UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

METRONIDAZOL NA ANGIOGÊNESE CORNEAL EM RATOS (*Rattus norvegicus*, variação *albinus, Wistar*)

Flor Diana Yokoay Claros Chacaltana

Médica Veterinária

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Orientador: Prof. Dr. José Luiz Laus

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para obtenção do título de Doutor em Cirurgia Veterinária.

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Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

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DADOS CURRICULARES DO AUTOR

Flor Diana Yokoay Claros Chacaltana nascida em 28 de novembro de 1984 na cidade de Lima, Perú, filha de Jesús Manuel Claros Muñoz e Flor Esther Melchora Chacaltana Tanloc. Graduou-se em Medicina Veterinária pela Universidad Alas Peruanas (Lima-Perú) em 2007. Atuou como médica veterinária na Clínica Veterinária Walac (Lima-Perú) até agosto de 2008. Em março 2011 ingressou no curso de Mestrado do Programa de Pós-Graduação em Ciências Veterinárias da Universidade Federal do Rio Grande do Sul, RS/Brasil, sob orientação do professor doutor João Antonio Tadeu Pigatto, concluindo-o em fevereiro de 2013. Deu início ao curso de Doutorado no programa Pós-graduação em Cirurgia Veterinária da Universidade Estadual Paulista, Câmpus Jaboticabal, SP/Brasil, em março de 2014, sob orientação do professor doutor José Luiz Laus. Realizou doutorado sanduiche na “University of Illinois Urbana-Champaign” no 2017, sob orientação da professora Bianca Da Costa Martins.

Voici mon secret. Il est très simple: on ne voit bien qu'avec le cœur. L'essentiel est invisible pour les yeux.

Eis o meu segredo: só se vê bem com o coração. O essencial é invisível aos olhos.

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CERTIFICADO

Certificamos que o Protocolo nº 06174/14 do trabalho de pesquisa intitulado **"Metronidazol na angiogênese corneal em ratos (*Rattus norvegicus*, variação *albinus, Wistar*)"**, sob a responsabilidade do Prof. Dr. José Luiz Laus está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 08 de maio de 2014.

Jaboticabal, 08 de maio de 2014.

Prof.ª Dr.ª Paola Castro Moraes
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LISTA DE ABREVIATURAS

ANOVA	análise de variância.
ARVO	*Association for Research in Vision and Ophthalmology*.
AVG rank	posição de um valor especificado em um conjunto de dados
CEUA	Comissão de Ética no Uso de Animais.
CNV	corneal neovascularization
FGF	fator de crescimento de fibroblastos.
FGFb	fator de crescimento fibroblástico básico
G1	grupo 1
G2	grupo 2
G3	grupo 3
G4	grupo 4
GFs	fatores de crescimento.
KW	Kruskal–Wallis.
Max	valor máximo
Met_0.1%	grupo tratado com colírio de metronidazol 0,1%.
Met_0.5%	grupo tratado com colírio de metronidazol 0,5%.
Min	valor mínimo
PBS	tampão fosfato salino
PC	pontos que tocam as córneas
PDGF	fator de crescimento derivado de plaquetas
PV	pontos que tocam os vasos.
SD	standard deviation
± sd	± standard deviation
Sham	grupo placebo, tratado com tampão fosfato salino.
Untreated	grupo não tratado.
VEGFA	fator de crescimento endotelial vascular A.
VEGF	fator de crescimento endotelial vascular.
X	mediana

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METRONIDAZOL NA ANGIOGÊNESE CORNEAL EM RATOS
(*Rattus norvegicus*, variação *albinus, Wistar*)

RESUMO – Visou-se avaliar os efeitos do metronidazol sobre a neovascularização corneal. A pesquisa foi dividida em dois artigos. No primeiro, compararam-se modelos de angiogênese corneal induzida por cauterização alcalina. Vinte e quatro ratos *Wistar* foram distribuídos em quatro grupos, que diferiram apenas quanto aos procedimentos para cauterização alcalina. Um fragmento circular de papel filtro de 3mm, embebido em solução aquosa de nitratos de prata e de potássio (3:1, vol/vol), foi pressionado sobre a região axial da córnea (olho direito). Os tempos de contato do papel com a córnea foram de 10 segundos para os grupos 1 e 4 (G1 e G4) e de 20 segundos para os grupos 2 e 3 (G2 e G3). Após cauterização, as córneas foram lavadas, por 1 min. Em G1 e em G2 os papéis filtro foram removidos imediatamente antes da lavagem. Em G3 e em G4, as córneas foram lavadas com os papeis filtro *in loco*. Parâmetros de neovascularização corneal foram estudados em diferentes momentos pós-cauterização. Diferenças foram significativas quando $p < 0,05$. As córneas em G1 apresentaram menos áreas vascularizadas (12,63 ± 12,59%), em comparação às córneas em G3 (41,95 ± 17,32%) e ém G4 (33 ± 11,74%) ($p < 0,05$). Os protocolos adotados para G2, G3 e G4 mostraram excelente reprodutibilidade, com 100% de córneas vascularizadas. No segundo, objetivou-se monitorar efeitos de instilações de soluções oftálmicas de metronidazol, 0,1% e 0,5%, sobre a neovascularização corneal, induziram-se lesões por cauterização alcalina em córneas (olho direito) de 40 ratos *Wistar* como descrito para G3. Após cauterização, os ratos foram distribuídos em quatro grupos (Met_0.1%, Met_0.5%, Sham, e não tratado). Os grupos Met_0.1% e Met_0.5% receberam, por instilação, soluções de metronidazol ao 0,1% ou de 0,5%, respectivamente, à intervalos de 6 horas, por 30 dias. O grupo Sham recebeu tampão fosfato salino (diluente de metronidazol). O grupo não tratado não recebeu qualquer instilação. Córneas em todos os grupos foram avaliadas em diferentes momentos pós-cauterização, quanto às intensidades das queimaduras, aos índices de neovascularização e aos percentuais de áreas vascularizadas. Nos dias 15 e 30, cinco ratos de cada grupo foram submetidos à eutanásia, para colheita de córneas que foram avaliadas à histopatologia. Diferenças foram significativas quando $p < 0,05$. Os índices de neovascularização corneal calculados para os grupos Met_0.1% e Met_0.5% foram menores que os obtidos para os grupos Sham e não tratado ($p < 0.05$). Córneas tratadas com 0,1% ou 0,5% de metronidazol apresentaram menos áreas vascularizadas. Há como admitir que a instilação de soluções de metronidazol, 0,1% ou 0,5%, inibiu o crescimento de vasos e a progressão de neovascularização corneal, em ratos albinos de laboratório.
Palavras-chave: cauterização alcalina, neovascularização corneal, metronidazol, rato

