, retinal excitotoxicity and Bdnf pathway.

Methods: The experiment was conducted in seven groups of male rats, over 6 months old, weight between 350g to 450g. One group was a control group with 6 animals. The other groups were submitted to acute ischemia / reperfusion (I/R) and treated or not with sildenafil citrate (SC) for 7, 14 and 21 days. The eyeballs were used for gene and protein expression for Caspase-7, Tnf-alpha, Fas-l, Bcl-2, Bax, Nmda-GluN2B, Nmda-GluN2D and Bdnf by RT-PCR and Western blotting,

Results: Eye drops SC treatment did not show statistical difference for the genes and proteins studied, when compared to PG. Moreover, eye drops SC treatment reduced the levels of excitotoxic stress (Nmda-GluN2B and GluN2D) and increased the anti-apoptotic gene and protein (Bcl-2) expression and decreased the pro-apoptotic gene and protein (Bax) expression, accompanied by the decrease of Caspase-7, Tnf-alpha, Fas-l and there was higher Bdnf protein expression in the SG than in PG.

Conclusion: Although there was no statistical difference in the genes and proteins studied, eye drops Sildenafild showed a tendency of neuroprotective effect in retinal I/R model, through enhancing anti-excitotoxic, anti-apoptotic and Bdnf pathways.

Key Words: Glaucoma, Sildenafil citrate, Ischemia-reperfusion, Retinal ganglion cell, Apoptosis; neurons, Nmda, Bdnf.

INTRODUCTION

Retinal cell apoptosis may occur following ischemia/reperfusion (I/R) injury as a result of the oxidative stress and expression of apoptosis-related proteins in a process leading to physiological and pathological changes. Retinal ischemia likely contributes to the etiology of several retinal diseases, including diabetic retinopathy, optic neuropathy, glaucoma, and retinal artery occlusion (OSBORNE et al., 2004). Several mechanisms including inflammation, oxidative stress, excitotoxicity, and apoptosis are responsible for the retinal damage after I/R insult, which ultimately leads to retinal neuronal loss through necrosis, apoptosis, and autophagy (D'ONOFRIO; KOEBERLE, 2013; SCHMID et al., 2014; ZANONI et al., 2017). Among the various retinal neurons, retinal ganglion cells (RGCs) are thought to be the most vulnerable to I/R (D'ONOFRIO; KOEBERLE, 2013; LAM et al., 1999a; SCHMID et al., 2014).

Increased evidence suggests that retinal neuronal cells are damaged by apoptosis after retinal I/R injury, and retinal glial activation during the process of an acute intraocular pressure (IOP) elevation might play an important role in retinal neuronal cell death (LAM et al., 1999; JOACHIM et al., 2017; ZANONI et al., 2017). RGCs loss is irreversible, causing visual impairment. Previous studies have shown a positive correlation of the loss of retinal cells, RGC and optic nerve with up regulation of the pro-apoptotic pathway (e.g. Tnf-α, Fas-l, Bax, Caspase-7, Caspase-9, Caspase-3) and down regulation of the anti-apoptotic pathway (e.g. Bcl-2, p-53) (ANTONSSON, 2004; KIM; JU; NEUFELD, 2004; ZHANG et al., 2005; NIE; ET AL, 2009; KIM et al., 2013; EZRA-ELIA et al., 2017; ZANONI et al., 2017).

Glutamate excitotoxicity has also been proposed to be an important contributor to the death of retinal ganglion cells (RGCs) in glaucoma and ischemia (BAI et al., 2013; LAM et al., 1999b) The central hypothesis of excitotoxicity injury by N-metil-D-aspartato receptor (Nmdar), mainly of N-methyl-D-aspartate (Nmda), triggering a massive activation and influx of Calcium, resulted in a pro-apoptotic signaling in neurons and RGC (BAI et al., 2013; PAPOUIN; OLIET, 2014).

Bdnf is a member of the neurotrophin family, which is richly expressed during embryonic development and contributes greatly to the development of the nervous

system by participating in axonal and dendritic growth. In adults, Bdnf is expressed at relatively low levels while it regulates synaptic transmission and plasticity. Bdnf stimulates the growth of neurites from regenerating RGCs, and can protect optic nerve (ON) axons and RGCs from damage in various models of ocular injury. However, endogenous Bdnf was persistently down-regulated in the ischemic retina.(GUPTA et al., 2014; QUIGLEY et al., 2000; UNOKI; LAVAIL, 1994; XU et al., 2016). Therefore, neuroprotection is always the aim to reduce or prevent RGC damage, with pharmacological intervention (OSBORNE et al., 1999; ZHANG et al., 2016; NUCCI et al., 2018).

The Pde5 inhibitor, Sildenafil Citrate (SC), was initially approved for the treatment of erectile dysfunction and nowadays also for pulmonary arterial hypertension (WIROSTKO et al., 2012). It was observed that SC showed a protective effect neuronal survival, and even neurogenesis, in models of brain, spinal cord and cerebral injury (OZDEGIRMENCI et al., 2011; SERARSLAN et al., 2010). Sildenafil has cardioprotective effects and could reduce apoptosis and necrosis in cardiac tissues after I/R injury (DAS et al., 2009) as well as a neuroprotective role in the retinal I/R model (EZRA-ELIA et al., 2017; ZANONI et al., 2017).

