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Efeitos da guanosina sobre a captação de glutamato em retinas de ratos Wistar submetidos a um modelo experimental de isquemia e reperfusão ocular

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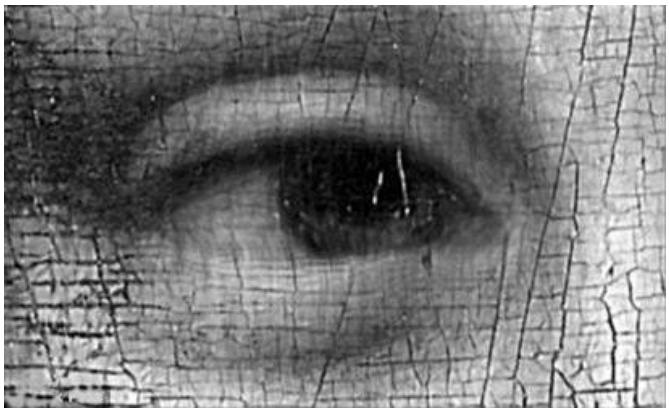
Ao singular renascentista e humanista italiano que tanto me inspira com suas múltiplas facetas de pensador, cientista, anatomista ...

Estudo dos olhos humanos e suas relações com o sistema nervoso central.
Provavelmente em 1490.

... e artista: Leonardo Da Vinci.



Mona Lisa, Olho direito. Entre 1503 e 1507.

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Dedico este trabalho a toda a minha família, em especial aos meus pais, Gabriel e Maria Dalva, ao meu irmão Carlo, à minha esposa Graciela e às minhas filhas, Isabela e Sofia. Também dedico aos pacientes que atendemos em nossa prática diária, como médicos oftalmologistas, e que, talvez um dia, se beneficiem dos resultados destas pesquisas.

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ÉTICA EM PESQUISA

Todos os experimentos realizados por nós e que compõem o conjunto desta obra foram aprovados pelo Comitê de Ética em Pesquisa da UFRGS e estão em concordância com a legislação brasileira. Todos os procedimentos realizados por nós também estão em concordância com as normas internacionais de experimentação em animais exigidas pela *Association for Research in Vision and Ophthalmology (ARVO)*, dos Estados Unidos da América.

ABREVIATURAS

AIC: ângulo iridocorneano

AICs: ângulos iridocorneanos

ATP: adenosina trifosfato

CECs: células endoteliais corneanas

CGR: células ganglionares retinianas

CoQ10: coenzima Q10

CV: campo de visão

DTT: ditiotreitol

EAAT: *excitatory amino acid transporter* (do inglês)

EAO: espécies ativas de oxigênio

GLU: glutamato

GPAA: glaucoma primário de ângulo aberto

GPAF: glaucoma primário de ângulo fechado

GTP: guanosina trifosfato

GUA: guanosina

I-R: isquemia e reperfusão

MEV: microscopia electrônica de varredura

NMDA: N-metil-D-aspartato

ON: óxido nítrico

ONS: óxido nítrico sintetase

PIO: pressão intraocular

SNC: sistema nervoso central

TCO-SP: tomografia de coerência óptica de segmento posterior

VEGF: *vascular endothelial growth fator* (do inglês)

RESUMO

Objetivos: Desenvolver um modelo de isquemia e reperfusão (I-R) ocular baseado no aumento da pressão intraocular (PIO) em ratos Wistar, e utilizar este modelo para investigar o efeito da guanosina (GUA) na captação de glutamato (GLU) nas retinas destes ratos em condições de I-R.

Métodos: Desenvolvemos um modelo de I-R ocular e utilizamos este modelo para investigar 30 ratos Wistar, divididos em 3 grupos de 10 animais. Em cada rato, o olho direito foi submetido à elevação da PIO, gerando isquemia retiniana por 45 minutos, sem nenhuma intervenção no olho esquerdo (controle). No grupo 1, os animais não receberam GUA. No grupo 2, os animais receberam injeção intraperitoneal de GUA 30 minutos antes da isquemia e, no grupo 3, os animais receberam GUA na água durante 1 semana antes e 1 semana após a isquemia. Todos os animais foram mortos 7 dias após a isquemia e suas retinas foram coletadas para quantificar a captação de GLU.

Resultados: As captações de GLU nas retinas controle foram semelhantes em todos os grupos. No grupo 1, a captação de GLU foi reduzida pela I-R. Esta redução foi abolida pela GUA administrada na água (grupo 3) e, no grupo 2, a captação de GLU aumentou com a administração intraperitoneal de GUA ($P<0.001$; ANOVA).

Conclusões: Estes resultados sugerem que a I-R ocular gerada em nosso modelo experimental diminuiu a captação de GLU nas retinas de ratos Wistar e que a GUA aboliu tal redução ou, até mesmo, aumentou a captação de GLU. Este efeito da GUA está de acordo com estudos prévios que revelaram comportamento

neuroprotetor da GUA no sistema nervoso central, por estimular a captação de GLU por astrócitos. Na retina, este efeito pode ser devido à ação da GUA estimulando a captação de GLU pelas células de Müller.

PALAVRAS-CHAVE:

Guanosina, glutamato, isquemia, reperfusão, pressão intraocular, ratos Wistar.

ABSTRACT

Purpose: To devise an experimental model of ocular ischemia-reperfusion (I-R) based on intraocular pressure (IOP) elevation in Wistar rats, and use this model to investigate the effect of guanosine (GUA) on glutamate (GLU) uptake in retinas of Wistar rats submitted to such ocular I-R injuries.

Methods: We devised an experimental model of ocular I-R and applied this model to investigate 30 Wistar rats, divided in 3 groups of 10 rats. Each rat was submitted to IOP elevation in the right eye generating retinal ischemia during 45 minutes with no intervention in the left eye (control retina). In group 1, animals did not receive any GUA. In group 2, animals received an intraperitoneal injection of GUA 30 minutes before ischemia and, in group 3, animals received GUA in water during 1 week before and 1 week after ischemia. All animals were killed 7 days after ischemia and retina samples were obtained. Glutamate uptakes were performed from these retina samples.

Results: GLU uptake in control retina was similar in all groups. In group 1, GLU uptake was significantly reduced by I-R; this reduction was abolished by GUA administration in water (group 3) and GLU uptake increased with intraperitoneal GUA (group 2).($P<0.001$; ANOVA)

Conclusions: These results point that I-R generated by our experimental model decreased GLU uptake in retinas of Wistar rats and that GUA abolished or even overcomed this decrease. These GUA effects are in agreement to previous results, which show that GUA administration presents neuroprotection in central nervous

system by stimulating GLU uptake, mainly by astrocytes. In retina, this effect may be due to GUA stimulation of GLU uptake exerted mainly by Müller cells.

KEY-WORDS

Guanosine, glutamate, ischemia, reperfusion, intraocular pressure, Wistar rats.

INTRODUÇÃO

O glaucoma é uma das principais causas de cegueira no mundo e se caracteriza pela degeneração das células ganglionares retinianas (CGR), alterações na papila do nervo óptico e perda do campo de visão (CV) nos olhos afetados por esta doença[1]. A elevação da pressão intraocular (PIO) permanece como sendo o mais importante fator de risco conhecido para o desenvolvimento e progressão da neuropatia óptica e perda de CV associados ao glaucoma[2-16]. Somando-se a isto, já foi demonstrado que a redução da PIO diminui o risco de progressão do glaucoma[2, 15]. Além do glaucoma, outras causas de elevação da PIO incluem traumas oculares, uso de corticóides, cirurgias oculares e uveítes[17-25].

Também já é de amplo conhecimento da comunidade médica que a elevação da PIO observada no glaucoma agudo de fechamento angular e em modelos experimentais de aumento da PIO pode levar a danos isquêmicos ao nervo óptico e à retina[26-38]. Lesões isquêmicas ao nervo óptico e à retina, também podem ocorrer em situações de oclusões vasculares sem aumento da PIO, como nos casos de oclusão da artéria central da retina e em oclusões venosas da retina[39-48].

Assim, percebemos que a hipóxia aos tecidos oculares, em especial à retina e ao nervo óptico, representa o mecanismo que pode levar à cegueira em diversas doenças oculares, como as oclusões arteriais da retina, as tromboses venosas retinianas, as complicações da retinopatia diabética e vários tipos de glaucoma[49].

Além das lesões decorrentes da isquemia, há evidências de que a reperfusão também pode gerar danos à retina, como já demonstrado em modelos de isquemia e reperfusão (I-R)[50-57].

Neste sentido, considerando a grande incidência e prevalência na população do glaucoma e dos eventos vasculares oclusivos afetando a retina e o nervo óptico[5, 58-63], reveste-se de expressiva relevância o estudo de substâncias capazes de oferecer efeitos neuroprotetores contra estas lesões oculares decorrentes de I-R.

REVISÃO DA LITERATURA

Glaucoma e seus tipos

O termo glaucoma abrange um grupo de doenças que compartilham em comum três características principais, quais sejam: (a) degeneração das células ganglionares retinianas (CGR); (b) alterações na papila do nervo óptico, e; (c) perda do campo de visão (CV)[1]. A pressão intraocular (PIO) encontra-se elevada na maioria dos casos de glaucoma, representando o principal fator de risco conhecido para o desenvolvimento e progressão da neuropatia óptica e perda de CV associados ao glaucoma[2-16], embora também exista uma forma menos comum da doença, conhecida como glaucoma de pressão normal[64-67]. Além da PIO, história familiar positiva (familiar em primeiro grau com glaucoma) e aumento da idade também representam fatores de risco para o glaucoma[5, 58, 59].

Quanto à prevalência do glaucoma, esta varia de acordo com as características do grupo estudado (é maior em idades crescentes) e com o tipo de glaucoma avaliado[5, 58, 59]. Assim, por exemplo, a prevalência do glaucoma primário de ângulo aberto (GPAA) em indivíduos com mais de 40 anos de idade varia de 2,8% a 6,5%[5, 68, 69], enquanto o glaucoma primário de ângulo fechado (GPAF) apresenta prevalência variando de 0,5% a 3,0% na mesma faixa etária[58, 59, 68, 69].

Existem vários critérios para classificar os diferentes tipos de glaucoma. Assim, os glaucomas podem ser classificados quanto:

a) à etiologia da doença;
b) às características anatômicas do segmento anterior do globo ocular, levando-se especialmente em consideração o ângulo iridocorneano;
c) aos níveis de PIO;
d) ao caráter agudo ou crônico da doença;
e) ao início da doença.

A seguir, apresentaremos, brevemente, os diferentes tipos de glaucoma, segundo os critérios acima expostos.

a) Quanto à etiologia da doença:

Segundo este critério, os glaucomas podem ser classificados em primários e secundários. Os glaucomas primários representam a grande maioria dos casos de glaucoma e ocorrem em decorrência de fatores genéticos e ambientais ainda não bem entendidos pela ciência, sendo o aumento da PIO o seu principal fator de risco[2-16]. Nestes casos, o glaucoma não é decorrente de alguma outra condição (cirurgia ocular, por exemplo) ou doença. Já os glaucomas secundários, ocorrem em decorrência de algum evento (trauma ou cirurgia ocular, por exemplo), do uso de algum medicamento (corticosteroides, por exemplo) ou de alguma outra doença (uveítes ou endoftalmites, por exemplo) que provoquem o aumento da PIO[17-21, 24, 25, 70, 71].

b) Quanto ao ângulo iridocorneano:

O ângulo iridocorneano (AIC) é o ângulo compreendido entre a íris e a córnea. Quanto menor for o AIC, maior a probabilidade de haver uma crise aguda de fechamento angular, com o consequente rápido aumento da PIO, uma vez que o espaço compreendido entre a íris e a córnea é justamente o local de drenagem do humor aquoso para fora do globo ocular (especificamente, através da malha trabecular). Segundo este critério (abertura do AIC), os glaucomas podem ser classificados em glaucomas de ângulo aberto ou de ângulo fechado[26, 30, 31, 72-74]. Existem vários níveis de fechamento angular, variando desde AICs mais agudos (menores) até AICs mais abertos (maiores). Os glaucomas de ângulo aberto representam a maioria dos casos de glaucoma.

c) Quanto aos níveis de PIO:

Segundo este critério, os glaucomas podem ser classificados em glaucomas acompanhados de aumento da PIO e glaucomas de pressão normal[64-67, 69]. Os glaucomas com aumento da PIO representam a grande maioria dos casos de glaucoma, enquanto os glaucomas de pressão normal são menos frequentes, apresentando prevalência de 0,73% na população[69]. Por outro lado, o glaucoma de pressão normal é mais frequente em portadores de apnéia obstrutiva do sono, nos quais atinge prevalência de até 5,7%[75].

d) *Quanto ao caráter agudo ou crônico da doença:*

Segundo este critério, os glaucomas podem ser classificados em agudos ou crônicos, dependendo do tempo de evolução da doença. Os glaucomas crônicos representam a grande maioria dos casos de glaucoma.

e) *Quanto ao início da doença:*

Segundo este critério, o glaucoma pode ser congênito, quando presente desde o nascimento, ou adquirido, quando se manifesta anos após o nascimento, geralmente após os 40 anos de idade. O glaucoma congênito muitas vezes está associado com outras alterações congênitas, como a aniridia e, mesmo com adequado tratamento cirúrgico, apresenta prognóstico limitado em grande parte dos casos[76-78].

De tudo o que foi apresentado acima, constata-se que o tipo mais comum de glaucoma é o glaucoma primário crônico de ângulo aberto, mais comumente chamado apenas de glaucoma primário de ângulo aberto (GPAA).

Tratamentos para o glaucoma

Considerando os diversos tipos de glaucoma, existem diferentes abordagens terapêuticas, evidentemente. Contudo, mesmo com tamanha variedade de apresentações clínicas para o glaucoma, todos os tratamentos disponíveis

atualmente têm, em última análise, o mesmo objetivo: reduzir a PIO. A diminuição da PIO, por sua vez, pode ser obtida através de meios farmacológicos[79-81] ou cirúrgicos[82-84].

A abordagem farmacológica para o tratamento do glaucoma evoluiu muito nas últimas duas décadas, especialmente com o desenvolvimento de drogas análogas de prostaglandinas, as quais são capazes de reduzir consideravelmente a PIO[79-81, 85, 86]. Quando apenas um fármaco não reduz suficientemente a PIO, podemos associar diferentes drogas, a fim de obter uma redução adicional da mesma. Em geral, considera-se aceitável associar até 3 drogas antiglaucomatosas de diferentes mecanismos de ação (de diferentes classes farmacológicas). Apesar destes avanços no campo farmacológico, existem efeitos adversos associados às drogas anti-glaucomatosas e, em muitos casos, não se obtém uma redução satisfatória da PIO apenas com o uso de medicações, havendo a necessidade de ser empregada uma abordagem cirúrgica. Já dentre os procedimentos cirúrgicos disponíveis para reduzir a PIO, destacam-se as cirurgias antiglaucomatosas conhecidas como trabeculotomias, trabeculectomias, iridectomias e os implantes valvulares[82-84, 87, 88]. Contudo, tais procedimentos cirúrgicos apresentam considerável imprevisibilidade na redução e controle da PIO, além de riscos de complicações durante e após o ato cirúrgico[82-84, 87, 88].