METRONIDAZOLE IN CORNEAL ANGIOGENESIS IN RATS
(*Rattus norvegicus*, Albinus variation, *Wistar*)

ABSTRACT- The effect of metronidazole on corneal neovascularization were evaluated. The research was divided into two articles. In the first, models of corneal angiogenesis by alkaline cauterization were developed. Twenty-Four Wistar rats were divided into four groups, which differed in procedures for alkaline cauterization. A circular piece of filter paper of 3mm, soaked in a solution of silver and potassium nitrates (3:1, vol/vol) was pressed onto the axial region of the cornea (right eye). Cauterization times were 10 (G1 and G4), or 20 seconds (G2 and G3). After cauterization, the corneas were washed or 1 min. In G1 and G2, the filter papers were removed just prior to washing. In G3 and G4, the corneas were washed with the filter paper in loco. The parameters of corneal neovascularization were studied at different moments of after cauterization. Differences with $p < 0.05$ were considered significant. On day 15, G1 corneas showed smaller vascularized areas (12.63 ± 12.59%) compared to those in the G3 (41.95 ± 17.32%) and G4 (33 ± 11.74%) groups ($p < 0.05$). The G2, G3, and G4 protocols showed excellent reproducibility, and induced vascularization in 100% of corneas. In the second, in order to monitor the effects of 0.1% and 0.5% instillations of metronidazole ophthalmic solutions on corneal neovascularization. After cauterization, rats were distributed in four groups (Met_0.1%, Met_0.5%, Sham, and untreated). The groups Met_0.1% and Met_0.5% received, by instillation, solutions of 0.1% and 0.5% metronidazole solution, respectively, at intervals of 6 hours, for 30 days. The Sham group received phosphate-buffered saline (metronidazole diluent). Untreated group received no treatment or instillation. The corneas in all groups were evaluated at different post-cauterization moments, regarding burn intensitiy, indices of CNV and percentages of vascularized areas. On days 15 e 30 after the burn, five rats from each group were euthanized for the harvest of corneas which were processed for histopathological examinations. Differences with $p < 0.05$ were considered significant. However, the indices of corneal neovascularization (CNV) for Met_0.1% and Met_0.5% groups were lower than those for Sham and untreated groups ($p < 0.05$). Furthermore, corneas treated with 0.1% or 0.5% metronidazole have fewer vascularized areas compared to control corneas. We can admit that regular instillation of 0.1% or 0.5% metronidazole inhibits blood vessel growth and progression of neovascularization in alkali-burned corneas of laboratory albino rats.

Key words: alkaline cauterization, corneal neovascularization, metronidazole, rat

CAPÍTULO 1 – Considerações Gerais

1 INTRODUÇÃO

A neovascularização corneal é condição patológica que ocorre em resposta a injúrias, como as infecciosas e as inflamatórias. Caracteriza-se pela gênese e o crescimento de vasos sanguíneos na córnea. Quando não controlada, a neovascularização corneal ocasiona déficil visual importante.

Relativamente aos mecanismos de neovascularização corneal, grande parte do conhecimento advém de estudos envolvendo modelos experimentais de angiogênese patológica induzida. Há, em literatura, diferentes protocolos para indução de lesões corneais que culminam em neovascularização. O mais difundido deles é o de cauterização alcalina pontual. No entanto, a cauterização alcalina pontual está associada com padrões variáveis de resposta vascular, a depender de variantes metodológicas, que incluem o formato do instrumento cauterizante e o tempo de exposição da córnea a ele. Para assegurar homogeneidade e evitar *viéses* em estudos comparativos sobre neovascularização corneal, notadamente nos que envolvem fármacos ou procedimentos cirúrgicos em teste, recomenda-se que o modelo experimental seja validado.

As diferentes modalidades farmacológicas e cirúrgicas, atualmente disponíveis para tratamento da neovascularização corneal, não são totalmente efetivas e estão associadas com índices variáveis de sucesso clínico. Relativamente ao uso de fármacos, congregam-se, entre outros, os agentes anti-inflamatórios e os biofárrmacos. Enquanto os anti-inflamatórios estão associados com efeitos adversos, os biofármacos são de uso *off label*. Há, por conseguinte, a necessidade científica e econômica pela descoberta de novos fármacos, capazes de modularem vias anti-angiogênicas em córneas neovascularizadas.

O metronidazol pertence à classe dos nitroimidazóis e é empregado, inclusive em oftalmologia, no tratamento de infecções causadas por bactérias anaeróbias e por protozoários. Há evidências de que ele pode modular a angiogênese patológica. Todavia, estudos prévios conduzidos em tecidos não oculares resultaram em achados controversos. Os efeitos de colírios de metronidazol sobre a neovascularização

corneal não são conhecidos, porém, podem ser investigados em modelos experimentais de angiogênese.

No presente estudo, avaliaram-se padrões de resposta vascular decorrentes de diferentes procedimentos para cauterização alcalina em córneas de ratos. Ademais, monitoraram-se efeitos de instilações regulares de colírios de metronidazol, 0,1% e 0,5%, sobre a gênese e o crescimento de vasos em modelos experimentais de angiogênese corneal.

2 REVISÃO DE LITERATURA

2.1 Metronidazol

O metronidazol é um fármaco sintético de ação antimicrobiana, derivado heterocíclico do 5-nitroimidazol (VAN DER BIJL et al., 2004; KODYM et al., 2011; DE CASTRO; DE GOUVEIA SANTOS, 2015) que contém, em sua estrutura, um radical NO_2 ligado a um núcleo de cinco átomos (Figura 1). No Brasil, o metronidazol representa o fármaco antimicrobiano mais prescrito para uso sistêmico (GONÇALVES; HEINECK, 2016). Na Inglaterra, em 2015, o metronidazol foi o fármaco antimicrobiano mais prescrito para usos tópico e sistêmico (CHAPLIN, 2016).