In the eye, SC can affect ocular blood flow and choroidal volume due to its effects on smooth vascular muscles. It may also have vasodilatory effects on choroidal sinusoids and retinal vessels similar to vasodilatation of the corpus cavernosum (MARMOR; KESSLER, 1999; EGAN; POMERANZ, 2000).

In the present study, we investigated whether eye drops SC can play a neuroprotective role in retina against I/R and explore its potential mechanism. In particular, we focused our study on apoptosis pathway, Bdnf and Nmda.

METHODS

Animals

A total of thirty-six male rats (*Rattus norvegicus*), over 6 months old, average weighing between 350g to 450g, were used in this study. The animals were clinically and ophthalmically evaluated by routine methods and allocated in appropriate cages,

clean, sanitized, ventilated environment and, with access to food and water "*ad libitum*" and were kept under 12-hour cycles of light and controlled temperature.

Ethical approval statement

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures performed in the present study involving animals were in accordance with the ethical standards of the Ethics Committee on Animal Use (CEUA), Universidade Estadual Paulista (UNESP), Botucatu Medical School (Protocol No. 1199-2016).

Groups and experimental protocols

The animals were divided into seven groups: a control group (CG) consisting of twelve animals without any intervention, and other six groups with induced retinal I/R injury, treated with sildenafil (SG) for 7 days (n = 6), 14 days (n = 6) and 21 days (n = 6)or placebo (PG) for 7 days (n = 6), 14 days (n = 6) and 21 days (n = 6). Topical proparacaine hydrochloride (0.5%), and a combination of intramuscular ketamine (75 mg/kg) and xylazine (10 mg/kg) were used for anesthesia.

A 30-gauge needle connected to saline reservoir was inserted into the anterior chamber through the cornea of right eyes, coupled to a bag of 0.9% saline, positioned at a height of 150 cm above the eye (according to Produit-Zengaffinen et al. 2009 and Schmid et al. 2014). Intraocular pressure was raised to 45 mmHg for 60 min, and IOP was measured using a rebound tonometer (TonoVet Icare, Vantaa, Finland). Afterwards, the animals were treated with dipyrone (200 mg/kg) and morphine (1 mg/kg) by hypodermic injection as analgesics. Sildenafil citrate was (pH 4,7; vehicle - buffer with sodium chloride, monosodium phosphate, disodium phosphate, benzalkonium chloride and water for injectables) (Eye Pharma Company, São Paulo - Brazil) and was instilled as eye drops BID (between 8:00 a.m. - 8:30 a.m. and, between 8:00 p.m. - 8:30 p.m. in the right eyes) on the day of I/R injury induction and during

every day until euthanasia, while PG were some solution (without SC) and with equal criteria.

All animals (SC and PG) were euthanized by intramuscular injection of a combination of ketamine (75 mg/kg) and xylazine (10 mg/kg) and a subsequent intracardiac injection of sodium pentobarbital (120 mg/kg) 1 h after the last eye drop instillation. The CG animals were euthanized using the same method as SC and PG after attesting eye health by clinical and ophthalmological evaluation.

Collection, storage and preparation of samples

After euthanasia, six animals in each group had their right eyeball excised with the optic never. The eyeballs were cut in half and add in another two Eppendorf for quantitative real time PCR (qRT-PCR) and Western Blot (WB) and stored at -80 °C until gene expression and Western blot.

Quantitative real time PCR (qRT-PCR)

We evaluated *Caspase-7*, *Tnf-alpha*, *Fas-l*, *Bcl-2*, *Bax*, *Nmda-GluN2B, Nmda-GluN2D* and *Bdnf*. For that, the samples were processed with the assistance Precellys 24 in a special RNA free tube. Total RNA was extracted using the TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was measured by spectrophotometer (NanoDrop ND-8000 UV-Vis Spectrophotometer, NanoDrop Technologies) using absorbance at 260 nm. To eliminate any contamination with genomic DNA, the total RNA extracted was treated with DNase. Total RNA (1 μg) was added 1 μL of DNase (1u/ μL) (Invitrogen) 1 μL of buffer (with MgCl2 10x) and DEPC water to complete the final volume of 10 μL. The samples were incubated at 25 ° C for 15 minutes and the enzyme inactivated by adding 1 μL of EDTA (25mM) and incubated for 10 minutes at 65 °C.

For the reverse transcription of RNA, each sample was treated with DNase and added 1 μL of oligodT (stock 10 mM), 1 μL of dNTP mix (dGTP, dCTP, dATP, dTTP - the stock 10mM) and 1 μL random primer (30μg / μL). The total RNA was denatured at 65°C for 5 minutes (followed by incubation on ice for 1 minute) and added to the tube 2 μL of DTT 0.1 M, 1.0 μL enzyme SuperScript III Reverse Transcriptase (200u/μL; Invitrogen) and 3 μL of reaction buffer (5x).