Assim, verificamos que os tratamentos atualmente disponíveis para os diversos tipos de glaucoma se baseiam na redução da PIO, tendo diversas limitações, efeitos adversos, riscos e complicações. Não há, portanto, um tratamento que possa ser considerado intrinsecamente neuroprotetor, independente da redução da PIO.

Oclusões vasculares retinianas

Os episódios oclusivos no território retiniano podem ocorrer nos leitos arterial ou venoso[39-43]. Em ambos os casos, as oclusões vasculares podem determinar severa diminuição da acuidade visual (visão central), que ocorre geralmente de forma abrupta. Na fase aguda, esta baixa da visão pode decorrer de diferentes mecanismos, como o efeito de barreira exercido por áreas de hemorragia ou como consequência do edema na região macular[39-43, 89, 90]. A recuperação da visão nos dias, semanas e meses subsequentes é variável, mas, infelizmente, em muitos casos não ocorre recuperação significativa da mesma, devido ao dano celular causado pela hipóxia gerada pela oclusão vascular.

A investigação das oclusões vasculares retinianas costuma ser feita, principalmente, com dois exames complementares ao exame oftalmológico, quais sejam: a angiografia fluoresceínica e a tomografia de coerência óptica de segmento posterior (TCO-SP). A angiografia fluoresceínica nos permite localizar e delimitar hemorragias retinianas, áreas de isquemia, edemas retinianos, regiões com neovasos (vasos retinianos anômalos que se formam em resposta à isquemia retiniana) e áreas preservadas (poupadas do evento isquêmico)[42, 91-93]. Já a TCO-SP é um exame extremamente preciso, gerando imagens das diversas camadas da retina com resolução equiparável a cortes histológicos [41, 43, 91, 92, 94-96]. Este exame é de grande utilidade para avaliar os danos isquêmicos decorrentes das oclusões vasculares retinianas, especialmente em relação à quantificação do edema na região macular da retina[41, 43, 91, 92, 94-96].

Em relação às complicações tardias dos eventos isquêmicos que acometem a retina, destacam-se a perda da visão e o glaucoma neovascular. Nas fases tardias,

a perda da visão pode decorrer: (a) do edema crônico na região macular da retina; (b) da morte celular de fotorreceptores, células bipolares e CGR, devido à isquemia, e; (c) do descolamento tracional da retina com substituição do tecido retiniano por tecido fibroso[39-43, 89, 90]. Já o glaucoma neovascular, por sua vez, consiste na elevação da PIO, causada por alteração no AIC e na malha trabecular, decorrentes da formação de neovasos nesta região, comprometendo, assim, a drenagem do humor aquoso[97-100]. Acredita-se que estes neovasos sejam formados como uma resposta do organismo à isquemia retiniana causada pela oclusão vascular e que tal neovascularização seja mediada por fatores de crescimento, em especial pelo fator de crescimento endotelial vascular, mais conhecido pela sigla VEGF, derivada de seu nome em inglês: *vascular endothelial growth factor*[101-103].

Tratamento das oclusões vasculares retinianas

O tratamento das oclusões vasculares retinianas visa recuperar a visão e evitar as suas complicações tardias, como o glaucoma neovascular. Neste sentido, existem, basicamente, duas abordagens terapêuticas: (a) a fotocoagulação retiniana com laser e (b) a terapia antiangiogênica. Estas duas linhas de tratamento podem ser associadas ou empregadas isoladamente[90, 104-106].

Os tratamentos com laser (geralmente do tipo argônio) surgiram antes das terapias antiangiogênicas e consistem em destruir áreas isquêmicas da retina através do fenômeno conhecido como fotocoagulação retiniana, que é, em última

análise, uma cauterização bastante precisa da retina (com o laser), gerando uma cicatriz coriorretiniana. Ao destruir áreas isquêmicas, o laser diminui o estímulo isquêmico gerador de mediadores inflamatórios que promovem a formação de neovasos, mediados pelo VEGF[104-107]. Assim, podemos dizer que a fotocoagulação com laser sacrifica as áreas isquêmicas da retina para preservar as áreas que ainda encontram-se sadias.

Já a terapia antiangiogênica, por sua vez, consiste no uso de drogas que visam combater os efeitos do VEGF na circulação retiniana, através da ação de anticorpos direcionados contra o VEGF. Dentre as drogas que já foram testadas para esta finalidade, as mais empregadas são o bevacizumab e o ranibizumab, as quais precisam ser injetadas dentro do olho (na cavidade vítrea), através de injeções transesclerais realizadas na região correspondente à *pars plana*[92, 101, 108-110]. Sabemos que o VEGF, além de promover a formação de neovasos, também favorece o aumento da permeabilidade vascular no território retiniano, o que contribui para a formação e manutenção de edemas retinianos, especialmente na região macular. Assim, ao se oporem aos efeitos do VEGF, o bevacizumab e o ranibizumab combatem não apenas a neovascularização retiniana, mas também o edema macular, que é o grande responsável pela baixa da acuidade visual na maioria dos casos de oclusões vasculares retinianas[92, 101, 108-110].

Contudo, mesmo quando ocorre recuperação da visão com o uso das drogas antiangiogênicas, o ganho de visão costuma ser transitório, devido ao efeito limitado das mesmas. Desta forma, na maioria das vezes, é necessário repetir a dose da medicação, por meio de novas injeções intravítreas do agente antiangiogênico.

Assim, podemos dizer que os tratamentos atualmente disponíveis para os diversos tipos de oclusões vasculares que acometem a retina se baseiam na

destruição de áreas retinianas isquêmicas com laser e/ou no combate aos efeitos do VEGF com as drogas antiangiogênicas. Portanto, assim como nos diversos tipos de glaucoma, também não existe um tratamento para as oclusões vasculares retinianas que possa ser considerado intrinsecamente neuroprotetor para as células retinianas.

Neuroproteção

Discutir amplamente o conceito de neuroproteção é bastante complexo e extrapola o escopo da presente Tese de Doutorado. Assim, empregaremos o termo neuroproteção e seus derivados (agentes neuroprotetores, por exemplo), no sentido de proteção dos tecidos neuronais contra agressões diversas, como os insultos isquêmicos, por exemplo. Em relação aos tecidos neuronais, por sua vez, estamos nos referindo às células nervosas (neurônios) do sistema nervoso central e periférico (neurônios do nervo óptico, por exemplo), bem como às células nervosas modificadas encontradas na retina (fotorreceptores, células bipolares e células ganglionares da retina). Neste sentido do termo neuroproteção, não existem tratamentos (disponíveis para uso clínico) para o glaucoma e para as oclusões vasculares da retina que possam ser considerados diretamente neuroprotetores para as células (neurônios) do nervo óptico e para as células da retina (fotorreceptores, células bipolares e células ganglionares da retina), o que torna relevante a pesquisa por drogas que possam exercer tal efeito neuroprotetor.

Neuroproteção e o metabolismo do glutamato no sistema nervoso central

A neuroproteção vem emergindo como uma área de muito interesse para pesquisas médicas e vários estudos nesta área têm sido dedicados ao entendimento do metabolismo do glutamato (GLU), uma vez que o GLU é o principal neurotransmissor excitatório do sistema nervoso central (SNC) e está envolvido em diversas funções cerebrais, como os processos de aprendizagem e memória, por exemplo[111-114]. Embora o GLU seja essencial para o funcionamento normal do SNC, o aumento do GLU na fenda sináptica pode levar a uma neurotoxicidade, devido à excessiva estimulação dos receptores de GLU, o que é conhecido como excitotoxicidade do GLU[115]. Os eventos excitotóxicos, por sua vez, estão envolvidos em várias doenças agudas (hipóxia, isquemia e convulsões) e crônicas (epilepsia, doenças de Parkinson e Alzheimer) que afetam o SNC[112, 114, 116, 117].

No SNC, o principal processo endógeno responsável pela manutenção da concentração de GLU, na fenda sináptica, abaixo dos níveis tóxicos consiste na captação de GLU exercida por transportadores localizados, principalmente, nas membranas plasmáticas dos astrócitos[112, 116, 117, 118]. Portanto, ao manter a concentração de GLU na fenda sináptica em níveis normais, os astrócitos contribuem para a neuroproteção no SNC.

Neuroproteção e o metabolismo do glutamato na retina

Uma vez que o GLU também é o principal neurotransmissor excitatório encontrado na retina[119], o estudo do GLU representa uma oportunidade para investigar as lesões retinianas decorrentes de I-R. De fato, múltiplas evidências se somam, sustentando a idéia de que o GLU está envolvido nos danos isquêmicos retinianos[119-126].

A I-R provocada na retina devido à elevação experimental da PIO leva a danos nas CGR e à morte das mesmas por apoptose ou necrose[121, 123]. Nestas condições, ocorrem alterações estruturais, funcionais e bioquímicas que resultam no acúmulo extracelular de GLU, o qual desencadeia a cascata excitotóxica, devido à ativação de receptores glutamatérgicos do tipo N-metil-D-aspartato (NMDA) e também de receptores não-NMDA[121-123, 125]. A excessiva ativação destes receptores pode aumentar a atividade da enzima óxido nítrico sintetase (ONS)[49, 123], elevando a produção de óxido nítrico (ON), o qual pode ser tóxico para as células, resultando na morte celular[49]. A ativação dos receptores de GLU do tipo NMDA também leva ao excessivo fluxo de Ca^{2+} para o interior dos neurônios, o que é uma etapa crucial para a excitotoxicidade do GLU, contribuindo para a morte celular[122, 123, 125, 127]. Portanto, acredita-se que a excessiva estimulação dos receptores glutamatérgicos possa danificar o tecido retiniano por uma cascata de eventos bioquímicos, como a ativação da ONS e o aumento do Ca^{2+} intracelular, o que já foi descrito como sendo o evento bioquímico de maior contribuição para a perda de CGR[49].

O acúmulo extracelular de GLU verificado nas lesões retinianas causadas por I-R pode estar relacionado com o aumento da liberação de GLU e/ou com a

diminuição da sua captação[128] . Na retina, esta captação de GLU parece ser exercida, principalmente, pelas células de Müller, as quais expressam um tipo de transportador de GLU chamado GLAST[119]. De fato, há evidência de que a capacidade dos transportadores GLAST de sustentar o fluxo de GLU para o espaço intracelular é particularmente suscetível a um evento isquêmico agudo[119].

De um modo geral, a captação de GLU é mediada por uma família de genes de transportadores sódio-dependentes de alta afinidade que incluem 5 subtipos em mamíferos; em humanos, estes transportadores de GLU são mais conhecidos na literatura científica pela sigla EAAT, advinda da língua inglesa (*Excitatory Amino Acid Transporter*), e são numerados de 1 a 5[129–132]. Na retina, o EAAT1 (também chamado GLAST) é encontrado nas células de Müller e em astrocitos[133]; o EAAT2 (também chamado GLT-1) está localizado em cones e em células bipolares[134]; o EAAT3 (também chamado EAAC1) é encontrado em células ganglionares, células amácrinas e células horizontais, e raramente em células bipolares[135]; o EAAT5 está localizado em fotorreceptores e células bipolares[136]; e o EAAT4 ainda não foi identificado na retina[132].

Em relação às células ganglionares retinianas, há dados sugerindo que os transportadores EAAT1 (GLAST) e EAAT2 (GLT-1) desempenham importante papel no sentido de limitar a excitotoxicidade por GLU e que a interferência em suas funções aumenta o GLU extracelular, impactando adversamente a sobrevivência das CGR[132]. Considerando a retina como um todo, a captação de GLU é dominada pela atividade dos transportadores GLAST[137] e sabemos que eventos isquêmicos agudos podem comprometer a função destes transportadores, como já mencionado previamente[119]. Assim, situações de I-R que afetem a retina podem comprometer a captação de GLU pelas células de Müller, por interferir nestes transportadores. A

perda (ou diminuição) da função dos transportadores GLAST parece estar relacionada com alterações metabólicas agudas induzidas pelo evento isquêmico, como a perda do gradiente de sódio, a acidose ou o estado de fosforilação do transportador, e não com a diminuição (*down regulation*, em inglês) da expressão do transportador[138-140]. Além disso, já foi descrita a redução do GLAST e do GLT-1 na retina de ratos em um modelo experimental de glaucoma[141]. Em humanos com glaucoma, também foi demonstrada a diminuição do GLAST, o que é consistente com a elevação do GLU encontrado no humor vítreo destes indivíduos com glaucoma[142]. Portanto, substâncias capazes de estimular a captação de GLU, evitando o aumento excessivo do GLU no espaço extracelular, poderiam oferecer um efeito neuroprotetor contra a excitotoxicidade do GLU na retina.

Outra potencial via bioquímica para oferecer neuroproteção retiniana contra a excessiva estimulação por GLU, consiste no uso de antagonistas de receptores glutamatérgicos. Neste contexto, ação neuroprotetora contra a excitotoxicidade por GLU já foi demonstrada em modelos experimentais de isquemia retiniana, com o uso do MK-801, um antagonista de receptor NMDA[122, 123, 125]. Esta neuroproteção parece ser exercida pelas células de Müller, que são ativadas durante o episódio isquêmico e desempenham um papel protetor contra a morte celular retiniana mediada por receptores NMDA[125].

O papel do estresse oxidativo no metabolismo do glutamato

O metabolismo do GLU está envolvido com o estresse oxidativo e acredita-se que espécies ativas de oxigênio (EAO) produzidas durante eventos de I-R na retina estejam implicadas na excitotoxicidade do GLU[128, 143]. Neste sentido, EAO parecem estar relacionadas com o acúmulo extracelular de GLU[143, 144] e com os danos celulares induzidos pela ativação de receptores glutamatérgicos[145]. Também já foi descrito que sistemas geradores de radicais livres podem alterar transportadores de GLU sódio-dependentes em astrócitos[146-148]. Adicionando-se a isso, quando células retinianas foram incubadas na presença de H_2O_2, foi observada uma redução na captação de GLU, assim como com outro agente oxidativo (5,5-ditio-bis-2-nitro-benzoato), sugerindo que a redução na captação de GLU seja devida a alguma ação oxidativa[128]. Neste mesmo estudo, foi demonstrado que os agentes redutores ditiotreitol (DTT) e β-mercaptoetanol foram capazes de reverter parcialmente os efeitos do H_2O_2[128].