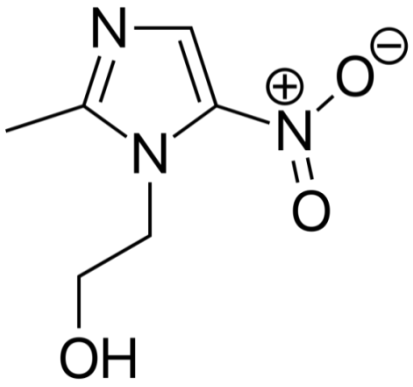

Figura 1. Estrutura química do metronidazol. *National Institutes of Health, Health & Human Services.*

A penetração do metronidazol na célula alvo ocorre por difusão passiva, sob a forma de pró-fármaco, cuja ativação se dá por processos de redução no citoplasma

bacteriano ou, no caso dos protozoários, em organelas específicas. A redução intracelular do metronidazol gera um grupo nitro de curta duração, que atua como receptor de elétrons. Por possuir efeitos citotóxicos, o grupo nitro enseja desestabilização e quebra da dupla hélice de DNA, o que desencadeia a inibição da síntese de ácidos nucléicos e gera compostos citotóxicos, causando morte celular (PÉREZ-TRALLERO; IGLESIAS, 2003; LÖFMARK; EDLUND; NORD, 2010).

Estudos mostraram que o metronidazol é hábil em penetrar células ou tecidos tumorais; portanto, ele tem sido explorado como agente quimioterápico e antimicrobiano (QIAN et al., 2010). Outro campo de aplicação refere-se ao controle do odor em feridas (DA COSTA SANTOS; DE MATTOS PIMENTA; NOBRE, 2010; DE CASTRO; DE GOUVEIA SANTOS, 2015). Ademais, trata-se de fármaco com potencial antioxidante, eficaz no tratamento de feridas por queimadura e infectadas (RAO et al., 2002). Em oftalmologia, o metronidazol, na forma de colírio, está recomendado em infecções causadas por bactérias anaeróbias e protozoarios. Colírio de metronidazol 0,5%, associado com outros fármacos antiprotozoários, é também utilizado em ceratites por *Acanthamoeba* na medicina humana (VAN DER BIJL et al., 2004; KODYM et al., 2011).

Evidências indicam que o metronidazol é capaz de estimular a contração de miofibroblastos, a migração e a proliferação de células epiteliais, acelerando reparação tecidual (RAO et al., 2002; NICHOLSON; ARMSTRONG, 2004; SAMPAIO et al., 2009). Efeitos anti-inflamatórios também têm sido a ele atribuídos (NISHIMUTA; ITO, 2003; YPSILANTIS; CARAPETI; CHAN, 2015). Estudos *in vitro* mostraram que o metronidazol inibe mediadores inflamatórios gerados por neutrófilos, reduzindo danos oxidativos teciduais (MCCLELLAN; NOBLE, 2000).

Alguns pesquisadores atribuíram ao metronidazol efeitos moduladores importantes sobre a angiogênese tecidual. Todavia, há controversas e ainda não se estabeleceram, com níveis suficientes de evidência, se o metronidazol possui ação pró-angiogênica ou anti-angiogênica ou ambas (dependendo do tecido alvo). Pezo (2012) percebeu que o fármaco, quando aplicado a membrana corioalantóica de frangos, ensejou efeitos anti-angiogênicos. Michalska et al. (2011), ao estudarem os efeitos da combinação de metronidazol com clindamicina sobre tumores ginecológicos benignos, observaram que a angiogênese foi inibida. No entanto, Sampaio et al.

(2009), ao estudarem os efeitos do metronidazol sobre a cicatrização de feridas por segunda intenção em ratos, observaram aumento na produção de colágeno e promoção de angiogênese.

2.2 Angiogênese

O termo angiogênese foi cunhado por HERTIG, em 1935, para descrever a formação de novos vasos sanguíneos na placenta (SAFATLE et al., 2002). Atualmente, conceitua-se angiogênese (ou neovascularização) como o crescimento de novos capilares a partir de capilares e vênulas pré-existentes (KWON et al., 2005; AMBATI et al., 2006; ELLENBERG et al., 2010). Sob condições fisiológicas adequadas, ela se manifesta como um processo molecular orquestrado (Figura 2 A). A angiogênese é essencial durante o desenvolvimento embrionário e fetal. Em adultos, ela ocorre durante a cicatrização de lesões, o crescimento do esqueleto, o ciclo menstrual e a gravidez, bem como em doenças específicas, incluindo desordens intraoculares neovasculares, artrite reumatoide imunogênica, psoríase e tumorigênese (SAFATLE et al., 2002; BROWN; HUDLICKA, 2003; OLIVEIRA et al., 2010; CHUNG; FERRARA, 2011). O crescimento de capilares também ocorre em resposta à atividade física, notadamente no coração e no músculo esquelético. Também em tecidos musculares esqueléticos, a angiogênese pode representar adaptação fisiológica a certas condições prolongadas de hipóxia ou de baixas temperaturas (BROWN; HUDLICKA, 2003).