The sample were incubated at 25 °C for 5 minutes and subsequently at 50 °C for 60 minutes and the enzyme was inactivated at 70 °C for 15 minutes. The cDNA thus obtained was measured by spectrophotometer (NanoDrop ND-8000 UV-Vis Spectrophotometer, NanoDrop Technologies) using absorbance at 260 nm.

Western Blot

Total protein was obtained from eyeball tissues after extraction with the RIPA lysis buffer. The supernatant was extracted for determination of protein content using Bradford method. The electrophoresis and transfer to the nitrocellulose membrane were standardized previously by our research group (FONSECA-ALVES et al., 2017). The blots were blocked with 3% bovine serum albumin in TBS-T (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 2 hour and incubated overnight with the respective primary antibodies (Tnf-alfa- 1:2500; Fas-L - 1:1000; Bax – 1:1000; Bcl-2 – 1:1000; Caspase 7 – 1:1000; Nmda- GluN2B-1:1000; Nmda- GluN2D – 1:1000 and Bdnf – 1:1000). We used the goat anti-β-actin antibody (1:1000; sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies, the blots were detected by means of chemiluminescence (Amersham ECL Select Western Blotting Detection Reagent, GE Helthcare). The protein expression was quantified by densitometric analysis of the bands and was expressed as integrated optical density (IOD). The protein expression was normalized to the β-actin values. Normalized data are expressed as the means±SD.

Statistics

T-test were used to evaluate RT-PCR and WB results. We used t-test to evaluate statistical difference between two variables (e.g. SG 7 days vs. PG 7 days). The results statistical significance set at $p < 0.05$.

RESULTS

RT-PCR

Concerning gene expression, there was no difference in *Tnf-alpha*, *Fas-L*, *Bax*, *Caspase-7*, Nm*da-GluN2D*, and *Bdnf* expression among SG and PG, in each treatment period. *Bcl-2* showed higher expression in PG at 7 days treatment, and lower in 21 days

treatment. *Nmda-GluN2B* was higher in PG at 14 days of treatment, when compared to SG 14 days (Figure 1).

For Tnf-alpha, PG had a tendency of higher gene expression, in all experiment times, and the treatment of rats with eye drops SC caused decreases Tnf-alpha gene expression. The expression of *Fas-L* was progressively increased in PG when compared to SG, with no statistical difference.

In experimental I/R injury, the expression of *Caspase-7* did not differ significantly, but there was a tendency for this gene expression in PG when compared to SGs (7 and 21 days) (Figure 1).

After I/R injury, the pro-apoptotic gene *Bax* was progressive up-regulated in PG compared to SG. Contrary, the anti-apoptotic *Bcl-2* levels were up-regulated in PG at 7 days ($p<0.01$) and down regulated at 21 days at PG ($p<0.04$). At 21 days, there was an anti apoptotic effect in SG, probably due to the treatment, and fewer histopathological lesions in the treated group (data not shown).

The expression of *Nmda-GluN2B* did differ significantly in 14 days treatment group ($p<0,02$), but there was a tendency for this gene to be more expressed in PG at 21days of treatment, when compared to SG. Eye drops SC treatment had no effect on *Bdnf* gene expression after retinal I/R injury.

PROTEIN EXPRESSION

Figure 2 shows a representative Western blotting band of protein expression of Tnf-alpha, Fas-L, Bax, Bcl-2, Caspase-7, Nmda-GluN2D, Nmda-GluN2D, Bdnf and β-actin. Figure 3 shows the protein levels in the retinal I/R injury in different groups of the study. The protein expression was significantly different only in Nmda-GluN2D at 7 days of treatment, with lower expression in SG 7 days and higher expression in PG 7 days. The level of Nmda-GluN2D and Nmda-GluN2B proteins was lower in the SG compared with the PG.

Tnf-alpha, Fas-L and Caspase-7 were not significantly different in all groups, but there was a tendency of higher expression in PG than SG. In contrast, Bdnf and Bcl-2 expression levels were higher in the SG treated animals compared with PG.

DISCUSSION

In this study, we investigated the protective effect of eye drops SC on the survival of retinal cells following I/R challenge caused by an acute IOP elevation. We

found that eye drops SC had an effect in inhibit the activation of the cell pro-apoptotic factors and Nmda exocytosis in the retinal I/R model in rats, suggesting the mechanism of protection, and effects on Bdnf levels in the SG. Neuronal cell death in the retina during I/R may be an important factor that contributes to damage to visual function. It is a complicated process, and the underlying mechanisms are unclear. In a previous study, ganglion cells died in a way that was morphologically and biochemically consistent with apoptosis (ZHANG et al., 2005; REICHSTEIN et al., 2007; D'ONOFRIO; KOEBERLE, 2013; SCHMID et al., 2014). qRT-PCR and Western blot results showed that expression of Tnf-α, Fas-L, Caspase-7, Bax, Nmda-GluN2D and Nmda-GluN2b was low in SG than PG. However, expression of Bcl-2 and Bdnf was increased in SG than PG.