Outra evidência de que o estresse oxidativo participa na excitotoxicidade do GLU foi revelada ao estudar a coenzima Q10 (CoQ10), que é um cofator essencial para a cadeia transportadora de elétrons[126]. Este estudo demonstrou que a administração intraocular de CoQ10 diminuiu o aumento de GLU em um modelo de I-R em retinas, sugerindo que o estresse oxidativo e a falência energética devam estar implicados nos mecanismos celulares que resultam na morte das CGR[126]. Além destas evidências, também foi demonstrado que a sobrecarga de Ca^{2+} induzida por GLU causa falência mitocondrial, excessiva formação de EAO e

ativação de complexas cascatas de proteases, nucleases e lipases, que podem resultar em morte neuronal por apoptose[137, 149].

O papel da guanosina na neuroproteção

A guanosina (GUA) é um nucleosídeo que apresenta efeitos neuroprotetores contra a excessiva estimulação dos receptores de GLU no SNC, como já demonstrado em vários estudos[150-157]. No SNC, a neuroproteção da GUA parece estar relacionada com a sua capacidade de estimular a captação de GLU[158, 159]. O aumento da captação de GLU pelos astrócitos no SNC mediado pela GUA parece ser independente da ativação do receptor de adenosina[158] e o local de ligação da GUA está situado na membrana plasmática dos astrócitos[159]. Outras purinas derivadas da guanina, como o GMP e o GTP, também têm demonstrado efeito estimulatório sobre a captação de GLU, porém as evidências sugerem fortemente que a GUA é o mediador comum dos efeitos destas purinas[159].

Este efeito da GUA na captação de GLU parece estar relacionado com a concentração de GLU. De fato, a captação de GLU foi significativamente estimulada em altas concentrações (1mM), mas não em baixas concentrações (10-100μM) extracelulares de GLU em fatias de cérebro de rato expostas à GUA[160]. A GUA não tem efeito na captação de GABA, sugerindo um efeito específico da GUA na captação de GLU pelos astrocitos[159]. Considerando que a captação de GABA também é sódio-dependente, isto poderia indicar que o efeito estimulante da GUA

provavelmente não envolve mecanismos relacionados ao aumento do gradiente de sódio[159]. Esta especificidade em relação ao sistema glutamatérgico é consistente com estudos comportamentais, onde a GUA teve propriedades anticonvulsivantes contra a excessiva estimulação do sistema glutamatérgico pelo ácido quinolínico e pela alfa-dendrotoxina, mas não contra o antagonista GABA-A picrotoxina[151, 153].

O efeito da GUA na captação de GLU tem, provavelmente, relevância sob o aspecto fisiopatológico, uma vez que há situações (*in vivo* e *in vitro*) nas quais a elevação do GLU extracelular é acompanhada de aumento da GUA[161-163]. Este aumento da GUA pode estar acoplado ao estado energético da célula. Níveis basais de GUA intracelular e de adenosina em células neurais estão na faixa nanomolar, sendo bem maiores para o ATP (3 mM) e o GTP (300 mM)[161]. Assim como ocorre com o ATP, os níveis de GTP são mantidos relativamente estáveis durante o metabolismo energético normal. Em condições de hipóxia, ocorrem relativamente grandes aumentos (da ordem de 10 vezes) na concentração dos nucleosídeos, para mínimos decréscimos (cerca de 0,1 a 1%) nas concentrações de ATP e GTP[159]. Considerando que os transportadores de nucleosídeos mediam as trocas entre os compartimentos intra e extracelulares, a liberação de quantias biologicamente significativas de GUA e de adenosina podem ocorrer em condições patológicas[159]. Estes nucleosídeos podem diminuir os níveis de GLU por inibir a liberação de GLU, no caso da adenosina[164, 165], e por aumentar a remoção do GLU (captação de GLU) pelos astrócitos, no caso da GUA[158]. Este efeito da GUA é, provavelmente, de particular relevância em condições de excitotoxicidade, uma vez que a GUA não afeta a captação de GLU pelos astrócitos em baixas concentrações de GLU[160].

Assim como no SNC, a GUA também pode ter um efeito neuroprotetor contra a excessiva estimulação dos receptores de GLU na retina. Estes efeitos

neuroprotetores na retina podem decorrer da ação da GUA sobre a captação do GLU pelas células de Müller. Contudo, apesar da conhecida neuroproteção da GUA no SNC, nós não encontramos nenhum estudo avaliando o papel da GUA como neuroprotetor contra as lesões retinianas decorrentes de I-R.

Modelos de isquemia e reperfusão ocular em ratos

A fim de estudar os danos decorrentes da isquemia e reperfusão ocular, vários modelos experimentais foram desenvolvidos nos últimos anos[50-57]. A isquemia ocular pode ser obtida através de diferentes métodos, como a oclusão cirúrgica de vasos arteriais (artérias carótidas ou a artéria central da retina), por exemplo. Embora representem bons modelos de isquemia, estas oclusões vasculares produzidas cirurgicamente apresentam a desvantagem de serem tecnicamente mais difíceis de serem realizadas, pois envolvem dissecções muitas vezes complexas. Além disso, uma vez ocluído o vaso arterial, sua reperfusão pode ser mais difícil de ser obtida, limitando o estudo das lesões decorrentes da reperfusão ocular.

Neste sentido, modelos experimentais baseados na elevação da pressão intraocular constituem uma abordagem interessante para gerar isquemia ocular, uma vez que podem ser facilmente revertidos, permitindo, assim, também o estudo das lesões de reperfusão. Além disso, a isquemia induzida pelo aumento da PIO representa um bom modelo para estudar o glaucoma agudo de fechamento angular[123]. Tendo isto em mente, vários autores sugeriram diversos modelos de I-R ocular baseados na elevação da PIO[36, 37, 53, 54, 120, 122, 123, 166].

Revisando estes modelos, nós observamos que o tempo de isquemia usado por diferentes autores variou de 30 a 180 minutos, sendo que a maioria destes utilizou um período de 30 a 60 minutos[36, 37, 53, 120, 166]. Em relação ao tempo necessário para caracterizar dano às estruturas retinianas, há evidências de lesões às células ganglionares retinianas após 45 minutos de isquemia[37, 123]. Portanto, 45 minutos de isquemia parece ser um período de tempo suficiente para ser empregado em modelos experimentais, com o intuito de investigar lesões oculares retinianas de origem isquêmica, sem incorrer em excessivos tempos de aumento da PIO, o que poderia gerar danos extras decorrentes da excessiva PIO por si só (danos barométricos). Por esta razão, escolhemos o tempo de 45 minutos de isquemia para ser usado em nosso modelo experimental de I-R.

Em relação ao tempo de reperfusão, este variou de 12 horas a 14 dias nos diferentes modelos experimentais avaliados em nossa revisão da literatura[36, 37, 53, 54, 120, 122, 123]. Em nosso modelo experimental, testamos diversos tempos de reperfusão, como será visto mais adiante no artigo 2.

Já no que diz respeito ao nível máximo da PIO necessária para obter a isquemia total da retina, esta variou de 110 a 130 mmHg nos diversos modelos por nós analisados[36, 37, 166]. Alguma variabilidade na PIO necessária para obter a isquemia total da retina em ratos já é esperada e pode ser explicada devido ao fato de que existem variações genéticas e anatômicas nos animais criados para serem utilizados nos centros de pesquisa. Em nosso modelo experimental, utilizamos uma PIO maxima de 150 mmHg, a fim de termos a certeza de estarmos trabalhando com a oclusão total da retina.

Desenvolvimento de um modelo de isquemia e reperfusão ocular baseado no aumento da pressão intraocular em ratos Wistar

Ratos Wistar representam uma raça de ratos albinos, pertencentes à espécie *Rattus norvegicus*. Uma vez que esta raça de ratos é amplamente utilizada em pesquisas médicas em diversos países, optamos por utilizar estes animais em nosso modelo experimental, a fim de facilitar a reprodução dos nossos estudos por outros pesquisadores em diferentes centros de pesquisa. Além disso, em ratos Wistar, a isquemia total da retina pode ser facilmente observada, uma vez que, neste momento, o olho fica completamente pálido (branco), devido à interrupção do fluxo sanguíneo, mudando o aspecto normal do olho deste tipo de rato, que se caracteriza por ser vermelho, já que são ratos albinos. Esta facilidade em observar a isquemia total da retina em ratos Wistar representa uma vantagem metodológica no uso destes ratos em relação a outros animais, por conferir maior precisão na determinação da PIO necessária para a isquemia retiniana.

Para desenvolver e testar um método de I-R ocular baseado na elevação da PIO em ratos Wistar, decidimos avaliar inicialmente o comportamento das células endoteliais corneanas (CECs), uma vez que tais células são bastante sensíveis à elevação da PIO e à isquemia ocular. As CECs desempenham um papel crucial para o funcionamento normal da córnea, já que a espessura e a transparência corneanas são mantidas graças à ação de bombeamento ativo do humor aquoso (para fora da córnea, evitando o edema corneano), realizado por estas células[167]. A análise (em vertebrados) das CECs por microscopia electrônica de varredura (MEV) revela tartar-se de uma camada contínua de células poligonais[168-171] , tendo formato

predominantemente hexagonal (em 59 a 80% das CECs) em mamíferos[170, 172]. A densidade das CECs (por mm^2) costuma diminuir com a progressão da idade do animal, uma vez que não se observa divisão celular de tais células (após o nascimento) em várias espécies de mamíferos[168, 169, 173]. Neste sentido, em nosso modelo experimental, utilizamos animais (ratos Wistar) de mesma idade e considerados como sendo adultos jovens.

A estrutura normal das CECs já foi descrita em humanos e em outras espécies animais[168-170, 172], porém, em nossa revisão da literatura, não encontramos nenhuma referência ao comportamento ou à descrição das CECs de ratos Wistar com MEV em condições de I-R ocular. Para avaliar as CECs em nosso modelo experimental, optamos por utilizar a MEV para mensurar o tamanho e os aspectos morfológicos destas células endoteliais. A microscopia eletrônica de varredura é considerada bastante precisa para avaliar as estruturas celulares, porém devemos lembrar que os valores de área celular e de densidade celular obtidos por MEV não representam os valores reais observados in vivo, uma vez que os métodos de fixação usados na MEV induzem ao encolhimento (diminuição) do tecidos corneanos[174]. Neste contexto, estima-se uma diminuição de 60% dos tecidos corneanos com o método de dissecar e depois fixar, e de 50% com o método de fixar primeiro todo o globo ocular para depois dissecar[174]. Em nosso modelo, utilizamos o método de dissecar e depois fixar, por facilitar a dissecção ocular, já que a fixação torna os tecidos mais rígidos e quebradiços.

Considerações finais acerca da revisão da literatura

Com base na revisão da literatura acima apresentada, percebe-se que:

a) As lesões teciduais impostas ao nervo óptico e à retina, associadas com as diversas formas de glaucoma e oclusões vasculares retinianas, envolvem, em maior ou menor proporção, eventos de I-R;

b) Os tratamentos disponíveis atualmente para estas doenças (glaucoma e oclusões vasculares retinianas) visam combater fatores relacionados às mesmas, como a redução da PIO e o antagonismo dos efeitos do VEGF, porém não podem ser considerados como sendo diretamente neuroprotetores para as células da retina e do nervo óptico;

c) Existe, portanto, a necessidade de realizarmos estudos a fim de encontrarmos ou desenvolvermos drogas com efeitos neuroprotetores para as células da retina e do nervo óptico em condições de I-R;

d) Já foi demonstrado que a GUA interfere na captação da GLU no SNC, comportamento este que pode ser considerado neuroprotetor para os neurônios no SNC;

e) Apesar disto, ainda não há estudos evidenciando o efeito neuroprotetor da GUA para o nervo óptico e para a retina em situações de I-R ocular;

f) Neste contexto, seria profícuo testar o papel da GUA na captação de GLU nos tecidos retinianos em condições de I-R;

g) Para testar esta hipóstese, podemos utilizar modelos experimentais, gerando I-R ocular através da elevação reversível da PIO em ratos Wistar;

h) Ratos Wistar são animais amplamente estudados em laboratório em diversos países, permitindo a reprodução de nossos testes em outros centros de pesquisa;

i) O endotélio corneano dos ratos Wistar pode ser estudado inicialmente para desenvolver e testar o nosso modelo experimental de I-R ocular baseado na elevação reversível da PIO.

Assim, o propósito do presente estudo é o de desenvolver um modelo experimental de I-R retiniana em ratos Wistar baseado no aumento da PIO e utilizar este modelo para investigar o papel da GUA na captação de GLU em condições de I-R retiniana.

OBJETIVOS

Objetivo principal:

Investigar o papel da guanosina na captação de glutamato em condições de isquemia e reperfusão retiniana em olhos de ratos Wistar.

Objetivo secundário:

Desenvolver um modelo experimental de isquemia e reperfusão ocular em olhos de ratos Wistar, baseado no aumento reversível da pressão intraocular.

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Artigo 1 em inglês:

TITLE

Analysis of the corneal endothelium in eyes of Wistar rats using scanning electron microscopy

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ARTICLE TYPE

Original Article - Laboratory Science

FINANCIAL DISCLOSURE AND CONFLICT OF INTEREST

None of the authors have financial interests with materials or devices used in this article. The authors have no conflict of interest to disclose.

FINANCIAL SUPPORT

This work was supported by financial grants from Brazilian agencies: CNPq, CAPES, INCT/CNPq, IBN.Net/CNPq/FINEP, FAPERGS, UFRGS.

ABSTRACT

Purpose: To investigate the ultrastructure, morphology and morphometry of the corneal endothelium of normal Wistar rats using scanning electron microscopy.(SEM) Our study also aims to compare central versus peripheral corneal regions and right versus left eyes with respect to endothelial polygonality, cell area and cell density.

Methods: Twenty eyes from 10 healthy Wistar rats were studied. The posterior endothelial surfaces of corneas were examined with SEM in central and peripheral corneal regions. The photomicrographs were analyzed with help of an image analyzer software, determining polygonality, cell area and cell density.

Results: Posterior endothelium surface of Wistar rats revealed a continuous layer of polygonal cells, with interdigitations, microvilosities and cilia. The majority of endothelial cells were six-sided (66.5%), with five-sided (27.4%), seven-sided (2.8%), four-sided (2.6%) and eight-sided (0.7%) cells constituting the remaining corneal endothelium. The mean cell area was 134.5 μm^2 and mean endothelial cell density was 7,499.8 cells/mm^2. The coefficient of variation of mean cell area was 0.214. There were no statistical differences between right and left eyes, nor between central and peripheral corneal regions with respect to polygonality, mean cell area and mean cell density.

Discussion: Our results suggest that corneal endothelium of Wistar rats is a continuous layer of polygonal cells, which is in accordance with other vertebrates. Endothelial cells ultrastructure also seems to be similar to other mammals. With respect to endothelial cell area and cell density, we must remember that values obtained from SEM can be different according to the fixation method used.