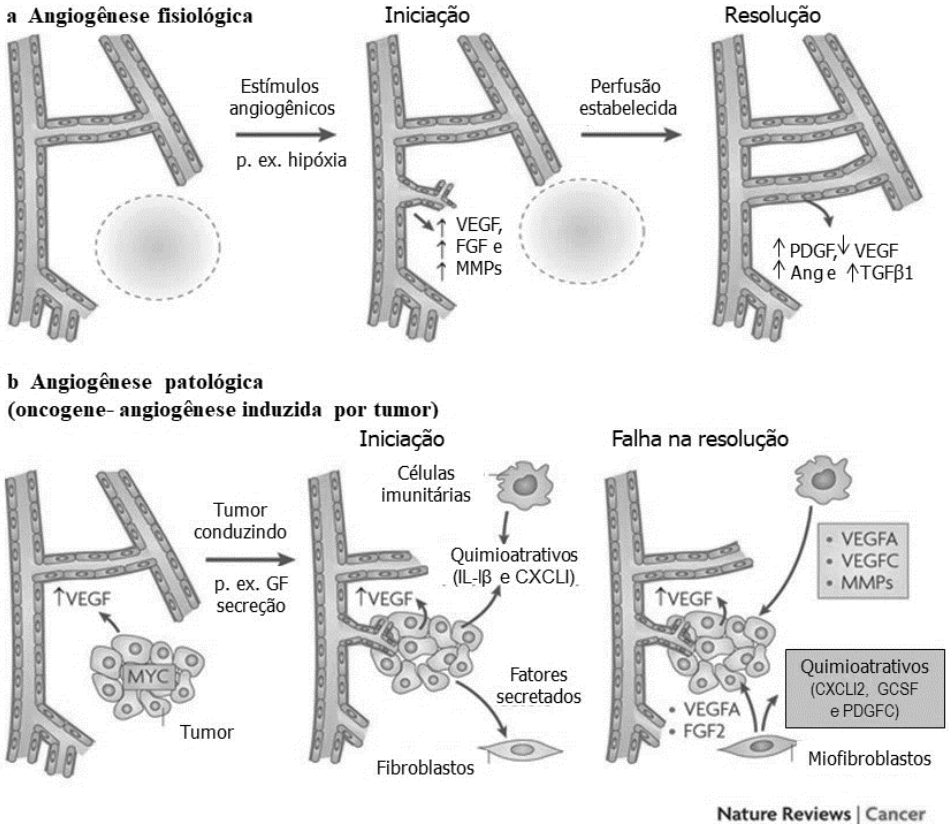

Figura 2. Angiogênese fisiológica e angiogênese patológica. (a), sob condições fisiológicas adequadas, alguns estímulos, como a hipóxia (---), podem ativar a cascata angiogênica. Na fase de iniciação, alguns fatores de crescimento (GFs), como o fator de crescimento endotelial vascular A (VEGFA) e o fator de crescimento de fibroblastos (FGF), ensejam aumento das células endoteliais e desestabilização do vaso sanguíneo, o que inicia a germinação de um novo vaso e a proliferação endotelial vascular. No estabelecimento e perfusão do novo vaso, os níveis de VEGF diminuem e a fase de resolução resulta coincidente com um aumento no fator de crescimento derivado de plaquetas (PDGF), entre outros. (b) sob condições patológicas, como na neovascularização tumoral impulsionada por oncogenes, o VEGFA secretado pelo tumor pode ativar a cascata angiogênica. O oncogene MYC, por exemplo, expresso em células tumorais, eleva a expressão de fatores solúveis envolvidos na ativação de fibroblastos residentes e responsáveis por atrairem células do sistema imunológico para o sítio angiogênico (Modificado de CHUNG; LEE; FERRARA, 2010).

A angiogênese é requerida para a cicatrização de lesões e estimulada em processos inflamatórios (SAFATLE et al., 2002; OLIVEIRA et al., 2010). Quando descontrolada, representa condição patológica que contribui para o estabelecimento e a propagação de neoplasias (Figura 2B), bem como para o desenvolvimento de doenças inflamatórias, de neovascularização corneal, de placas ateroscleróticas, de hemangiomas, de endometriose e de obesidade, entre outras (OLIVEIRA et al., 2010).

O equilíbrio na expressão de fatores pró- e anti-angiogênicos é o responsável pela regulação da angiogênese. Quando há estímulo pró-angiogênico, as células endoteliais alteram-se na sua forma, provocam vasodilatação, degradam a membrana basal endotelial, proliferam e migram, formando capilares (AUSPRUNK; FOLKMAN, 1977; SAFATLE et al., 2002). Este padrão de crescimento tem sido bem documentado sob condições normais e patológicas (BROWN; HUDLICKA, 2003).

A angiogênese patológica compreende muitos dos eventos da angiogênese fisiológica (CHUNG; FERRARA, 2011). Em ambas, uma cascata de eventos celulares impulsiona o estabelecimento de novos vasos em resposta à crescente demanda por oxigênio e por nutrientes. Uma grande diferença entre a angiogênese fisiológica e a patólógica é que a última não atinge a resolução, após o estabelecimento da perfusão vascular. A cascata da angiogênese patológica é persistente, não resolvida, e “alimentada” pela condição gênese patológica (CHUNG; FERRARA, 2011). Sob condições que envolvem inflamação, como crescimento de tumores e aterosclerose, por exemplo, o crescimento de novos vasos está frequentemente associado com resultados clínicos negativos (JETTEN et al., 2014).

2.3 Modelos Biológicos para Estudos sobre Angiogênese

Modelos biológicos são essenciais para a investigação de mecanismos associados à angiogênese, bem como para se avaliarem os efeitos de fármacos. Não há consenso sobre o modelo ideal para se estudar a modulação angiogênica (NORRBY, 2006). Uma vez que tecidos orgânicos são heterogêneos e que reações celulares envolvidas com a angiogênese são complexas, a escolha pelo modelo deve considerar os objetivos da pesquisa e o potencial do fármaco modulador. Fármacos anti-angiogênicos, importantes na terapia clínica de tumores e da degeneração macular, por exemplo, devem ser estudados em tecidos avasculares (NORRBY, 2006).

Dentre os modelos biológicos empregados em estudos sobre a angiogênese destacam-se os tecidos transparentes, por permitirem fácil visibilização (muitas vezes sem necessidade de procedimentos invasivos) dos vasos em formação (ROGERS; BIRSNER; D´AMATO, 2007). Para se estudarem os mecanismos de angiogênese

patológica, os modelos mais indicados são o mesentério, a cartilagem auricular, a membrana corioalantóica de frango e a córnea (COCKERILL; GAMBLE; VADAS, 1995; KENYON et al., 1996). Para se estudarem os mecanismos de angiogênese fisiológica associada ao desenvolvimento, organismos semitransparentes, como os alevinos do peixe-zebra (*Danio rerio*) e os girinos da rã de unhas africana (*Xenopus laevis*), podem ser utilizados (NORRBY, 2006).