The lack of statistically significant differences in gene expression could be due to the low number of samples, but there was a tendency for the animals treated with eye drops SC to have lower expression in the pro-apoptotic pathway than those of the PG, and higher in the anti-apoptotic (*Bcl-2*). According to these results, the apoptosis factors could be related to the deregulations of the apoptosis pathway, after ischemic effects.

In our study, apoptotic genes and proteins expression were positive correlation with IOP, once the elevated IOP up-regulated pro-apoptotic factors including Tnf-α, Fas-L, Caspase-7 and Bax in PG. In contrast, it correlated negatively with Bcl-2. Eye drops SC up-regulatied Bcl-2 and Bdnf resulting in low levels of pro-apoptotic factors in SG when compared with PG, suggesting that this may be an important mechanism in neuroprotection acting in apoptotic pathway.

Retinal cells and RGC loss caused by retinal I/R occur primarily by apoptosis, and it may also involve other cell death pathways (KUEHN; FINGERT; KWON, 2005). There are two main apoptotic pathways: the extrinsic death receptor pathway, and the intrinsic, or mitochondrial, pathway. Both are linked, and considerable interplay occurs between them. In general, protein and gene over expression has been detected in many experimental glaucoma models associated with RGC apoptosis, as is a pro-inflammatory cytokine with multiple functions in the immune response. Tnf-alpha and Fas-L are rapidly up-regulated following experimental elevation of intraocular pressure (IOP), and their signaling is involved in the RGC death process during

neurodegeneration in ocular hypertensive eyes, suggesting an important role of this cytokine in modifying the neurodegenerative process (YANG et al., 2003; TEZEL, 2008; YANG et al., 2011; D'ONOFRIO; KOEBERLE, 2013).

In our model, although there was no statistical difference in Tnf-alpha and Fas-L expression, between SG and PG, but there was a tendency of higher gene and protein expression in PG than in SG, what is in accordance to elevation of IOP and the protective role of eye drops SC.

The Bcl-2 family of proteins consists of anti-apoptotic (Bcl-2 and Bcl-xL) and pro-apoptotic members (Bax and Bak) (ANTONSSON, 2004). Bcl-2 and Bcl-xL could inhibit apoptosis by preventing the release of cytochrome C from mitochondria into the cytoplasm. The dynamic balance between the anti-apoptotic and pro-apoptotic members of the Bcl-2 family may determine the susceptibility of a cell to apoptosis (ANTONSSON, 2004; KIM; JU; NEUFELD, 2004; ZHANG et al., 2005; NIE; ET AL, 2009; KIM et al., 2013; EZRA-ELIA et al., 2017; ZANONI et al., 2017). The present study showed that retinal I/R caused a significant suppression of Bcl-2 in the PG, which was not seen in the group treated with eye drops SC. This finding could be explained by the presence of more cellular death in the PG than in the SG (data not shown).

Previously our group found statistical differences in *Fas-L, Bax, Bcl-2* and *Caspase-7* gene expression with up regulation in the PG 7 and PG 21 days, when compared to SG ZANONI et al., 2017. In this study we did not detect gene expression changes, although we saw a neuroprotection tendency in the eye drops SC group, as well as a more preserved RGC layer (higher number of ganglionar cells) and retina at 21 days treatment than oral SC (data not shown).

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. However, its accumulation in extracellular spaces kills neurons through excitotoxic mechanisms via activation of glutamate receptors (CROZIER et al., 2008; ZHOU; BAI et al., 2013; SHENG, 2013). In the retina, excitotoxicity is believed to play an important role in retinal I/R injury and more recently in neuronal loss in glaucoma. However, there are contradicting reports regarding the involvement of apoptosis in excitotoxicity of cerebral neurons (LAM et al., 1999; BAI et al., 2013; PAPOUIN; OLIET, 2014).

Although we observed significantly difference in gene expression (*Nnmda-GluN2B*, 14 days) and protein expression (Nnmda-GluN2D, 7 days), we found low protein expression in SG 7 days and progressive higher expression of Nmda-GluN2B and Nmda-GluN2D in PG than in SG. These results suggest that both Nmda-GluN2B and Nmda-GluN2D play a critical role in retinal degeneration by glutamate excitotoxicity. Therefore, a Nmda-GluN2B-selective antagonist in combination with an Nmda-GluN2D-selective antagonist could be an effective strategy for the management of glaucoma and various forms of retinopathy (LAM et al., 1999b; OSBORNE et al., 1999b; CROZIER et al., 2008; SCHMIDT; BERGERT; FUNK, 2008). Our results suggested that eye drops SC treatment helped in suppressing the over-expressions of oxidative stress markers (NMDA subunits) induced by retinal I/R.

Consistent with this, BAI et al., (2013) showed that GluN2B- and GluN2D-containing NMDARs played a critical role in NMDA-induced excitotoxic retinal cell death and RGC degeneration in GLAST KO mice. Inhibition of GluN2B and GluN2D activity is a potential therapeutic strategy for the treatment of several retinal diseases, including retinal ischemia, diabetic retinopathy, and glaucoma.