KEY-WORDS: Cornea, endothelium, wistar rat, *Rattus norvegicus*, scanning electron microscopy.

INTRODUCTION

The corneal endothelium is a single layer of polygonal cells covering the corneal posterior surface.[1] Studying the corneal endothelium of animals is of veterinary interest. Moreover, the knowledge about the corneal endothelium of animals has great interest for research purposes, since it can be applied to study corneal diseases, intra-ocular drugs and intra-ocular surgical procedures. The structure of normal corneal endothelium has been described in humans[2], dogs[3, 4, 5], horses[6] and other animal species[7, 8, 9, 10, 11] with electron microscopy. Electron microscopy also has been used to study corneal structures in rats, such as Descemet's membrane[12], and to investigate corneal changes in unhealthy rats.[13, 14] Despite this, the normal corneal endothelium of Wistar rats has not been described using scanning electron microscopy (SEM) with respect to surface morphology, cell area, cell density and coefficient of variation of mean cell area.

Wistar rats are a strain of albino rats belonging to the species *Rattus norvegicus*. Since Wistar rats are largely used in laboratory and animal researches, it is of great interest to know the normal corneal endothelium of these animals. In this regard, our study aims to investigate the ultrastructure, morphology and morphometry of the corneal endothelium of normal Wistar rats using SEM. Our study also aims to compare central versus peripheral corneal regions and right versus left eyes with respect to endothelial polygonality, cell area and cell density.

METHODS

Animals and Ethics

All experiments were in agreement with Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil, and also in accordance to Association for Research in Vision and Ophthalmology, USA, regarding the use of animals in ophthalmic and vision research. Twenty eyes from 10 healthy Wistar rats were studied. The Wistar rats were males, 120 days of age, 250-350 g, kept in 12-hours light/dark cycles. All animals were killed by standard decapitation protocol.

Samples preparation

Samples preparation for SEM was done following the method reported by Virtanen et al.[15] Immediately after death, eyes were enucleated and the corneas (corneal buttons) were excised, transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 and stored for 24 hours at 4ºC for fixation. Corneal buttons were obtained by 360º scleral section 1 mm from corneal limbus. Subsequently, the corneas were washed again in cacodylate buffer and dehydrated through an increasing series of ethanol solutions. Thereafter, the specimens were submitted to critical point drying using liquid carbon dioxide. Corneas were placed on 10 mm aluminum stubs with double-sided adhesive tape and sputter coated with gold-palladium.

Scanning electron microscopy and image analysis

The posterior endothelial surfaces of each cornea were examined and photographed using a scanning electron microscope (JEOLJSM 5410, Tokyo, Japan) operated at 10 kV. Forty photomicrographs were obtained from each cornea with magnifications of 750x, 1,800x, 2,000x, 2,500x and 4,500x. Photomicrographs were obtained from two regions of each cornea: 20 photomicrographs from central region (center of the cornea) and 20 photomicrographs from peripheral region.(1 mm from corneal limbus) The photomicrographs were introduced into a computer in which they were analyzed with help of an image analyzer software.(Image Tool, *UTHSCSA, San Antonio, USA*) With this software, 200 endothelial cells of each cornea were analyzed (100 from central region and 100 from peripheral region), determining polygonality (number of sides of each endothelial cell), cell area and cell density. The coefficient of variation of mean cell area was calculated by dividing the standard deviation (SD) of the cell area by mean cell area.

Statistical analysis

Statistical analysis was conducted with SPSS 12.0, using paired Student T test. Values of $P<0.05$ (two-tailed) were considered significant.

RESULTS

Morphology

In regard to morphology, SEM revealed that posterior endothelium surface of wistar rats is a continuous layer of polygonal cells of almost uniform size and shape (Figure 1). Our study also revealed that endothelial cell borders have discrete interdigitations and posterior cell surfaces have microvilosities which appeared as multiple protrusions (Figure 2). Small pits were observed over the posterior cell surface as shown in Figure 3. We also detected the presence of cilia protruding from the posterior surface of endothelial cells into the anterior chamber (Figure 4).

General quantitative results

The majority of endothelial cells were six-sided (66.5%), with five-sided (27.4%), seven-sided (2.8%), four-sided (2.6%) and eight-sided (0.7%) cells constituting the remaining corneal endothelium. The mean cell area of corneal endothelium was 134.5 μm^2 (SD: 28.8 μm^2) and mean endothelial cell density was 7,499.8 cells/mm^2.(SD: 1,599.5 cells/mm^2) The coefficient of variation of mean cell area was 0.214.

Right versus left eyes

There were no statistical significant differences between right and left eyes, with respect to polygonality, mean cell area and mean cell density, as shown in Tables 1 and 2.

Central versus peripheral regions

There were no statistical significant differences between central and peripheral regions of the cornea, with respect to polygonality, mean cell area and mean cell density, as shown in Tables 3 and 4.

DISCUSSION

Corneal thickness and corneal transparency are maintained by the barrier function and active fluid pump of corneal endothelium.[1, 16] In most vertebrates the normal corneal endothelium is formed by a mosaic like pattern of homogenous polygonal cells arranged regularly on cornea innermost layer.[1, 6, 8, 17] Similarly, in the current study, SEM revealed that posterior endothelium surface of Wistar rats is a continuous layer of polygonal cells, which is in accordance with other vertebrates.[1, 3-11, 17] The majority of endothelial cells were six-sided, with five-sided cells being the second most frequent kind of polygonality found in our study. This pattern of distribution for polygonality is in accordance with studies conducted in humans, rabbits and other vertebrates, where 59-80% of corneal endothelial cells were hexagonal.[10, 18-20]

In regard to cell morphology, our study revealed that endothelial cell borders have interdigitations and posterior cell surfaces have microvilosities and small pits (maybe representing pinocytotic vesicles), which are in accordance with studies in humans, monkeys and rabbits.[21, 22] We also detected the presence of cilia, which have been seen in humans, monkeys and rabbits [21, 22] but which have not been observed in other species, such as ostrich.[20]

With respect to endothelial cell area and cell density, our results revealed higher cell density and corresponding less cell area when compared to some species, such as rabbits and ostrich.[20, 21] We did not find any statistical significant difference between right and left eyes, which is in accordance to other animals.[20] Again, we did not find statistical significant difference between central and peripheral corneal regions with respect to cell area and cell density. For this kind of comparison

(central *vs* peripheral regions) the literature show conflicting results, sometimes with and other times without statistical significant difference.[21] In fact, cell area and cell density values obtained from SEM can be very different according to the fixation method used.[21] For example, Doughty [21] estimated that shrinkage in the dissect-then-fix method is around 60% and for the whole-globe fixation method it is less, but still around 50%. In our study, we used the dissect-then-fix approach. In another study with corneal endothelial cells of rabbits, Schutten and Van Horn [23] found an average of tissue shrinkage of 29.7% when they measured the same corneal endothelial cells before fixation and after processing for SEM. In addition to that, Doughty and colleagues [24] found that shrinkage caused by fixation methods used for SEM may be different depending on the cell type. In this regard, they showed that 5-sided cells were 11.1% smaller, but 6-sided cells were 17.6% smaller.[24] Hence, it is not appropriate to compare cell area (nor cell density) values obtained from SEM with values obtained from methods that does not interfere in cell dimensions, such as specular microscopy.[4] As an example, using specular microscopy (SM) Yee et al[7] found a mean cell area of 452.3 μm^2 and a mean cell density of 2,211 cells/mm^2 in rats with 34 weeks of age. In our study, we could not estimate cell shrinkage, since cell area and cell density values were obtained only from SEM, without any other method to compare.

Endothelial cell density declines with age progression in some mammalian species including cats, dogs, rabbits and humans.[3, 9, 25-27] In most species the endothelium low regenerative ability is compensated by the growing in size of endothelial cells while endothelial cell density declines.[16, 26, 28] The Wistar rats used in our study were all the same age and were considered to be young adults. Since

endothelial cell density is expected to decline with age in other mammalian species, it is expected that older rats would have lower cell density than our animals.

In summary, our study provided valuable information about the normal corneal endothelium of Wistar rats with respect to surface morphology, cell area, cell density and coefficient of variation of mean cell area. In conclusion, the ultrastructure and the morphometric parameters of the normal corneal endothelium of Wistar rats seems to be similar to those described in other vertebrates.

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LEGENDS

TABLES

Table 1. Comparison of central regions between right versus left eyes.

Table 2. Comparison of peripheral regions between right versus left eyes.

Table 3. Comparison between central and peripheral regions of right eyes.

Table 4. Comparison between central and peripheral regions of left eyes.

FIGURES

Figure 1. Scanning electron microscopy showing that posterior endothelium surface of wistar rats is a continuous layer of polygonal cells of almost uniform size and shape.

Figure 2. Scanning electron microscopy showing endothelial cell borders with discrete interdigitations and posterior cell surfaces with microvilosities which appeared as multiple protrusions.

Figure 3. Scanning electron microscopy showing small pits (arrows) over the posterior cell surface of endothelial cells.

Figure 4. Scanning electron microscopy showing the presence of cilia protruding from the posterior surface of endothelial cells into the anterior chamber.

Table 1

Variable	Central regions of right eyes (n = 10 eyes)	Central regions of left eyes (n = 10 eyes)	P value *
Polygonality			
Six-sided cells	66.9 %	65.7 %	0.496
Five-sided cells	27.1 %	28.1 %	0.585
Other	6.0 %	6.2 %	0.168
Cell area (μm^2)	133.8 ± 30.1	134.4 ± 31.2	0.822
Cell density (cells/mm^2)	7,502.2 ± 1,662.7	7,500.7 ± 1,669.9	0.864

Other: group composed by four, seven and eight-sided cells. Cell area and cell density are expressed as means ± standard deviation. * Paired Student T test.

Table 2

Variable	Peripheral regions of right eyes (n = 10 eyes)	Peripheral regions of left eyes (n = 10 eyes)	P value *
Polygonality			
Six-sided cells	66.8 %	66.4 %	0.833
Five-sided cells	27.0 %	27.6 %	0.761
Other	6.2 %	6.0 %	0.168
Cell area (μm^2)	134.6 ± 29.2	135.2 ± 29.6	0.858
Cell density (cells/mm^2)	7,498.9 ± 1,661.5	7,497.5 ± 1,665.0	0.883

Other: group composed by four, seven and eight-sided cells. Cell area and cell density are expressed as means ± standard deviation. * Paired Student T test.

Table 3

Variable	Central regions of right eyes (n = 10 eyes)	Peripheral regions of right eyes (n = 10 eyes)	P value *
Polygonality			
Six-sided cells	66.9 %	66.8 %	0.961
Five-sided cells	27.1 %	27.0 %	0.962
Other	6.0 %	6.2 %	0.168
Cell area (μm^2)	133.8 ± 30.1	134.6 ± 29.2	0.663
Cell density (cells/mm^2)	7,502.2 ± 1,662.7	7,498.9 ± 1,661.5	0.612

Other: group composed by four, seven and eight-sided cells. Cell area and cell density are expressed as means ± standard deviation. * Paired Student T test.

Table 4

Variable	Central regions of left eyes (n = 10 eyes)	Peripheral regions of left eyes (n = 10 eyes)	P value *
Polygonality			
Six-sided cells	65.7 %	66.4 %	0.691
Five-sided cells	28.1 %	27.6 %	0.785
Other	6.2 %	6.0 %	0.168
Cell area (μm^2)	134.4 ± 31.2	135.2 ± 29.6	0.529
Cell density (cells/mm^2)	7,500.7 ± 1,669.9	7,497.5 ± 1,665.0	0.596

Other: group composed by four, seven and eight-sided cells. Cell area and cell density are expressed as means ± standard deviation. * Paired Student T test.