2.4 Córnea

A córnea é a janela transparente do olho e essencial à acuidade visual. Ela representa 1/5 da túnica fibrosa dos olhos de animais domésticos. Sua espessura varia de acordo com a espécie. Nos ratos, a córnea tem entre 250–255 µm de espessura (NORRBY, 2006). Em mamíferos, com exceções, ela é formada, do meio externo para o interno, pelo filme lacrimal pré-corneal, epitélio, estroma, membrana de Descemet e endotélio (SLATTER, 2005).

Os fatores que mantêm a transparência corneal são bem conhecidos. Destacam-se a ausência de vasos sanguíneos e de pigmentos, a presença de epitélio anterior não queratinizado e de fibras nervosas não mielinizadas, o grau de organização e o pequeno diâmetro das fibras colágenas estromais, somados à relativa deturgescência do estroma (WHITLEY e GILGER, 1999; TEIXEIRA; BARROS; BARROS, 2009).

Qualquer insulto à córnea, que resulte em reações inflamatórias, não só pode ensejar destruição de tecido e formação de cicatrizes, mas também está associado à angiogênese e à linfangiogênese corneal. Portanto, na atualidade, a córnea representa um dos principais modelos para estudos sobre os mecanismos de angiogênese patológica (CURSIEFEN, 2007). Ademais, ela tem sido amplamente empregada em pesquisas que visam a investigar os efeitos clínicos de moléculas supostamente pró-angiogênicas ou anti-angiogênicas (CHO et al., 2009). A córnea, por suas caraterísticas macroscópicas (transparência) e localização, permite o monitoramento e a quantificação não invasiva, por biomicroscopia, da resposta vascular (SAFATLE et al., 2002).

2.5 Avascularidade Corneal

No curso da evolução dos animais vertebrados, a córnea dos mamíferos desenvolveu mecanismos para a prevenção e a modulação de reações inflamatórias e de estímulos pró-angiogênicos. A capacidade que a córnea tem de se manter avascular, na ausência de enfermidades, é designada de "privilégio angiogênico da córnea". Tal designação surge em paralelismo com o termo "privilégio imune corneal", o qual indica que a córnea é imuno-privilegiada e possui mecanismos que minimizam, por exemplo, o risco de rejeição à enxertia de materiais ou à transplantação de tecido corneal histologicamente incompatível (CURSIEFEN, 2007).

"O privilégio angiogênico corneal" é adquirido ainda no estágio fetal (CURSIEFEN, 2007). A manutenção da avascularidade corneal está parcialmente associada à baixa expressão de fatores pró-angiogênicos e à alta expressão de fatores anti-angiogênicos, sob condições fisiológicas basais (CHANG et al., 2001; SENTURK et al., 2016).

2.6 Neovascularização Corneal

Processos inflamatórios graves (CURSIEFEN, 2007) e certas doenças infecciosas, degenerativas ou traumáticas, podem resultar em perda do "privilégio angiogênico", ocasionando edema, deposição lipídica, formação de cicatrizes, vascularização e opacificação corneais (CHANG et al., 2001; SAMOLOV et al., 2005; BOCK et al., 2007). A neovascularização da córnea não só reduz a acuidade visual, como resulta na perda do privilégio imunogênico e em repercussões desfavoráveis, notadamente em pacientes que passaram por transplante de córnea (DASTJERDI et al., 2009). A angiogênese envolve o surgimento de novos vasos, a partir de capilares e de vênulas do plexo pericorneal (CHANG et al., 2001). O suprimento sanguíneo para a córnea emerge das artérias ciliares, que são ramos da artéria oftálmica que se dividem e terminam no plexo pericorneal na região do limbo. Os padrões de neovascularização corneal podem ser classificados em três categorias, segundo os aspectos clínicos: neovascularização profunda sobrejacente à membrana de Descemet, como ocorre em ceratite herpética; neovascularização estromal associada

à ceratite estromal; e neovascularização relacionada ao pannus vascular, por proliferação de tecido conjuntivo (AZAR, 2006; ELLENBERG et al., 2010). Os mecanismos que determinam se a neovascularização associada à inflamação atingirá áreas superficiais ou profundas da córnea, ou ambas, não são totalmente conhecidos (SAMOLOV et al., 2005).

Embora a neovascularização possa envolver diferentes profundidades ou áreas do estroma corneal, há evidências de que os botões corneais vascularizados formam-se principalmente nos terços superior e médio do estroma anterior (CHANG et al., 2001). Os botões corneais neovascularizados caracterizam-se por apresentarem concentrações elevadas do fator de crescimento endotelial vascular, comparativamente ao tecido corneal normal (HABOT-WILNER, et al., 2010).

A cinética de crescimento dos vasos sanguíneos que invadem a córnea pode ser didaticamente dividida em fases. Na fase que se inicia logo após o estímulo, ocorre liberação de fatores de crescimento vascular, os quais que se ligam a receptores específicos situados nas membranas das células endoteliais, em vasos pré-existentes. As células endoteliais mais sensíveis aos fatores angiogênicos são as das vênulas pós-capilares e as das vênulas terminais (POLVERINI, 1995).

Um dos eventos mais importantes da resposta angiogênica inicial é a ruptura dos contatos focais entre as células endoteliais, os pericitos e as células musculares lisas (POLVERINI, 1995). Desde a vênula mãe, as células endoteliais “ativadas” apresentam alterações na expressão de moléculas de adesão célula-célula ou célula-matriz, e reorganizam elementos do citoesqueleto. Além disso, elas produzem enzimas proteolíticas que degradam a matriz extracelular e as permitem migrar longas distâncias (POLVERINI, 1995; ELLENBERG et al., 2010).

O estágio inicial da resposta angiogênica pode evoluir sem proliferação endotelial, resultando na formação de papilas capilares que continuarão a crescer e a amadurecer, a menos que a cascata pró-angiogênica seja interrompida. Seguindo-se os acontecimentos migratórios iniciais, fases finais da gênese de microvasos incluem a expressão de citocinas e de fatores de crescimento específicos de células endoteliais. Uma vez formados, os novos capilares persistirão pelo tempo em que as demandas metabólicas do tecido exigirem, podendo permanecer como capilares ou se diferenciar em vênulas maduras ou em arteríolas (POLVERINI, 1995).