Bdnf is a major therapeutic target for ocular degenerative diseases that are characterized by ischemia-induced cellular damage in the retina (CARLOTTA et al., 2016; WU et al., 2015; XU et al., 2016). Delivery of exogenous Bdnf reduced the degree of retinal damage and increased the number of surviving ganglion cells under ischemic conditions (XU et al., 2016). Therefore, endogenous Bdnf levels play a key role in the survival of RGCs when exposed to ischemic insult (KHALIN et al., 2015; XU et al., 2016).

In the present study, eye drops SC treatment did not affect significantly the Bdnf after I/R injury. However, we found that Bdnf levels was elevated when treated with eye drops SC, suggesting that the underlying mechanism of SC induced protective effect against retinal I/R can be involve the Bdnf pathway. This finding was consistent with a previous reports, that foucus on the efficacy of Bdnf in promoting the survival of RGCs following injury to the retina or optic nerve (PUERTA et al., 2010; CUADRADO-TEJEDOR et al., 2011; D'ONOFRIO; KOEBERLE, 2013; XU et al., 2016). In addition, we also found that the Bdnf levels protein expression did not change in PG.

So far, researches had indicated that, among all the neurotrophic factors, Bdnf is the most effective at directing injured RGCs towards survival, a fact attributed to the high levels of expression of tyrosine receptor kinase B (TrkB; the Bdnf receptor) which is expressed by these cells (CROZIER et al., 2008; D'ONOFRIO; KOEBERLE, 2013; GUPTA et al., 2014; XU et al., 2016). Bdnf was found to be beneficial to RGCs in several injury models, including in promoting survival both *in vitro* and *in vivo*. Intravitreal injections of Bdnf have proven to slow the loss of RGCs in a rat chronic hypertension model, and to support the survival of retinal ganglion cells for up to 1 week following axotomy (CROZIER et al., 2008; D'ONOFRIO; KOEBERLE, 2013; GUPTA et al., 2014; PUERTA et al., 2010; XU et al., 2016) .

CONCLUSION

Our results demonstrated that eye drops SC had a neuroprotective role against retinal I/R injury through anti-oxidative and anti-apoptotic pathways. Due to the properties of non-toxic and high membrane permeability, eye drops SC may be a new therapeutic tool. In addition, it is easily administered and relatively stable. However, neuroprotective mechanisms of sildenafil on the retina remain to be elucidated and further studies should be made to confirm the efficacy of SC in the treatment of glaucoma.

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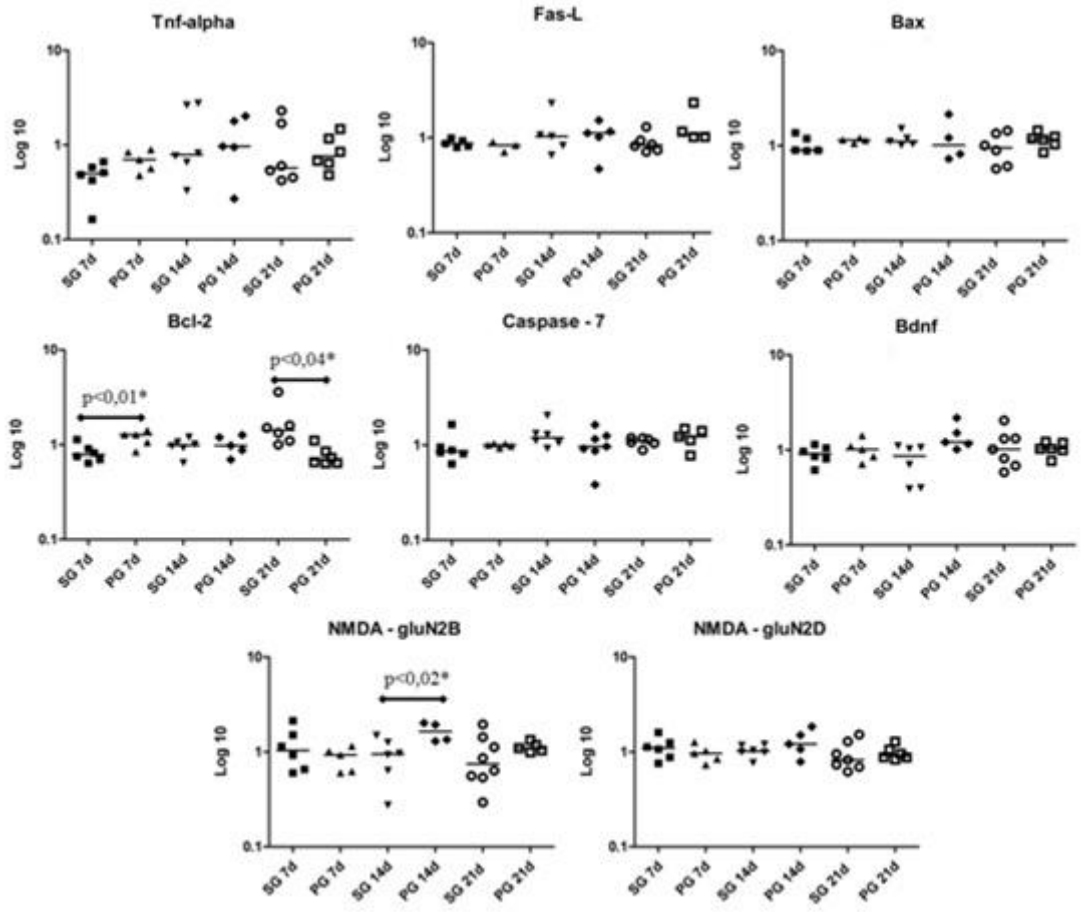