Figure 1

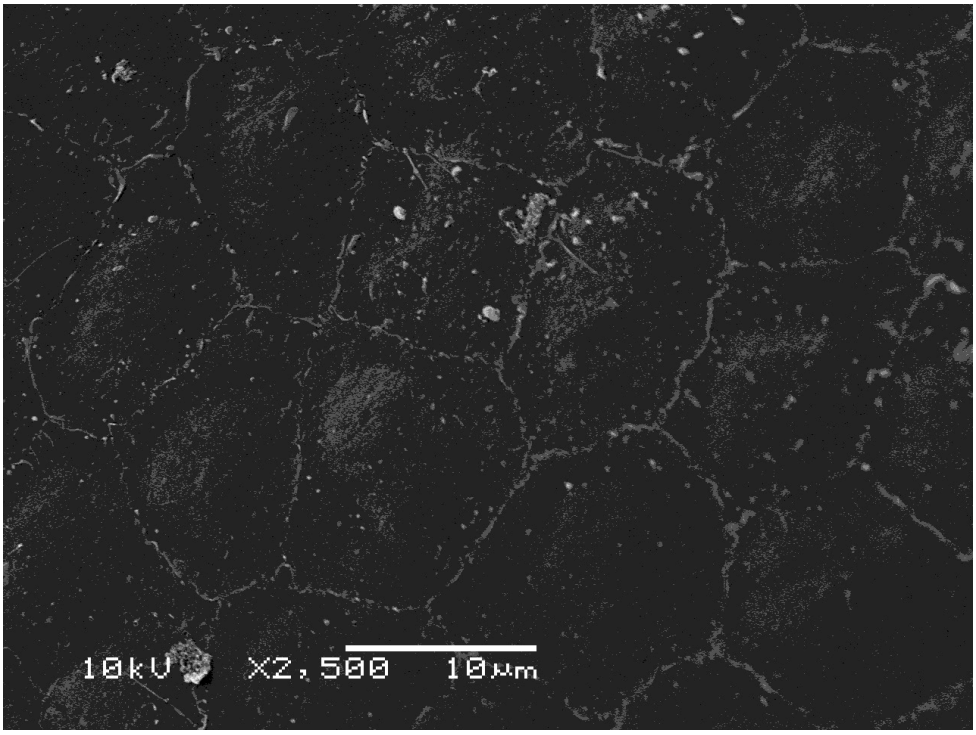


Figure 2

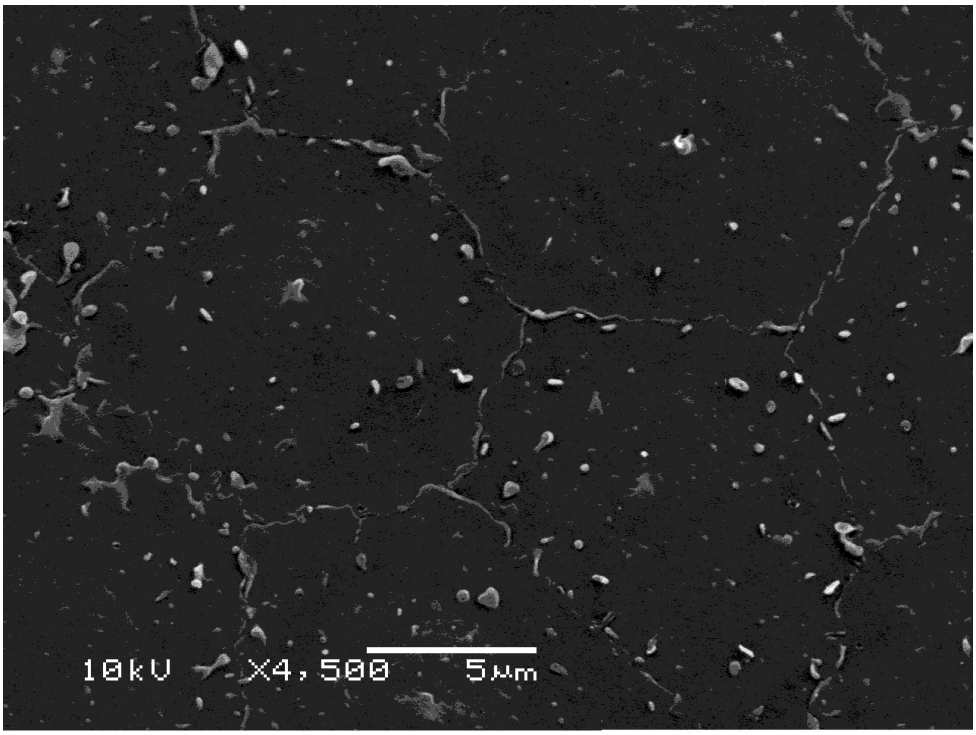


Figure 3

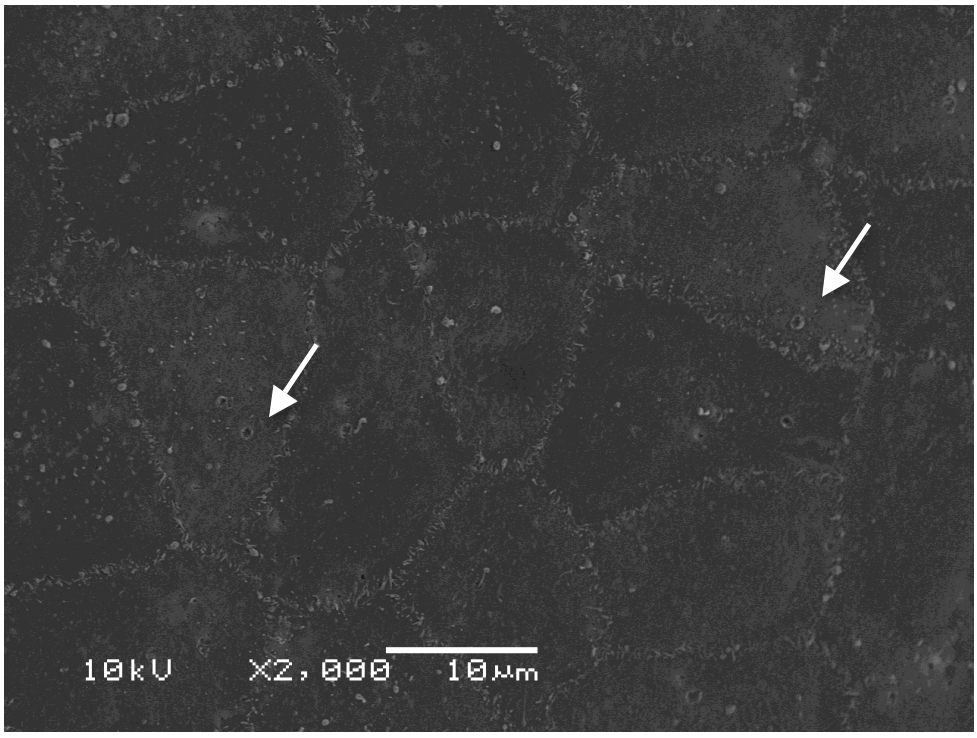
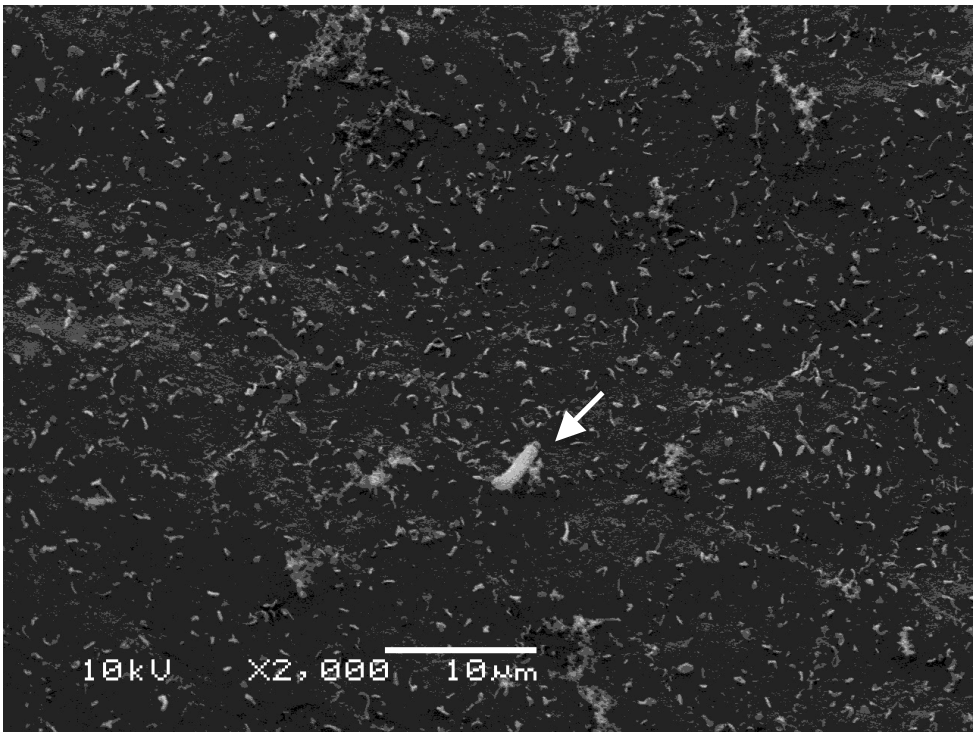


Figure 4



Artigo 2 em inglês:

TITLE

Use of an experimental model of ocular ischemia-reperfusion to investigate the corneal endothelium of Wistar rats

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ARTICLE TYPE

Original Article - Laboratory Science

SHORT RUNNING TITLE

Experimental ocular ischemia-reperfusion.

SYNOPSIS

This study presents a model of ocular ischemia-reperfusion (I-R) generated by intraocular pressure elevation. Using this model, we have shown corneal endothelial cells damaged in I-R eyes of Wistar rats.

FINANCIAL DISCLOSURE AND CONFLICT OF INTEREST

None of the authors have financial interests with materials or devices used in this article. The authors have no conflict of interest to disclose.

FINANCIAL SUPPORT

This work was supported by financial grants from Brazilian agencies: CNPq, CAPES, INCT/CNPq, IBN.Net/CNPq/FINEP, FAPERGS, UFRGS.

ABSTRACT

Purpose: To devise an experimental model of ocular ischemia-reperfusion (I-R) and apply this model to investigate the corneal endothelial cells (CECs) of Wistar rats using scanning electron microscopy.(SEM)

Methods: This is an experimental study in animals, in which thirty Wistar rats were divided in 3 groups, each one composed by 10 animals. Each rat was submitted to 45 minutes of ocular ischemia in its right eye, with no intervention in its left eye. The 3 groups differ with respect to the reperfusion period: (1) less than 30 seconds, (2) 24 hours, and (3) 7 days. The CECs were examined with SEM.

Results: In I-R eyes there were 15.4%, 16.2% and 16.5% of CECs damaged in groups 1, 2 and 3 respectively, differing from control eyes. The mean cell density reductions in I-R eyes were of 15.5%, 16.1% and 16.5% in groups 1, 2 and 3 respectively, differing from control eyes.

Conclusions: We presented a model of ocular I-R. Using this model, we have shown CEC density reduction and CEC damage in I-R eyes of Wistar rats, in different periods of reperfusion.

KEY-WORDS

Cornea, experimental model, ischemia, reperfusion, intraocular pressure.

INTRODUCTION

Intraocular pressure (IOP) elevation can lead to a variety of ocular injuries, such as optic nerve ischemia, corneal edema and loss of corneal endothelial cells. We know that IOP elevation observed in acute angle-closure glaucoma and in experimental models can lead to ischemic damage to the optic nerve and retina.[1,2] In addition to the ischemic injury, reperfusion also plays a role in ocular damage as shown in ischemia-reperfusion (I-R) models.[3-10] In this regard, it is of interest to develop experimental models of ocular I-R based on IOP elevation that can be used to study such I-R ocular injuries.

In order to devise such ocular I-R experimental model, we focused on corneal endothelial cells (CECs), since cornea and its endothelial cells are susceptible to IOP elevation. The structure of normal CECs has been described in humans and other animal species[11-13] with electron microscopy. In our study, we devised our experimental ocular I-R model working with corneal endothelium (CE) of Wistar rats, using scanning electron microscopy.(SEM)

Wistar rats are a strain of albino rats belonging to the species *Rattus norvegicus*. Since Wistar rats are largely used in animal researches, it is of great interest to know how CECs of these animals respond to IOP elevation conditions and I-R insults. Therefore, the purpose of this study is to devise an experimental model of ocular I-R based on IOP elevation and apply this model to investigate the CECs of normal Wistar rats using SEM, in different periods of reperfusion.

METHODS

Animals and Ethics:

All experiments were in agreement with Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil, and also in accordance to the Association for Research in Vision and Ophthalmology, USA, regarding the use of animals in ophthalmic and vision research. Thirty Wistar rats (males, 120 days of age, 250-350 g) were studied. All animals were killed by standard decapitation protocol.

Study design:

Experimental study in animals.

Groups:

The animals were divided in 3 groups, each one composed by 10 Wistar rats housed in an air-conditioned room (21–22°C), in 12-hours light/dark cycles with free access to food and water. In all groups, each rat was submitted to ocular ischemia in its right eye during 45 minutes with no intervention in its left eye.(control eye) The 3 groups differ from each other with respect to the reperfusion period after ocular ischemia as shown below.

Group 1: Animals were killed immediately (less than 30 seconds) after ocular ischemia.

Group 2: Animals were killed 24 hours after ocular ischemia.

Group 3: Animals were killed 7 days after ocular ischemia.

Experimental IOP elevation model:

The aim of our experimental IOP elevation model was to achieve an IOP capable to occlude completely the blood flow into the eye, generating a total retinal ischemia during 45 minutes. We assumed that this ischemia may represent almost total eye ischemia, although superficial corneal structures (corneal epithelium) probably were not under ischemic conditions, since O_2 supply from air over the cornea may be adequate to these superficial tissues. After 45 minutes, IOP was reduced to let reperfusion take place.

Preoperative procedures:

Systemic sedation was performed with intraperitoneal (IP) injection of xylazine (Coopazine, Schering-Plough Coopers, 2g/100ml) 3mg/Kg and ketamine (Dopalen, Vetbrands, 1g/10ml) 85mg/Kg, prior to ocular intervention. Ocular topical anesthesia was performed with proxymetacaine 0,5% eyedrops (Anestalcon, Alcon) stilled in right eye 8 minutes before intervention (cannulation procedure) and every 10 minutes during ischemia. Ocular antisepsis was conducted with aqueous povidone-iodine 5% eye drops (Ophthalmos) stilled once in right eye 5 minutes before intervention.

Operative procedures:

An insulin needle (26G½, BD) was used to cannulate anterior chamber by a direct insertion in the clear cornea region very close to the esclero-cornea limbus, without any previous incision.(Figure 1) The insulin needle was attached to a silicone tube in a closed system filled with NaCl 0,9% sterilized solution. It was also attached to a manometer allowing us to measure the IOP directly in real-time. This system was linked to a peristaltic pump which allowed us to control the infusion rate of NaCl solution into the anterior chamber. So, after cannulating the anterior chamber (AC), the needle was fixated to a support and the peristaltic pump began the IOP elevation. In the beginning of IOP elevation, the eye was red (normal Wistar rat eye color) because retinal artery was not occluded. Retinal artery occlusion was easily observed since the eye turned from red to white. It usually occurred around an IOP of 130-140 mmHg. So, to make sure that total occlusion was achieved, we used to elevate IOP until 150 mmHg. Once this IOP was achieved, it was maintained to complete 45 minutes of ischemia. Hereafter, the needle was removed and the eye became red again, since reperfusion occurred.

Post-operative procedures:

A combination of antibiotic (ciprofloxacin 3.5 mg/ml) and corticoid (dexamethasone 1 mg/ml) eye drops (Cylocort, Genom) was stilled in both eyes, every 6/6 hours, during the 24 post-operative (PO) hours in group 2 and during the 7 PO days in group 3.

Samples preparation:

Samples preparation for SEM was done following the method reported by Virtanen et al.[14] Immediately after death, eyes were enucleated and the corneas (corneal buttons) were excised by 360º scleral section 1 mm from corneal limbus. Corneal buttons were transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 and stored for 24 hours at 4ºC for fixation. Subsequently, the corneas were washed again in cacodylate buffer and dehydrated through an increasing series of ethanol solutions. Thereafter, the specimens were submitted to critical point drying using liquid carbon dioxide. Corneas were placed on 10 mm aluminum stubs with double-sided adhesive tape and sputter coated with gold-palladium.

Scanning electron microscopy and image analysis:

The posterior endothelial surfaces of each cornea were examined and photographed using a scanning electron microscope (JEOLJSM 5410, Tokyo, Japan) operated at 10 and 20 kV. Twenty photomicrographs were obtained from each cornea with magnifications of x1,000, x1,500, x1,600, x1,700 and x2,500. Photomicrographs were obtained from central corneal region. The photomicrographs were introduced into a computer in which they were analyzed with help of an image analyzer software.(Image Tool, UTHSCSA, San Antonio, USA) With this software, 100 CECs of each cornea were analyzed, determining polygonality (number of sides of each endothelial cell), cell area (μm^2), cell density (cells/mm^2) and the number of CECs damaged. One CEC was considered to be damaged when its ultrastructure was clearly modified, compared to normal CECs. Polygonality was the endpoint to

evaluate cell morphology. Polygonality, cell area and cell density determinations were conducted considering only CECs not damaged.

Statistical analysis:

Statistical analysis was conducted with SPSS 12.0 software and values of $P<0.05$ (95% confidence intervals; two-tailed) were considered significant. Paired Student T test was used to compare means of cell area and cell density between control and I-R eyes. One-way ANOVA followed by Tukey *post hoc* test was used to compare the number of CECs damaged, polygonality, cell area and cell density among all groups.