A abordagem terapêutica da neovascularização corneal pode envolver procedimentos de fotocoagulação a *laser*, diatermia, terapia fotodinâmica e enxertia de membrana amniótica, além do uso de fármacos esteroides e não esteroides, de sulfato de heparan de baixo peso molecular (HOSSEINI et al., 2007), e de biofármacos como a Rapamicina (KWON et al., 2005; HOSSEINI et al., 2007), o Bevacizumab (HOSSEINI et al., 2007; BARROS; BELFORT, 2007; KIM et al., 2008; PAPATHANASSIOU et al., 2008; DASTJERDI et al., 2009; KANG & CHUNG, 2010), o Pegaptanib (NG; ADAMIS, 2006) e o Pazopanib (AMPARO et al., 2013), entre outros. Não há consenso quanto ao melhor tratamento (HOSSEINI et al., 2007; KANG & CHUNG, 2010). Relativamente aos biofármacos, apesar dos resultados aparentemente bons, eles são de uso *off label* e possuem custo elevado, o que restringe a sua prescrição a uma pequena parcela da população. Há, portanto, necessidade científica e econômica de se descobrirem novos fármacos capazes de modular a angiogênese corneal. Visando-se à condução de estudos com tal escopo, consideram-se protocolos para indução de lesões que culminem com a angiogênese corneal.

2.7 Angiogênese Corneal Experimental

Protocolos para indução de angiogênese experimental em córneas incluem a produção de microbolsa para implantação intraestromal de *pellets* contendo o fator de crescimento fibroblástico básico (FGFb) (KENYON et al., 1996; ROGERS; BIRSNER; D´AMATO, 2007); a enxertia intraestromal de membranas biológicas, como o pericárdio e a amniótica (SAFATLE et al., 2002); a confecção de sutura corneal com nylon (CHO et al., 2009; PÉREZ-SANTONJA et al., 2013); as injeções intraestromais de algumas substâncias químicas; a implantação de células tumorais (KENYON et al., 1996); e cauterização alcalina pontual (HOSSEINI et al., 2007; PAPATHANASSIOU et al., 2008; AKAR et al., 2013; GIACOMINI et al., 2014; PISO et al., 2014).

Dos protocolos acima mencionados, os mais utilizados são a cauterização alcalina e a sutura corneal, notadamente pelo custo acessível e os baixos riscos de complicações, como perfuração da córnea. No entanto, tais protocolos, principalmente

o de cauterização alcalina pontual, possuem inúmeras variantes metodológicas (HOSSEINI et al., 2007; PAPATHANASSIOU et al., 2008; AKAR et al., 2013; GIACOMINI et al., 2014; PISO et al., 2014), as quais ensejam diferentes padrões de resposta vascular e dificultam a reprodutibilidade entre estudos (NORRBY, 2006; GIACOMINI et al., 2014). Enquanto alguns autores conseguiram criar modelos homogêneos de angiogênese corneal por cauterização alcalina, outros não lograram mesmo êxito (NORRBY, 2006).

3. HIPÓTESES

Duas hipóteses alicerçaram a realização do estudo. A primeira (Capítulo 2) é a de que variações nos procedimentos para cauterização alcalina pontual, notadamente nos tempos de exposição da córnea ao agente cauterizante, induzem diferentes padrões de resposta vascular. A segunda é a de que a instilação regular de colírio de metronidazol inibe a neovascularização corneal (Capítulo 3).

4. OBJETIVOS

Avaliarem-se padrões de resposta vascular associados a diferentes procedimentos para cauterização alcalina em córneas de ratos e monitorarem-se efeitos das instilações regulares de colírios de metronidazol, 0,1% e 0,5%, sobre a gênese e o crescimento de vasos em modelos experimentais de angiogênese corneal, em ratos.

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CAPÍTULO 2 – Artigo Científico[1]

Corneal Angiogenesis Based on Different Protocols of Alkaline Cauterization in Murine Models

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ABSTRACT

Purpose: Establish and compare protocols of alkaline cauterization for inducing corneal angiogenesis in murine models.

Methods: Twenty-four adult Wistar rats were distributed into four groups (G1, G2, G3, and G4). The right eye cornea from each rat was cauterized using filter paper (3 mm), soaked in a solution of silver and potassium nitrates (3:1). Cauterization times were 10 (G1 and G4), or 20 seconds (G2 and G3). Cauterized corneas were washed with Ringer's lactate solution. The filter paper was either removed before washing (G1 and G2), or kept on the corneas (G3 and G4). Corneas were photographed at multiple time points (2, 4, 6, 8, 11, 13, and 15 days after the procedure), and neovascularization parameters were assayed.

Results: Neovascularization was observed in 66% of G1 corneas, and 100% of G2, G3, and G4 corneas. On day 15, G1 corneas showed smaller vascularized areas (12.63 ± 12.59%) compared to those in the G3 (41.95 ± 17.32%) and G4 (33 ± 11.74%) groups ($P < 0.05$).

Conclusions: The silver and potassium nitrate solution effectively induced corneal angiogenesis. The G2, G3, and G4 protocols showed excellent reproducibility, and induced vascularization in 100% of corneas.

Keywords: Angiogenesis; alkaline cauterization; rat; cornea.