Figure 1. Gene expression graphs. There was no significantly difference in *Tnf-alpha*, *Fas-L*, *Bax*, *Caspase-7*, *Nmda-gluN2D* and *Bdnf* expression among SG and PG, in each treatment period. *Bcl-2* was significantly different, with higher expression in PG at 7 days treatment, and lower in 21 days treatment. *Nmda-gluN2B* was significantly difference in PG at 14 days of treatment, when compared to SG 14 days.

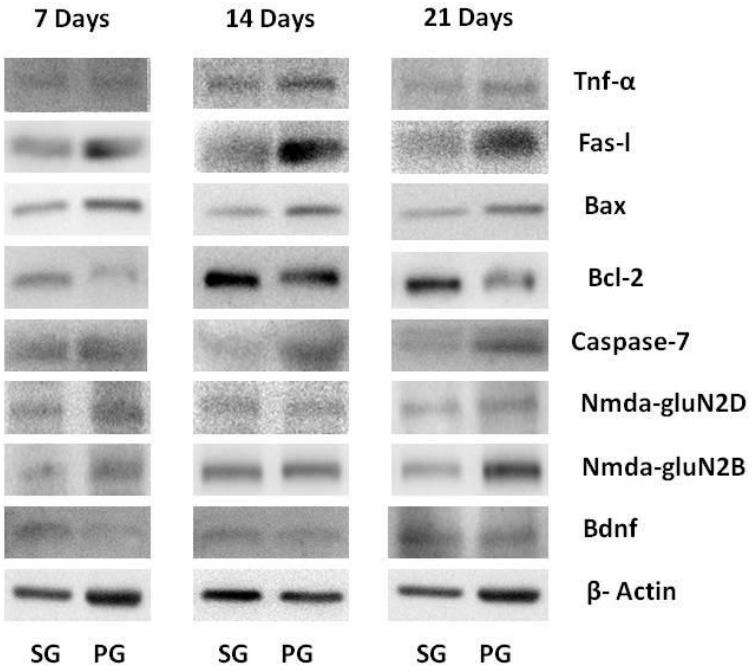

Figure 2. The representative Western blotting bands of protein expression of Tnf-alpha, Fas-L, Bax, Bcl-2, Caspase-7, Nmda-GluN2D, Nmda-GluN2D, Bdnf and β-actin.