RESULTS

Ultrastructure, morphology and cell damage:

Ultrastructure modifications of CECs were seen in I-R eyes of all groups, revealing damaged CECs, as shown in Figure 2. In Figure 2, we can see that CECs damaged presented ruptures in their cell plasmatic membranes. Figure 2 also show normal CECs in control eyes of all groups.

In control eyes there were no CECs damaged. In I-R eyes there were 15.4%, 16.2% and 16.5% of mean CECs damaged in groups 1, 2 and 3 respectively. Since no CECs damaged were seen in control eyes, significant differences between control and I-R eyes are evident and there is no sense in applying any statistical test in this regard. On the other hand, comparing only I-R eyes we did not find any statistically significant difference (P=0.780; one-way ANOVA) among all groups.

Cell morphology was studied evaluating the polygonality of CECs. Considering only CECs not damaged, there were no statistically significant differences among groups nor between control versus I-R eyes (P=0.833; one-way ANOVA), with respect to polygonality. In this regard, the majority of CECs were six-sided (64.7%), with five-sided (28.6%), seven-sided (3.3%), four-sided (2.9%) and eight-sided (0.5%) cells constituting the remaining CECs.

Cell density:

Intervention eyes presented less cell density compared to control eyes ($P<0.001$; paired Student T test) for all groups. The mean cell density reductions in I-R eyes were of 15.5%, 16.1% and 16.5% in groups 1, 2 and 3 respectively. Comparing only I-R eyes we did not find any statistically significant difference ($P>0.999$; one-way ANOVA) among all groups. These comparisons are shown in Graphic 1.

Cell area:

There were no statistically significant differences among groups nor between control versus I-R eyes ($P=0.986$; one-way ANOVA), with respect to cell area.

DISCUSSION

In order to study I-R damage to ocular tissues, several I-R models have been developed.[3-10] The elevation of IOP is an interesting approach to generate ocular ischemia since it can be easily reversed, allowing the study of reperfusion injuries as well. In addition to this, ischemia induced by high IOP may represent a model of acute angle-closure glaucoma.[15] With this in mind, many authors have suggested different models of ocular I-R based on IOP elevation.[2, 6, 7, 15-19]

Reviewing these models, we observed that the time of ischemia used by different authors varied from 30 to 180 minutes, but the majority of them used a period from 30 to 60 minutes.[2, 6, 16, 17, 19] Since there are evidences of damage to retinal ganglion cells after 45 minutes of ischemia,[15, 16] we adopted this time of ischemia in our experimental model, in order to devise a model that could be used in future studies involving not only corneal tissues, but also other ocular tissues. With respect to reperfusion period, it varied from 12 hours to 14 days in different models.[2, 6, 7, 15-18] In our study, we decided to investigate 3 different periods of reperfusion. Finally, in regard to IOP maximum level, it varied from 110 to 130 mmHg in some models[2, 16, 19] but we decided to use a cut-point of 150 mmHg in our study. This cut-point was obtained from a pilot study with 10 animals (data not shown) in which we observed that retinal artery occlusion usually occurred around an IOP of 130-140 mmHg. So, to make sure that total occlusion was achieved, we decided to elevate the IOP until 150 mmHg in the present study.

Using our experimental model of ocular I-R, we compared 3 groups with respect to endothelial cell damage, cell area and cell density. But why CECs are so important to be studied? CECs are very important to cornea, since corneal thickness

and corneal transparency are maintained by the barrier function and active fluid pump of CECs.[20] In the current study, SEM revealed that posterior CE surface of Wistar rats is a continuous layer of polygonal cells, which is in accordance with other vertebrates.[11, 12, 13, 21] In addition, SEM revealed that the majority of CECs were six-sided, which is also in accordance with studies conducted in other mammals, where 59-80% of CECs were hexagonal.[13, 22]

In regard to cell damage and cell density, we observed that intervention eyes presented more cell damage and less cell density than control eyes in all groups. We attribute these results not only to the ischemic insult, but also to the damage generated by IOP elevation in intervention eyes. Since no differences were observed in intervention eyes among the 3 groups, the reperfusion period seems to have had little or no influence in cell damage and cell density. We must remember that cell density determination was conducted considering only CECs not damaged and that endothelial cell density declines with age progression in mammalian species.[11, 12, 23] In this regard, the Wistar rats used in our study were all the same age and were considered to be young adults.

With respect to cell area, we did not observed differences among groups. Since there were more cell damage and less cell density in interventions eyes, an increase in cell area in the remaining CECs would be expected to happen. This increase in cell area is well known as a mechanism to compensate the cell density reduction and it has been documented in many species.[20, 23] So, we think that the periods of reperfusion studied were not sufficient to allow CECs to reorganize themselves in their mosaic like pattern, justifying the absence of differences in cell area among groups.

We also must remember that cell area and cell density values obtained from SEM does not correspond to real values observed in vivo, since the fixation methods used for SEM induce shrinkage in corneal tissues.[24] For example, Doughty[24] estimated that shrinkage in the dissect-then-fix method is around 60% and for the whole-globe fixation method it is less, but still around 50%. In our study, we used the dissect-then-fix approach.

In summary, in this study we presented a method that can be used to investigate I-R ocular injuries and also ocular damage generated by IOP elevation. Using this method, we have shown CEC density reduction and CEC damage in I-R eyes of Wistar rats, in different periods of reperfusion. Future studies may apply our method to investigate other ocular tissues in Wistar rats under I-R conditions generated by IOP elevation.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

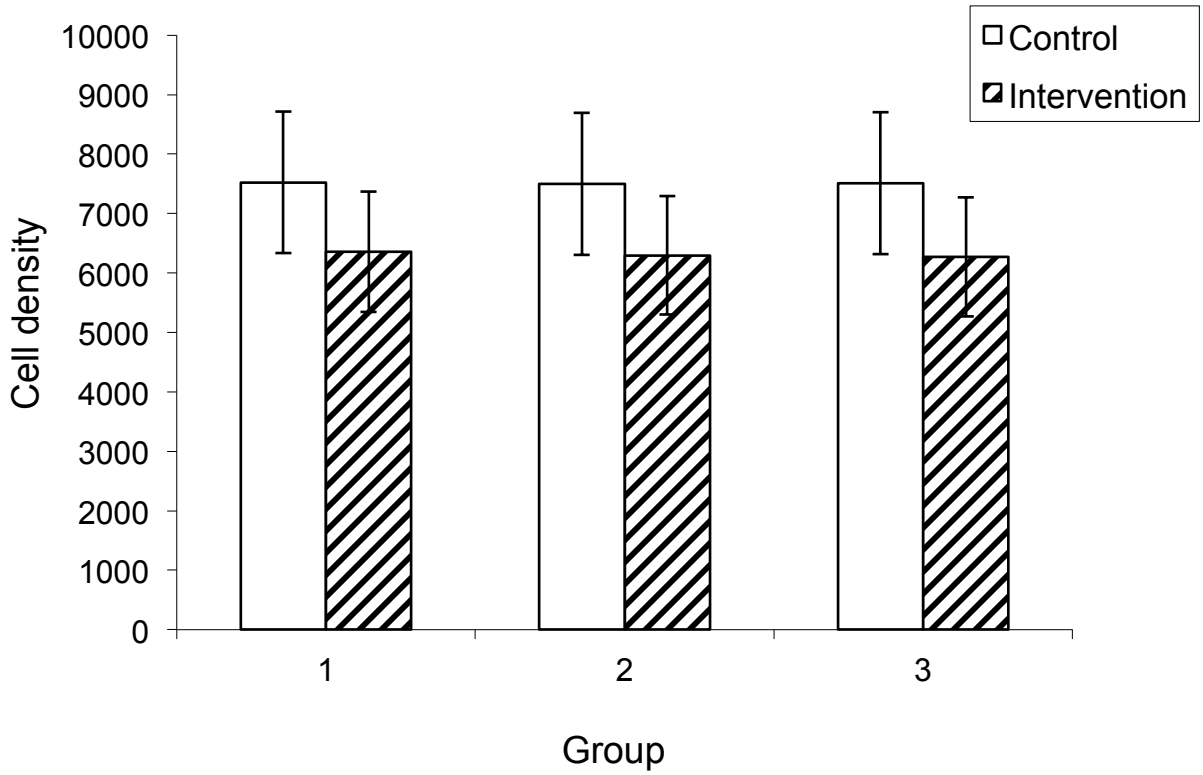
Figure 1.

An insulin needle (26G½, BD) is inside of the anterior chamber of the eye.

Figure 2.

All images (A to F) are photos obtained using a scanning electron microscope (SEM) operated at 10 or 20 kV. A, C and E are photos showing normal corneal endothelial cells (CECs), obtained from control eyes of groups 1, 2 and 3 respectively. B, D and F are photos obtained from intervention eyes of groups 1, 2 and 3 respectively. Arrows are showing ruptures in the cell plasmatic membranes of CECs damaged.

Graphic 1. Comparison of endothelial cell density in control and intervention eyes among the 3 groups.



Bars represent the endothelial cell density (cells/mm^2) in each group and are expressed as mean ± 95% confidence interval. Intervention eyes presented less cell density compared to control eyes (P<0.001; paired Student T test) for all groups. Comparing only I-R eyes we did not find any statistically significant difference.(P>0.999; one-way ANOVA)

Figure 1

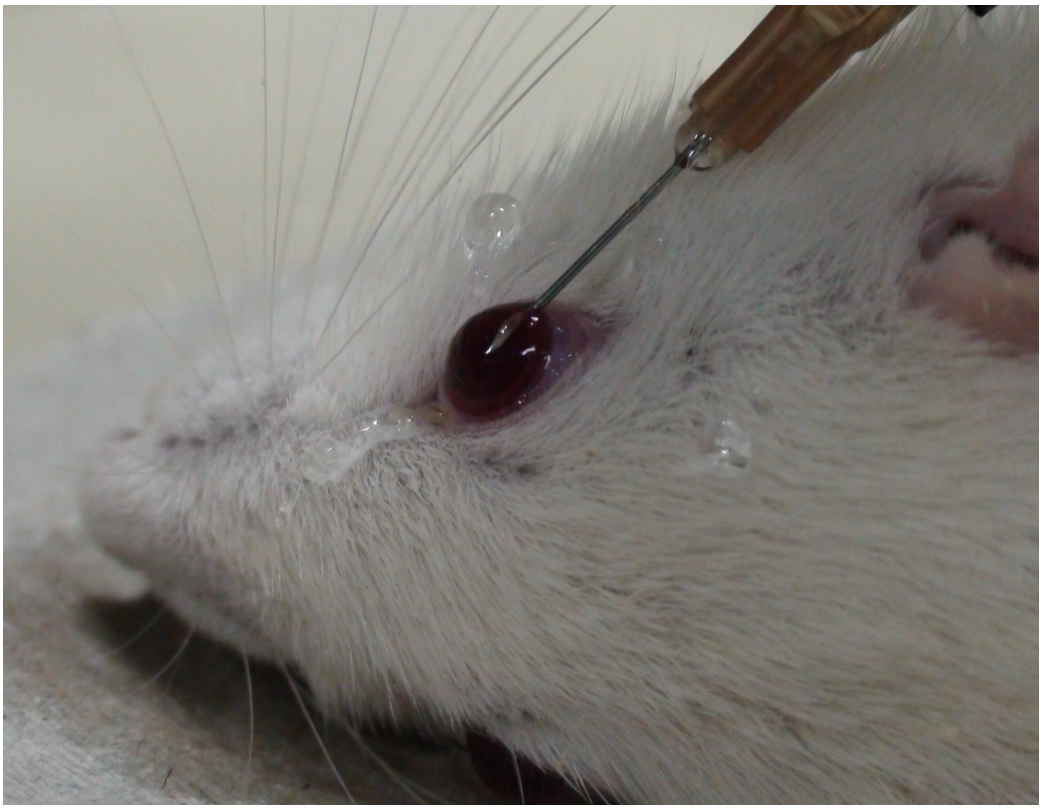
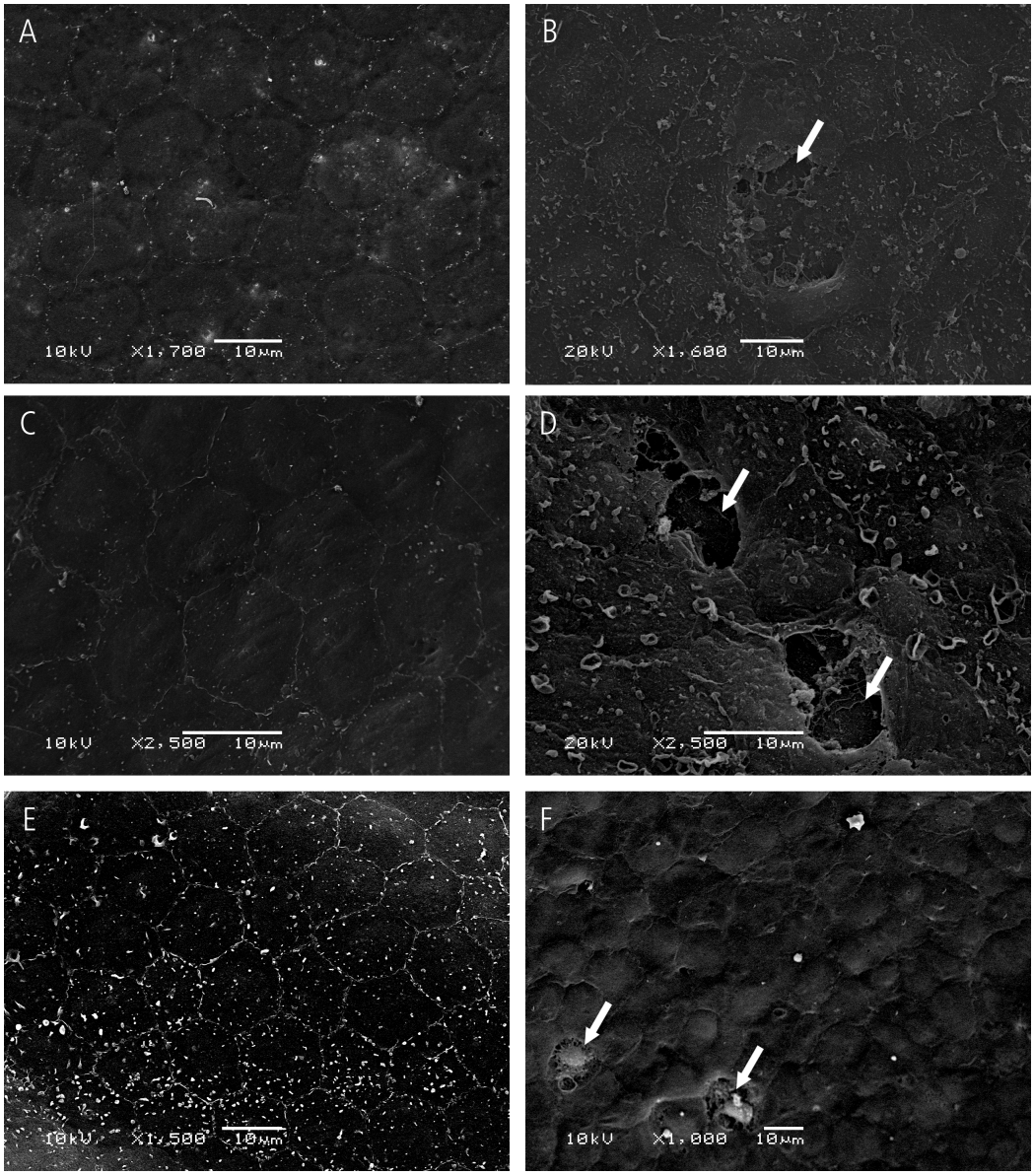


Figure 2


A
10kV X1,700 10μm
B
20kV X1,600 10μm
C
10kV X2,500 10μm
D
20kV X2,500 10μm
E
10kV X1,500 10μm
F
10kV X1,000 10μm

Artigo 3 em inglês:

TITLE

Effect of guanosine on glutamate uptake in retinas of adult Wistar rats submitted to ocular ischemia-reperfusion

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SHORT RUNNING TITLE

Guanosine on glutamate uptake in retina

SYNOPSIS

This study presents that ocular ischemia-reperfusion generated by our experimental model decreased glutamate uptake in retinas of Wistar rats and that guanosine abolished or even overcomed this decrease.