INTRODUCTION

Angiogenesis represents the formation of new blood vessels from the preexisting vasculature.[1-3] In mammals, angiogenesis occurs during embryonic development and continues, in some tissues and organs, during postnatal life. In adult animals, the angiogenic process is related to the cyclical events of the female reproductive cycle (ovulation and pregnancy). Under controlled physiological conditions, angiogenesis contributes to tissue repair and to inflammatory responses.[4,5]

The molecular mechanisms that regulate angiogenesis change during the development of many pathological conditions, which are collectively known as angiogenesis-dependent. In ophthalmology, for example, the exacerbation of the angiogenic process is associated with the development of many postoperative conditions or complications, such as corneal neovascularization, neovascular glaucoma, central retinal vein occlusion, corneal graft rejection, retinopathy of prematurity, diabetic retinopathy, age-related macular degeneration, and tumor progression.[2,3]

Many studies have focused on the elucidation of the mechanistic basis of angiogenesis, as well as on the development of drugs capable of blocking the progression of angiogenesis-dependent diseases, especially cancer.[2,3,6] Therefore, angiogenesis models are being utilized for in vitro applications, and in pre-clinical trials. The cornea has been widely used as a model for preclinical studies involving pro-angiogenic or anti-angiogenic molecules. Due to its transparency and anatomical location, it facilitates the monitorization of vascular responses, and allows their quantification using biomicroscopy. Different protocols have been proposed for the experimental induction of angiogenesis in the cornea.[6–9]

Considering the heterogeneity of organic tissues and the complexity of the cellular reactions involved in angiogenesis, the choice of the model should always consider the objectives of the study, and the characteristics (e.g., time of action) of the alleged modulator drug being tested.[10]

Protocols involving alkaline cauterization represent a good option for the induction of corneal angiogenesis, given the cost-benefit. However, some authors report unsatisfactory results after using these protocols, as not all corneas develop

angiogenesis. The reproducibility of corneal cauterization protocols using alkali remains controversial. While in some studies, alkaline cauterization appears to be very effective in the induction of corneal neovascularization, in others, the lack of reproducibility in the vascular response represented a limiting factor for the validation of the results.[10]

We hypothesized that variations in the alkaline cauterization steps, especially in the time of exposure of the cornea to the cauterizing agent, and in the type and size of the instrument used for cauterization, could lead to different vascular responses, thus compromising the reproducibility of the inter-observer results. In the literature, corneal cauterization times with alkali using a combination of 75% silver and 25% potassium nitrate range from 8 to 60 seconds.[8,11–16] The cauterizing instrument is usually a stick or filter paper, whose diameter ranges from 1.8 mm to 5 mm[8,11–16].

The aim of this study was to establish and compare the effectiveness of four alkaline cauterization protocols (with silver and potassium nitrate) in the induction of angiogenesis in rat corneas. Several parameters of corneal vascularization were evaluated and compared between the study groups and at different time points after the procedures.

METHODS

Animals and Procedures for Alkaline Cauterization

Twenty-four male rats (*Rattus norvegicus*, Wistar lineage), aged between 3 and 4 months, from the vivarium of the General Administration at São Paulo State University (Unesp), Botucatu, SP, Brazil, were used in this study. As inclusion criteria, the ocular examination included slit-lamp biomicroscopy (Nidek Co, SL-450, Aichi, Japan) and the fluorescein sodium dye test (Ophthalmos, São Paulo, Brazil). All animals were free of signs of eye disease.[17] The rats were divided into four groups (n = 6), designated as G1, G2, G3, and G4. The animals were fed a commercial pellet diet (65.82% carbohydrate, 5.36% fiber, 21.0% protein, and 4.96% fat) and they received water *ad libitum*.

The procedures for the induction of corneal angiogenesis were performed on animals under dissociative anesthesia.[18] Antisepsis was performed, and proxymetacaine eye drops (Alcon, São Paulo, Brazil) were instilled to promote desensitization of the ocular surface.

For alkaline cauterization, a circular piece of filter paper, with a diameter of 3 mm, embedded in a solution of 75% silver nitrate (Synth, Diadema, Brazil) and 25% potassium nitrate (Dinâmica, Contemporary Chemistry Ltda., Diadema Brazil), was pressed onto the axial region of the cornea, in the right eye of each animal. The contact time of the filter paper with the cornea was 10 seconds for the G1 and G4 groups and 20 seconds for the G2 and G3 groups. After cauterization, the corneas were washed with lactated Ringer's solution for 1 minute. In the G1 and G2 groups, the filter papers were removed immediately before washing the cornea. In the G3 and G4 groups, the corneas were washed in the presence of the filter paper. Post-cauterization, the animals subcutaneously received tramadol hydrochloride (5 mg/kg) as an analgesic, every 8 hours, for 10 days.

The eyes were examined after 2, 4, 6, 8, 11, 13, and 15 days post-cauterization, to monitor the vascular response. The examinations were performed after the instillation of anesthetic eye drops. Pupillary dilatation was achieved by instillation of 1% tropicamide (Alcon, São Paulo, Brazil). The fluorescein sodium dye test was also used (Ophthalmos, São Paulo, Brazil). The presence of edema, corneal ulcer, corneal melting, synechiae, and hypopyon were noted. During the clinical evaluations, the corneas were immediately photographed (laterally and frontally), using digital equipment (TRC-50DX, Topcon, Japan) with a green filter (red free) and without a filter.

Side view images of corneas, photographed at different time points during the postoperative evaluation, were studied by a single examiner, who measured the burn intensity.[19] This was scored based on the appearance of blisters and on the extent of their elevation from the corneal surface (0 = no blister; 1 = a small blister that is slightly raised above the surface; 2 = a medium blister that is moderately raised above the surface; 3 = a large blister).

Evaluation of Corneal Vascularization Parameters

Three parameters were evaluated: the development of neovascularization, the neovascularization profile, and the percentage of the corneal area occupied by vessels. Data were collected using frontal profile images of corneas and a green filter (red free). All evaluations were qualitative and quantitative, and were performed by a single examiner.

To assess the development of neovascularization, the extent of new vessel development in the cauterized eyes was graded using the following scores: negative (0), when no new vessels were visible; mild (1), when dense new blood vessels were present at the limbus; moderate (2), when the growth of new blood vessels extended from the limbus towards the periphery of the cauterized site; and severe (3), when new blood vessels reached the cauterized site.[20]

The neovascularization profile data received scores in the 0-3 range per corneal quadrant, with increments of 0.5, using a grid system based on the centripetal extent of the outgrowth of the neovascular branch from the corneoscleral limbus. The scores for each corneal quadrant were summed for each eye, to obtain the index of corneal neovascularization (range, 0-12).[21]

The percentages of the corneal areas that were occupied by vessels were calculated using the ImageJ software (http://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD, USA).[22] A test system containing 110 points was positioned over the photographic images.[9,23] The percentage of the area that was occupied by vessels was calculated with the (PV x 100)/PC formula, where PV represents the number of points that touch the vessels, and PC represents the number of points that touch the corneas.