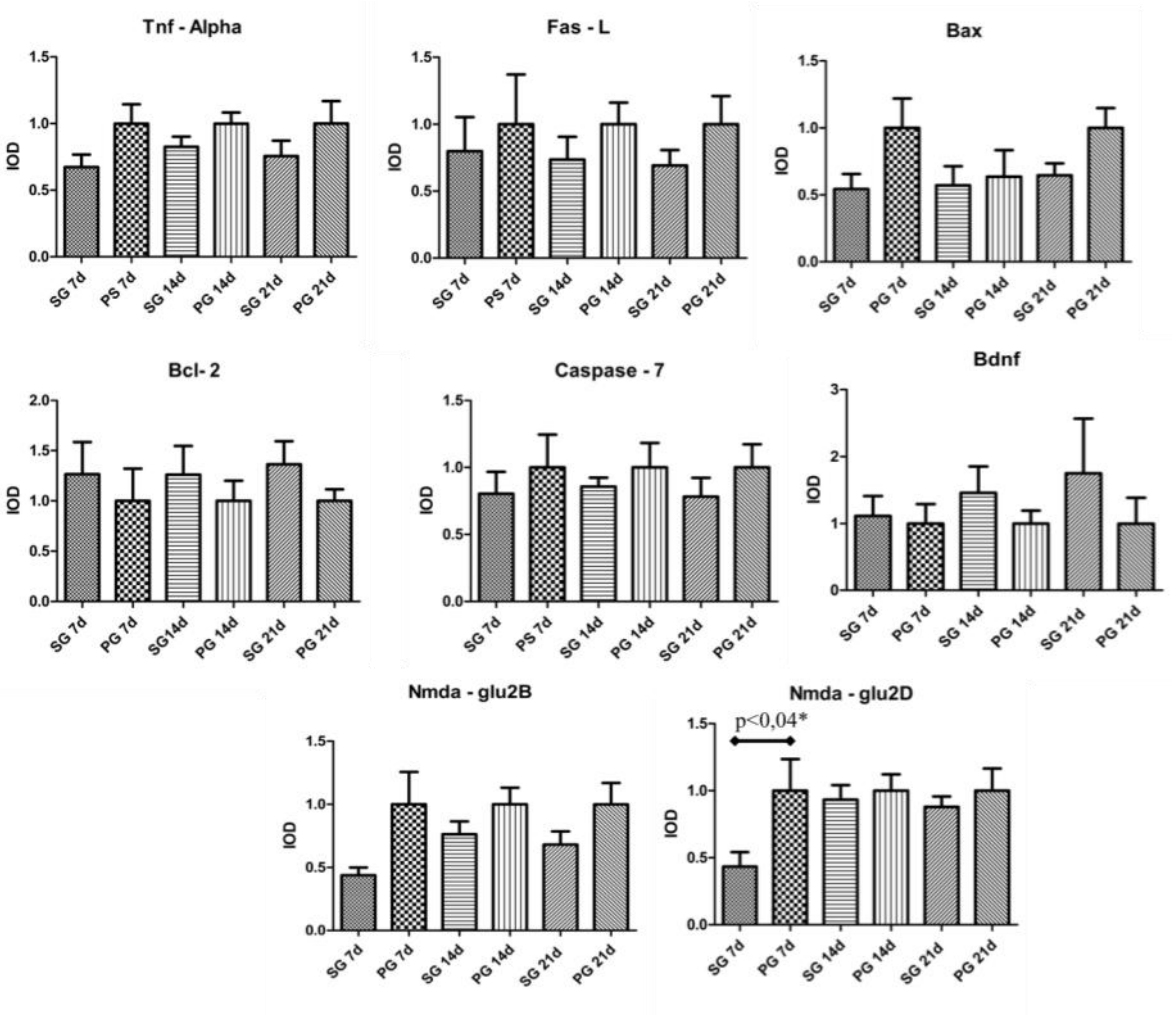

Figure 3. The quantitative analysis of protein with results normalized to β-actin. The protein expression, there was significantly difference only in Nmda-GluN2D 7 days and no significantly difference in Tnf-alpha, Fas-L, Bax, Bcl-2, Caspase-7, Nmda-GluN2D, Nmda-GluN2D and Bdnf. There are a profile of over expression in PG (Tnf-alpha, Fas-L, Bax, Caspase-7, Nmda-GluN2D and Nmda-GluN2B) than SG in each treatment period. Bcl-2 and Bdnf showed a profile of over expresion in the SG than PG. Note the profile of downregulation of SG and, higher protein expression in the PG.

5. DISCUSSÃO GERAL

No presente estudo, nossa hipótese baseou-se na ideia de que a SC não causa danos nas células da retina e possui ação neuroprotetora, sendo eficaz no tratamento de doenças caracterizadas por isquemia, associada ao aumento da perda de células da retina. O presente estudo demonstra, pela primeira vez, que o SC tópico a 2% apresentou resultados histológicos e morfológicos semelhantes à administração oral (EZRA-ELIA et al., 2017; ZANONI et al., 2017). Além disso, os resultados atuais, com a administração tópica de SC, foram melhores do que os SC orais, com maior preservação da camada de RGC e da retina aos 21 dias.

Nossos resultados, relativos à segurança da retina após o uso de CS, também foram descritos por WIROSTKO et al. (2012), que demonstrou que o tratamento oral crônico com sildenafil, não levou a nenhum sinal significativo no exame ocular, na avaliação da função visual, ou através de questionários de perturbação visual.

A espessura da retina e contagem de RGC em PG após I/R tiveram diminuições significativas e progressivas na morfologia e análise morfométrica após a lesão. Esse resultado foi semelhante a muitos estudos, demonstrando que houve perda de células neuronais, principalmente nas camadas retinianas, também na contagem de camadas de CGR, e isso foi bem descrito neste modelo (KALESNYKAS et al., 2012; SCHMID et al., 2014; UEDA et al., 2016; JOACHIM et al., 2017). Por outro lado, o tratamento com SC tópico, comparado ao nosso estudo anterior (ZANONI et al., 2017) teve efeito de neuroproteção significativamente melhor in vivo na morte celular induzida por I / R, redução da lesão celular da retina conforme demonstrado pelos resultados morfológicos e morfométricos em SG, com preservação no número de CGR e espessura em todos os grupos em comparação ao GP e controle.

Além disso, a degeneração retiniana progressiva após lesão de I/R, em nosso estudo, também foi fortemente correlacionada com perda celular significativa nas camadas de RGC e outras camadas no PG. Curiosamente, nosso estudo encontrou uma taxa de perda de células em dados histológicos (~ 40%) aos 21 dias no GP em comparação ao GC, o que se correlaciona com as alterações morfológicas das camadas retinianas lesadas por I/R. No entanto, o número de células na camada de RGC do SG diminuiu 13% após a lesão de I/R. KIM et al. (2013) encontraram taxa de perda de células em dados histológicos (~ 30%) aos 28 dias após a lesão de I / R em ratos. Estes

dados sugerem que a lesão por I / R causou perda celular de RGC e que o SC teve um efeito protetor na perda de células, uma vez que diminuiu 13% no grupo SG aos 21 dias.