FINANCIAL DISCLOSURE AND CONFLICT OF INTEREST

None of the authors have financial interests with materials or devices used in this article. The authors have no conflict of interest to disclose.

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ABSTRACT

Purpose: To investigate the effect of guanosine (GUA) on glutamate (GLU) uptake in retinas of Wistar rats submitted to ocular ischemia-reperfusion (I-R) injuries, using an experimental intraocular pressure (IOP) elevation model.

Methods: Thirty Wistar rats were divided in 3 groups of 10 rats. Each rat was submitted to IOP elevation in the right eye generating retinal ischemia during 45 minutes with no intervention in the left eye (control retina). In group 1, animals did not receive any GUA. In group 2, animals received an intraperitoneal injection of GUA 30 minutes before ischemia and, in group 3, animals received GUA in water during 1 week before and 1 week after ischemia. All animals were killed 7 days after ischemia and retina samples were obtained. Glutamate uptakes were performed from these retina samples.

Results: GLU uptake in control retina was similar in all groups. In group 1, GLU uptake was significantly reduced by I-R; this reduction was abolished by GUA administration in water (group 3) and GLU uptake increased with intraperitoneal GUA (group 2).($P<0.001$; ANOVA)

Conclusions: These results point that I-R generated by our experimental model decreased GLU uptake in retinas of Wistar rats and that GUA abolished or even overcomed this decrease. These GUA effects are in agreement to previous results, which show that GUA administration presents neuroprotection in central nervous system by stimulating GLU uptake, mainly by astrocytes. In retina, this effect may be due to GUA stimulation of GLU uptake exerted mainly by Müller cells.

KEY-WORDS

Guanosine, glutamate, ischemia, reperfusion, intraocular pressure.

INTRODUCTION

Glaucoma, one of the leading causes of blindness in the world, is characterized by retinal ganglion cell (RGC) degeneration, alterations in optic nerve head and visual field (VF) loss.[1] Intraocular pressure (IOP) elevation remains the most important known risk factor for development and progression of glaucomatous optic neuropathy and VF loss.[2-6] It is well known that IOP elevation observed in acute angle-closure glaucoma and in experimental models can lead to ischemic damage to optic nerve and retina.[7-10] Ischemic injury to optic nerve and retina can also result from occlusive vascular events without IOP elevation, such as cases of central retinal artery occlusion.[11] Therefore, retinal hypoxia is the potentially blinding mechanism underlying a number of sight-threatening disorders including central retinal artery occlusion, complications of diabetic eye disease and some types of glaucoma.[12] In addition to the ischemic injury, there are evidence supporting that reperfusion also plays a role in retina damage, as shown in ischemia-reperfusion (I-R) models.[13-16] Considering the great incidence and prevalence of glaucoma and occlusive vascular events affecting retina and optic nerve[17-18], it is relevant to study substances capable to offer neuroprotective effects against I-R injuries.

In regard to neuroprotection, many studies in this area focuse on the glutamatergic activity, since glutamate (GLU) is the major excitatory neurotransmitter in central nervous system (CNS).[19] Although GLU is essential for normal brain functions, increased GLU amounts in synaptic cleft can lead to neurotoxicity due to overstimulation of GLU receptors, which is called excitotoxicity.[20]

Once GLU is also the major excitatory neurotransmitter in retina[21], the study of glutamatergic activity is of great value in order to investigate retinal I-R injuries. In

fact, multiple evidences support that GLU is implicated in retinal ischemic damage.[21-26] Retinal I-R induced by experimental elevation of IOP leads to damage and loss of RGCs.[23, 25] Under these conditions, biochemical changes may cause an accumulation of extracellular GLU and activation of the excitotoxic cascades.[23, 25] Glutamate signaling mediated through the N-methyl-D-aspartate (NMDA)-type receptor (NMDAR) lead to excessive Ca^{2+} influx into neurons, which is a crucial step for its excitotoxicity.[24-26] In CNS, the main endogenous process responsible for maintaining GLU concentration in synaptic cleft below toxic levels is the GLU uptake, exerted by transporters located mainly in astrocytes.[27] In retina, this GLU uptake activity seems to be exerted mainly by Müller cells, which express the GLU transporter GLAST.[21]

Guanosine (GUA) is a nucleoside that has neuroprotective effects against glutamatergic excitotoxicity in CNS, as shown in several studies.[28-30] In CNS, GUA neuroprotection seems to be related to its stimulatory effect on GLU uptake, tending to decrease GLU concentration in the synaptic cleft.[31] In this regard, GUA could also presents neuroprotective effects against GLU-receptors overstimulation (excitotoxicity) in retina. These neuroprotective effects may occur by stimulatory effect of GUA on GLU uptake by Müller cells. Despite its documented neuroprotective effect in CNS, we did not find any study evaluating GUA neuroprotection in retinal I-R injuries. Therefore, the purpose of this study is to investigate the effect of GUA on GLU uptake in retinas of Wistar rats submitted to ocular I-R injuries, using an experimental IOP elevation model.

METHODS

Animals and Ethics:

All experiments were in agreement with Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil, and also in accordance to Association for Research in Vision and Ophthalmology, USA, regarding the use of animals in ophthalmic and vision research. Sixty eyes from 30 healthy Wistar adult rats (males, 120 days of age, 250-350 g) were studied. All animals were killed by standard decapitation protocol.

Study design:

Experimental study in animals.

Groups:

The animals were divided in 3 groups, each one composed by 10 healthy Wistar rats housed in an air-conditioned room (21–22°C), in 12-hours light/dark cycles with free access to food and water. In all groups, each rat was submitted to ocular ischemia in its right eye during 45 minutes with no intervention in its left eye (control eye). All animals were killed 7 days after ischemia (reperfusion period).

Group 1: CONTROL group: animals did not receive any GUA.

Group 2: GUA IP group: animals received an intraperitoneal (IP) injection of GUA 7.5 mg/Kg, 30 minutes before ischemia.

Group 3: GUA WATER group: animals received GUA dissolved in water (0.5 mg/ml) during 1 week before and 1 week after ischemia (total of 14 days drinking GUA).

Experimental IOP elevation model:

The aim of our experimental IOP elevation model was to achieve an IOP capable to completely occlude the blood flow into the right eye, generating a total retinal ischemia during 45 minutes. After 45 minutes, IOP was reduced to let reperfusion take place. In each animal right eye was submitted to intervention (I-R) and left eye was the control (no intervention occurred).

Preoperative procedures:

Systemic sedation was performed with IP injection of xylazine (Coopazine, Schering-Plough Coopers, 2g/100ml) 3mg/Kg and ketamine (Dopalen, Vetbrands, 1g/10ml) 85mg/Kg, prior to ocular intervention. Ocular topical anesthesia was performed with proxymetacaine 0.5% eye drops (Anestalcon, Alcon) stilled in right eye 8 minutes before the intervention (cannulation procedure) and every 10 minutes during ischemia.

Operative procedures:

An insulin needle (26G½, BD) was used to cannulate anterior chamber (AC) by a direct insertion in the clear cornea region very close to the esclero-cornea limbus without any previous incision.(Figure 1) If any intra-ocular bleeding occurred, the eye was not used for the study and the rat was immediately killed. The insulin needle was attached to a silicone tube in a closed system filled with NaCl 0.9% sterilized solution. It was also attached to a manometer allowing to measure the IOP directly in real-time. This system was linked to a peristaltic pump which allowed us to control the infusion rate of NaCl solution into the AC. After cannulating the AC, the needle was fixated to a support and the peristaltic pump began the IOP elevation. In

the beginning of IOP elevation the eye was red (normal Wistar rat eye color) because retinal artery was not occluded. Retinal artery occlusion was easily observed since the eye turned from red to white. It usually occurred around an IOP of 130-140 mmHg. So, to make sure that total occlusion was achieved, we decided to elevate the IOP until 150 mmHg in the present study. Once this IOP was achieved, it was maintained for 45 minutes of ischemia. Hereafter, the needle was removed and the eye became again red, since reperfusion occurred.

Post-operative procedures:

Antibiotic (ciprofloxacin 3.5 mg/ml) and corticoid (dexamethasone 1 mg/ml) eye drops (Cylocort, Genom) were stilled in both eyes 4 times a day (every 6/6 hours) during the 7 post-operative (PO) days.(reperfusion period)

Retina samples preparation:

Immediately after death, right and left eyes were kindly removed without any eye laceration. In each eye, scleral-corneal button was removed under surgical microscope by 360º scleral section 1 mm from corneal limbus. Sclera was then submitted to three radial cuts toward the optic nerve head without reaching it, allowing us to gently remove the retina with a fine spatula. Retina samples obtained with this method were almost the entire retina, since just *pars plana* (1 mm from limbus) was not present in each sample.

Glutamate uptake:

Na^+-dependent GLU uptake:

Glutamate uptake was performed as previously described.[32] Retina samples were preincubated at 33°C for 20 minutes in HBSS, followed by the addition of 100 µM [3H]glutamate. Incubation was stopped after 7 minutes with 2 ice-cold washes of 1 ml HBSS, immediately followed by the addition of 0.5N NaOH, which was then kept overnight. Na^+-independent uptake was measured using the same protocol described above, with differences in the temperature (4°C) and medium composition (N-methyl-D-glucamine instead of NaCl in HBBS). Na^+-dependent uptake (exert by astrocytic GLU transporters) was considered as the difference between the total uptake and the Na^+-independent uptake. Both uptake were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409). Glutamate uptake was expressed in nmol/mg of protein x min.

Protein determination:

Protein concentration was measured by the method of Lowry et al.[33] using bovine serum albumin as standard.

Statistical analysis:

Statistical analysis was conducted with SPSS 12.0 software and values of $P<0.05$ (95% confidence intervals; two-tailed) were considered significant. Glutamate uptake (mean±SD) was compared among all groups and subgroups (control and intervention eyes of each group) with one-way ANOVA followed by Tukey *post hoc* test.

RESULTS

Baseline:

Comparison of GLU uptake (mean±SD) among control eyes from the 3 groups revealed the presence of homogeneous subsets, since no significant differences were found among groups (one-way ANOVA; P=0.344).

Comparisons among all groups and subgroups:

Glutamate uptake values in each group and subgroup are summarized in Table 1. As we can see, GLU uptake in the intervention subgroups (I-R eyes) of group 1 (CONTROL), 2 (GUA IP) and 3 (GUA WATER) were statistically different (P<0.001; one-way ANOVA followed by Tukey *post hoc* test), as shown in Graphic 1. It means that the decrease of GLU uptake caused by I-R procedure (group 1) was abolished by the administration of GUA in the water (group 3) and overcomed by intraperitoneal GUA (group 2).

DISCUSSION

In order to study I-R damage to retina, different kinds of vascular occlusion-reperfusion models were developed.[13-16] Although different methods can be used to generate retinal ischemia, such as surgical vascular occlusions, the elevation of IOP is an interesting approach since it can be easily reversed, allowing the study of reperfusion injuries as well. Additionally, ischemia induced by high intraocular pressure is a model for retinal neurodegeneration presenting features similar to those seen in patients with central retinal artery occlusion and may represent a model of acute angle-closure glaucoma.[25] With this in mind, many authors suggested different models of retinal I-R based on IOP elevation.[8-10, 14, 15, 22, 24, 25, 34]

The time of IOP elevation (ischemia) used by different authors varied from 30 to 180 minutes, predominantly from 30 to 60 minutes.[8-10, 14, 22, 34] Since there are evidence of damage to RGCs after 45 minutes of ischemia,[9, 25] we adopted this time in our experimental model. With respect to reperfusion time, it varied from 12 hours to 14 days in different models [8-10, 14, 15, 22, 24, 25] and we decided to use 7 days of reperfusion.[9, 14, 22] Finally, in regard to IOP maximum level, it varied from 110 to 130 mmHg in some models[8, 9, 34] and we decided to use a cut-point of 150 mmHg in our study.