Neste estudo, investigamos o efeito protetor da SC tópica na sobrevivência de células da retina após desafio I/R causado por elevação aguda da PIO. Descobrimos que o SC tópico teve um efeito em inibir a ativação dos fatores pró-apoptóticos celulares e a exocitose Nmda no modelo de I/R da retina em ratos, sugerindo o mecanismo de proteção e os efeitos nos níveis de Bdnf no SC. A morte celular neuronal na retina durante a I/R pode ser um fator importante que contribui para o dano à função visual. Em um estudo anterior, as células ganglionares morreram de uma forma que era morfológica e bioquimicamente consistente com a apoptose (ZHANG et al., 2005; REICHSTEIN et al., 2007; D'ONOFRIO; KOEBERLE, 2013; SCHMID et al., 2014).

Os resultados de qRT-PCR e Western blot mostraram que a expressão de Tnf-α, Fas-L, Capsase-7, Bax, Nmda-GluN2D e Nmda-GluN2b foi baixa no SC em relação ao PG. Entretanto, a expressão de Bcl-2 e Bdnf foi aumentada no SC em relação ao GP. A falta de diferenças estatisticamente significativas na expressão gênica poderia ser devido ao baixo número de amostras, mas houve uma tendência para os animais tratados com SC tópico terem menor expressão na via pró-apoptótica do que os do PG, e maior em o anti-apoptótico (Bcl-2). De acordo com esses resultados, os fatores de apoptose poderiam estar relacionados às desregulações da via de apoptose, após os efeitos isquêmicos.

Em nosso estudo, a expressão de genes e proteínas apoptóticas correlacionou-se com a PIO, uma vez que os fatores pró-apoptóticos regulados positivamente pela PIO elevada, incluindo Tnf-α, Fas-L, Caspase-7 e Bax em PG. Em contraste, correlacionou negativamente com Bcl-2. Surpreendentemente, o SC tópico-up regulado por Bcl-2 resultou em baixos níveis de fatores pró-apoptóticos no GS quando comparado ao PG, sugerindo que este pode ser um mecanismo importante na neuroproteção que age na via apoptótica.

Embora tivéssemos diferenças significativas na expressão gênica (Nnmda-GluN2B, 14 dias) e na expressão proteica (Nnmda-GluN2D, 7 dias), encontramos baixa expressão protéica no GS 7 dias e progressivamente maior expressão de Nmda-GluN2B e Nmda-GluN2D PG do que no SG. Estes resultados sugerem que tanto o Nmda-

GluN2B quanto o Nmda-GluN2D desempenham um papel crítico na degeneração da retina pela excitotoxicidade do glutamato. Portanto, um antagonista seletivo de Nmda-GluN2B em combinação com um antagonista seletivo de Nmda-GluN2D poderia ser uma estratégia eficaz para o controle do glaucoma e várias formas de retinopatia (LAM et al., 1999b; OSBORNE et al., 1999b; CROZIER et al., 2008; SCHMIDT; BERGERT; FUNK, 2008). Nossos resultados sugerem que o tratamento tópico com SC ajudou a suprimir as expressões exageradas dos marcadores de estresse oxidativo (subunidades de NMDA) induzidas pela I / R da retina.

No presente estudo, o tratamento tópico com CS não apresentou diferença estatística significativa no Bdnf após a lesão de I / R. No entanto, descobrimos que os níveis de Bdnf estavam elevados quando tratados com SC tópico, sugerindo que o mecanismo subjacente da SC induzida pelo efeito protetor contra a retina I / R pode envolver a via Bdnf. Esse achado foi consistente com relatos prévios, que enfatizam a eficácia do Bdnf em promover a sobrevivência das CGR após lesões na retina ou no nervo óptico (PUERTA et al., 2010; CUADRADO-TEJEDOR et al., 2011; D'ONOFRIO). KOEBERLE, 2013; XU et al., 2016). Além disso, também descobrimos que a expressão protéica dos níveis de Bdnf não se alterou no PG.

6. CONCLUSÃO GERAL

Nossos resultados demonstraram que o SC tópico pode exercer um papel neuroprotetor contra a lesão I/R da retina através de vias anti-oxidativas, anti-apoptóticas e aumento dos níveis de Bdnf. Devido às propriedades de permeabilidade à membrana não tóxica, SC tópico pode ser uma nova estratégica terapêutica. Além disso, a fácil administração e relativamente estável, pode oferecer uma nova estratégia terapêutica para aplicação clínica nas doenças isquêmicas da retina. No entanto, os mecanismos neuroprotetores do sildenafil na retina ainda precisam ser elucidados e novos estudos devem ser feitos para confirmar a eficácia do SC no tratamento do glaucoma.

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