Using our experimental model of retinal I-R, we compared 3 groups with respect to GLU uptake. Baseline values of control subgroups were similar, validating the comparisons between intervention subgroups. But why GLU uptake is important in retinal I-R injuries? As mentioned before, GLU is the major excitatory neurotransmitter in retina and multiple evidences support that GLU is implicated in retinal ischemic damage.[12, 21-26] Retinal I-R leads to rise in extracellular GLU[23, 25]

which can cause excitotoxicity by activation of NMDA and non-NMDA glutamatergic receptors[24-26] resulting in RGCs death by apoptosis or necrosis.[12, 23-25] Over stimulation of these receptors can lead to increased nitric oxide synthase (NOS) activity[12, 25], rising nitric oxide (NO) production which may be toxic to cells resulting in their death.[12] This also lead to excessive Ca^{2+} influx into neurons, which is an important step for its excitotoxicity.[24-26] Thus, over stimulation of GLU receptors is thought to damage the retina by a cascade of biochemical effects such as neuronal NOS activation and increase in intracellular Ca^{2+}, which has been described as a major contributing factor to RGC loss.[12]

Extracellular GLU accumulation in retinal I-R injuries could be related to an increase in GLU release and/or a decrease of its uptake.[35] In retina, the main endogenous process maintaining synaptic GLU below toxic levels seems to be the GLU uptake mainly exerted by Müller cells, which express the GLU transporter GLAST.[21] In fact, there is evidence supporting that GLAST capability to sustain GLU inward transport is particularly susceptible to an acute ischemic process.[21] Overall, GLU uptake is mediated by a gene family of high affinity sodium-dependent transporters that includes 5 known mammalian subtypes; in humans, these GLU transporters have been called excitatory amino acid transporter (EAAT) 1 through 5.[36] In retina, EAAT1 (also called GLAST) is found in Müller cells and astrocytes[37]; EAAT2 (also called GLT-1) is localized in cones and in two types of bipolar cells[38]; EAAT3 (also called EAAC1) is found on horizontal, amacrine, and ganglion cells and, rarely, on bipolar cell[39]; EAAT5 is localized on photoreceptors and bipolar cells[40]; and EAAT4 has not been found in retina.[36]

In group 1 (CONTROL), we observed a reduction in GLU uptake in I-R eyes. But what does it mean? In our experimental model, GLU uptake values represent the

GLU present in intracellular space of total retina. As previously discussed, this intracellular GLU is probably located mainly inside Müller cells. Therefore, a reduction in GLU uptake in I-R eyes means that intracellular GLU can be reduced in these eyes. Now, the question is: why intracellular GLU is reduced in I-R eyes of group 1? One possible explanation could be that GLU uptake by Müller cells were compromised by retinal I-R injury. In fact, GLAST dominates GLU uptake in retina[41] and we know that the activity of this transporter can be compromised by an acute ischemic insult.[21] The loss of GLAST function seems to be due to acute metabolic changes induced by the ischemic attack, e.g. disruption of the necessary sodium gradient, acidosis or the phosphorylation state of the transporter[42], rather than rapid down regulation of transporter expression. In addition, it has been reported a reduction of retinal GLAST and GLT1 in an experimental rat model of glaucoma.[43] It also has been demonstrated GLAST loss in human glaucomatous tissue, consistent with the elevated GLU found in glaucomatous vitreous.[44]

Another possible explanation could be related to oxidative stress, since reactive oxygen species (ROS) produced in retinal I-R injuries have been implicated in GLU excitotoxicity.[35, 45] In this regard, ROS seem to be related to extracellular GLU accumulation and to cell injury induced by GLU receptors activation.[45, 46]

In groups 2 (GUA IP) and 3 (GUA WATER), we did not observed a GLU uptake reduction in I-R eyes; in fact it was observed an increase (compared to control) in group 2. These results could mean that extracellular GLU levels were maintained or even decreased by GUA administration. But how did GUA protect against such extracellular GLU increase? Moreover, can we say that avoiding this extracellular GLU increase GUA has shown a neuroprotective effect against retinal I-R injury?

In regard to the first question, we believe that GUA has avoided extracellular GLU increase by its action over GLU uptake. In our interpretation, GUA could be stimulating GLU uptake mainly by Müller cells, reducing extracellular GLU. In group 2, the intracellular GLU increase (extracellular GLU decrease) was significant as compared to control, but in group 3 it was not. Maybe, this different statistical behavior between groups 2 and 3 could be due to GUA bioavailability. Since we did not measured serological GUA, this bioavailability question remains to be elucidated by future studies.

Supporting our hypothesis that GUA stimulated GLU uptake, there is evidence showing that GUA increases GLU uptake in CNS.[31] Astrocytic GLU uptake increase mediated by GUA seems to be independent of adenosine receptor activation and GUA binding site is located on astrocyte membrane.[31] Other guanine-based purines, such as GMP and GTP, also had demonstrated stimulatory effect on GLU uptake, but evidences strongly indicate that GUA is the common mediator of this effect.[31] This GUA effect on GLU uptake seems to be related to GLU concentrations. In fact, GLU uptake was significantly stimulated at high (1 mM) but not at low (10-100 μM) GLU extracellular concentrations in rat brain slices exposed to GUA.[47] Guanosine has no effect on GABA uptake, suggesting a specific effect of GUA towards GLU uptake by astrocytes.[31] Considering that GABA uptake is also sodium dependent, this could indicate that GUA stimulatory effect probably do not involve mechanisms related to increase of sodium gradient.[31]

The effect of GUA on GLU uptake is of potential pathophysiological relevance, since there are *in vivo* and *in vitro* situations where extracellular GLU elevations are accompanied by an increase of GUA.[48] This increased release of GUA can be coupled to the energy status of the cell. Intracellular basal levels of GUA and

adenosine in neural cells are in the nanomolar range, being much higher for ATP (3 mM) and GTP (300 mM).[48] Similarly to ATP, GTP levels are maintained during normal energy metabolism. In hypoxic conditions, relatively large increases (e.g. 10-fold) in nucleosides concentration are produced from minimal decreases (e.g. 0.1 to 1%) in ATP and GTP concentration.[31] Since equilibrate nucleoside transporters mediate exchange between intracellular and extracellular compartments, the release of biologically relevant amounts of both GUA and adenosine may occur in some pathological conditions.[31] These nucleosides can decrease GLU levels both by inhibiting further GLU release in the case of adenosine[49] and by accelerating GLU removal by astrocytes in the case of GUA.[50] This effect of GUA is probably of particular relevance in excitotoxic situations, since GUA failed to affect astrocytic uptake of low (1 mM) GLU concentrations.[47]

Concerning the GUA acting as a neuroprotective factor against retinal I-R injuries, the present study has shown preliminary evidences supporting this idea, since GUA has demonstrated to avoid GLU uptake reduction (or even increasing) in I-R eyes. Our interpretation in favor of GUA neuroprotective effects is also based on the current opinion of several authors considering GUA as a neuroprotective factor against GLU-receptors overstimulation in CNS[28-30], due to its action increasing GLU uptake.[31] This neuroprotection seems to be exerted by Müller cells, which are activated during NMDA injuries and play a protective role against NMDA-induced retinal cell death.[26] Finally, there are data suggesting that both EAAT1 and EAAT2 GLU transporters are important for limiting excitotoxicity in the ganglion cell layer and that interference with their function increases extracellular GLU and adversely impacts RGC survival.[36]

In summary, our results suggest that GUA has avoided GLU uptake reduction in I-R eyes, in our experimental model. This effect may be due to GUA stimulation of GLU uptake exerted by Müller cells. As far as we know, this is the first study suggesting this effect of GUA on GLU uptake in retinas of Wistar rats submitted to ocular I-R injuries. At present, it is early to say if this GUA behavior may be considered some kind of neuroprotection against retinal I-R injuries. This theme is under investigation by our group.

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FIGURE LEGENDS

Figure 1.

An insulin needle (26G½, BD) is inside of the anterior chamber of the eye.

Figure 1.

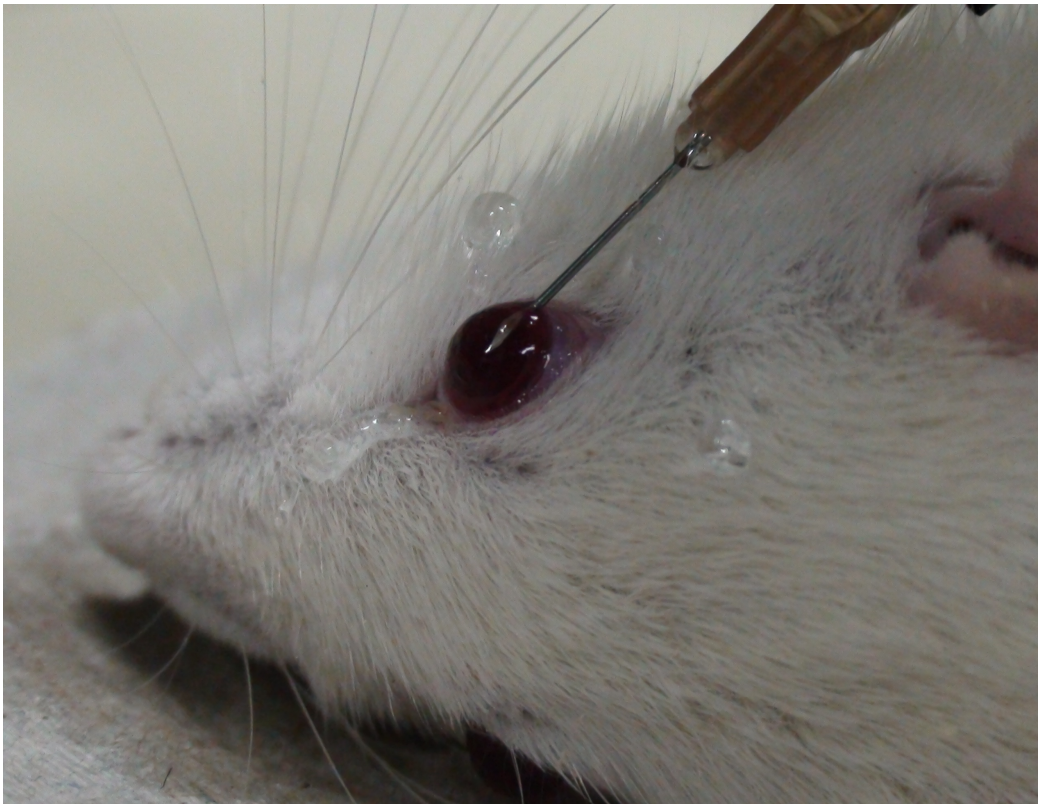
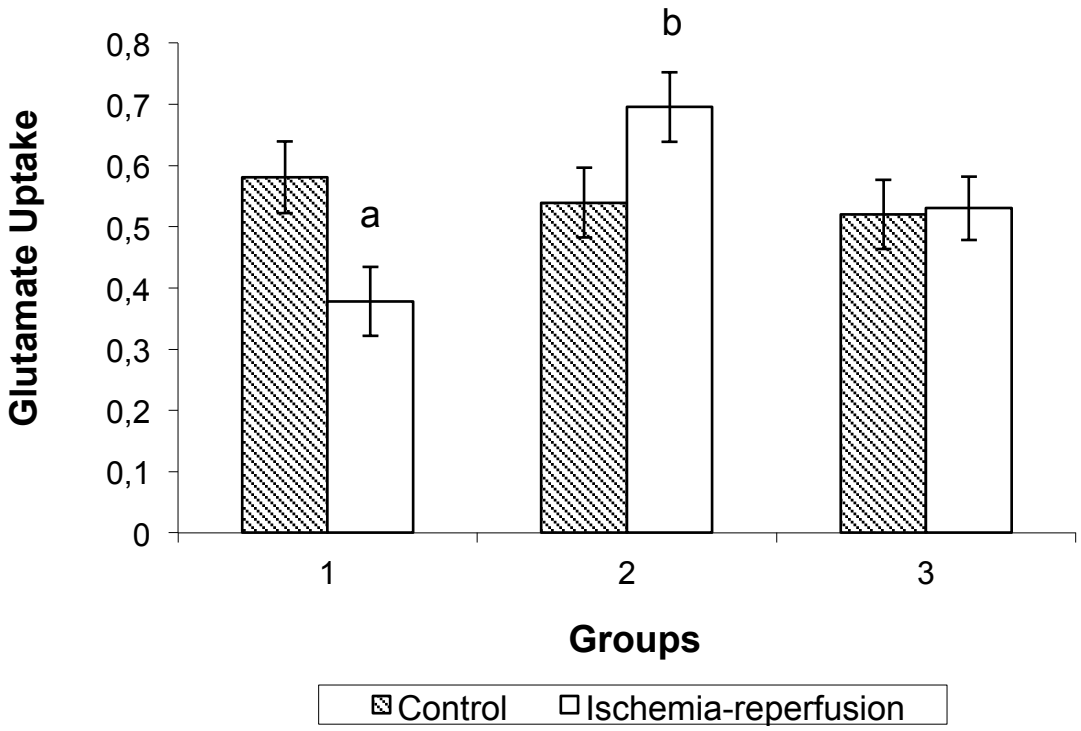


Table 1. Comparisons of glutamate uptake among all groups and subgroups.

Group	Control eyes n = 10	Ischemic-reperfusion eyes n = 10
Group 1: CONTROL	0.580 ± 0.095	0.378 ± 0.091 [(a)]
Group 2: GUA IP	0.539 ± 0.092	0.696 ± 0.092 [(b)]
Group 3: GUA WATER	0.520 ± 0.091	0.530 ± 0.084

Glutamate uptake was expressed in nmol/mg of protein x min (mean±SD). Subgroups: [(a)] [(b)] mean significant difference between them and among all the other subgroups ($P<0.001$; one-way ANOVA followed by Tukey *post hoc* test).

Graphic 1



Glutamate uptakes were measured in nmol/mg of protein x min, and values are expressed here as means with 95% confidence intervals. Groups: 1 = CONTROL; 2 = GUA IP; 3 = GUA WATER. Subgroups: [(a) (b)] These subgroups differed (statistically significant) from each other and from the rest of the subgroups tested.(P<0.001; one-way ANOVA followed by Tukey *post hoc* test)

CONSIDERAÇÕES FINAIS

No artigo 1, estudamos o endotélio normal de ratos Wistar, avaliando as células endoteliais quanto à morfologia, à área celular e à densidade celular por mm^2, concluindo-se que tais características são bastante parecidas com as encontradas em outras espécies de vertebrados. As informações geradas neste estudo foram de grande utilidade para que pudéssemos conhecer o endotélio normal dos ratos Wistar e, assim, compará-lo com o endotélio submetido a condições de I-R.

Esta comparação do endotélio normal com o endotélio de olhos submetidos a condições de I-R foi testada no artigo 2. Assim, o endotélio corneano nos permitiu avaliar o desempenho e a reprodutibilidade do modelo experimental de I-R ocular que desenvolvemos, baseado na elevaçãoo reversível da PIO. Os resultados deste estudo revelaram que, de fato, as células endoteliais corneanas sofreram lesões nos olhos submetidos a I-R com o aumento reversível da PIO.

No terceiro e último artigo desta série, utilizamos o nosso modelo de I-R ocular para testar o efeito da GUA na captação de GLU pela retina, verificando-se que os olhos submetidos a I-R dos animais que não receberam GUA, apresentaram diminuição na captação de GLU. Esta diminuição não ocorreu nos olhos de animais que ingeriram GUA na água e ocorreu, inclusive, uma elevação da captação de

GLU nos olhos dos animais que receberam GUA via intraperitoneal. Com base nestes resultados, acreditamos que a GUA tenha estimulado a captação de GLU pelas células de Müller na retina, o que pode ser interpretado como um indício preliminar de um possível efeito neuroprotetor da GUA para as células retinianas em condições de I-R ocular. Este potencial papel neuroprotetor da GUA na retina está em concordância com o já demonstrado papel neuroprotator da GUA no SNC. Portanto, acreditamos que seja interessante dar continuidade à investigação dos efeitos da GUA na captação de GLU na retina e no nervo óptico em condições de I-R ocular.