Statistical Analysis

Quantitative variables were analyzed using parametric [one-way analysis of variance (ANOVA), and repeated measures ANOVA, followed by Tukey's post-hoc test] and non-parametric tests [Kruskal-Wallis (KW), with Dunn’s post-test]. Differences were considered significant when $P < 0.05$. The evaluations were done using the MedCalc statistical software (MedCalc®, Mariakerke, Belgium).

Ethics

This study adhered to the rules of the Association for Research in Vision and Ophthalmology-ARVO (Statement for the Use of Animals in Ophthalmic and Visual Research). The ethical principles established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 2011) were followed. The study protocol was approved by the Ethics Committee on Animal Use (protocol number 06174/14) of FCAV/Unesp, Jaboticabal.

RESULTS

All eyes presented edema at days 2 and 4, during the evaluations. The occurrence of edema declined in subsequent evaluations. One eye in the G2 (16.6%) and one eye in the G3 groups (16.6%) displayed corneal melting in the evaluations at days 4 and 6, respectively. One animal in the G2 group was euthanized. Synechiae were observed in the G3 group, at day 11 (1 eye, 16.6%) and day 15 (2 eyes, 33.3%) of the evaluation. In all groups, some eyes presented corneal ulcer at the initial stages of the evaluation. Two eyes in the G4 group (33.3%) presented hypopyon, during the evaluation at day 2.

Neovascularization (measured as a cumulative rate) was present in 16.66% of the corneas on day 6, 50% of the corneas on day 8, and 66.66% of the corneas on day 11, in the G1 group. In the G2 group, neovascularization was present in 16.66%, 33.33%, 50%, and 100% of corneas on days 2, 4, 8, and 11, respectively. In the G3 group this occurred in 16.66%, 33.33%, 66.66%, and 100% of the corneas on days 2, 6, 11, and 13, respectively, whereas in the G4 group 16.66%, 83.33%, and 100% of the corneas presented neovascularization on days 4, 8 and 11, respectively.

The burn intensities (Figure 1), at the different evaluation times and in the various groups included in the study, are presented in Table 1. Corneas in the G2, G3, and G4 groups showed enhanced burn intensity when compared to the corneas in the G1 group ($P < 0.05$).

Table 1. Mean values with standard deviation, median, minimum, and maximum values, representing the burn intensity gradation in the G1, G2, G3, and G4 groups, on days 2, 4, 6, 8, 11, 13, and 15. Veterinary Ophthalmology Service, Unesp/FCAV, Jaboticabal, SP, Brazil, 2017

Day	G1 (n = 6)				G2 (n = 6)				G3 (n = 6)				G4 (n = 6)			
	Mean ± sd	X	Min	Max	Mean ± sd	X	Min	Max	Mean ± sd	X	Min	Max	Mean ± sd	X	Min	Max
2	0.67 ± 0.52	1	0	1	0.67± 0.52*	1	0	1	1.50 ± 0.84*	1	1	3	1.67 ± 0.82	1.5	1	3
4	0.33 ± 0.52	0	0	1	1.50 ± 1.23	1	0	3	1.33 ± 1.03	1	0	3	1.17 ± 0.99	1	0	3
6	0.17 ± 0.41	0	0	1	1.67 ± 1.21	1.5	0	3	1.83 ± 1.33	2	0	3	1.17 ± 0.99	1	0	3
8	0.33 ± 0.82**	0	0	2	1.33 ± 1.37	1	0	3	2.33 ± 0.82**	2.5	1	3	1.00 ± 1.10	1	0	3
11	0.33 ± 0.52**	0	0	1	1.20 ± 0.84	1	0	2	2.00 ± 1.1**	2	1	3	1.00 ± 1.10	1	0	3
13	0.17 ± 0.41**	0	0	1	1.20 ± 0.84	1	0	2	2.00 ± 0.9**	2	1	3	1.00 ± 1.10	1	0	3
15	0†	0	0	0	1.2- ± 0.84†	1	0	2	1.17 ± 0.41†	1	1	2	0.83 ± 0.75†	1	0	2

*P < 0.05, G2 versus G3

**P < 0.05, G1 versus G3

† P < 0.05, G1 versus G2, G3, and G4

Mean ± sd = mean ± standard deviation; x = median; min = minimum value; max = maximum value

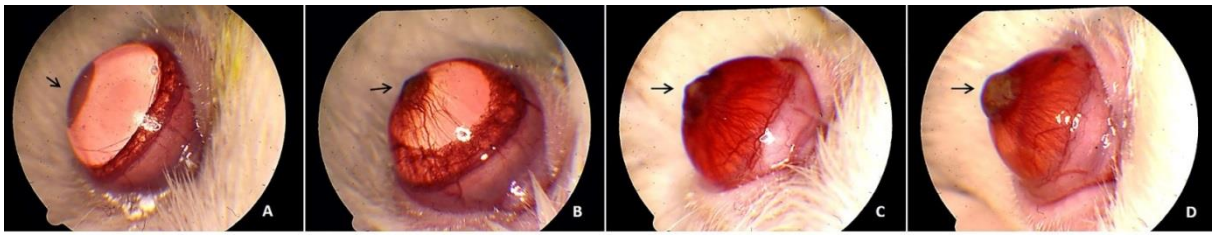

FIGURE 1. Images of corneas with burn intensity secondary to the corneal lesion induced by alkali (arrows). Grade 0 (A), 1 (B), 2 (C), and 3 (D). Veterinary Ophthalmology Service, Unesp/FCAV, Jaboticabal, SP, Brazil, 2017.

In the assessments at days 4, 6, 8, 11, and 13, the development of neovascularization in corneas from all groups was restricted to the limbal region (score 1), or extended from the limbal region to the edges of the cauterized site (score 2) (Table 2).

The results of the corneal neovascularization profiles in all groups and at various evaluation times are described in Table 3.

Our image analysis using the ImageJ software, showed that corneas in the G3 group showed a larger percentage of vessels, when compared with corneas in the G1, G2, and G4 groups ($P < 0.05$) (Figure 2).

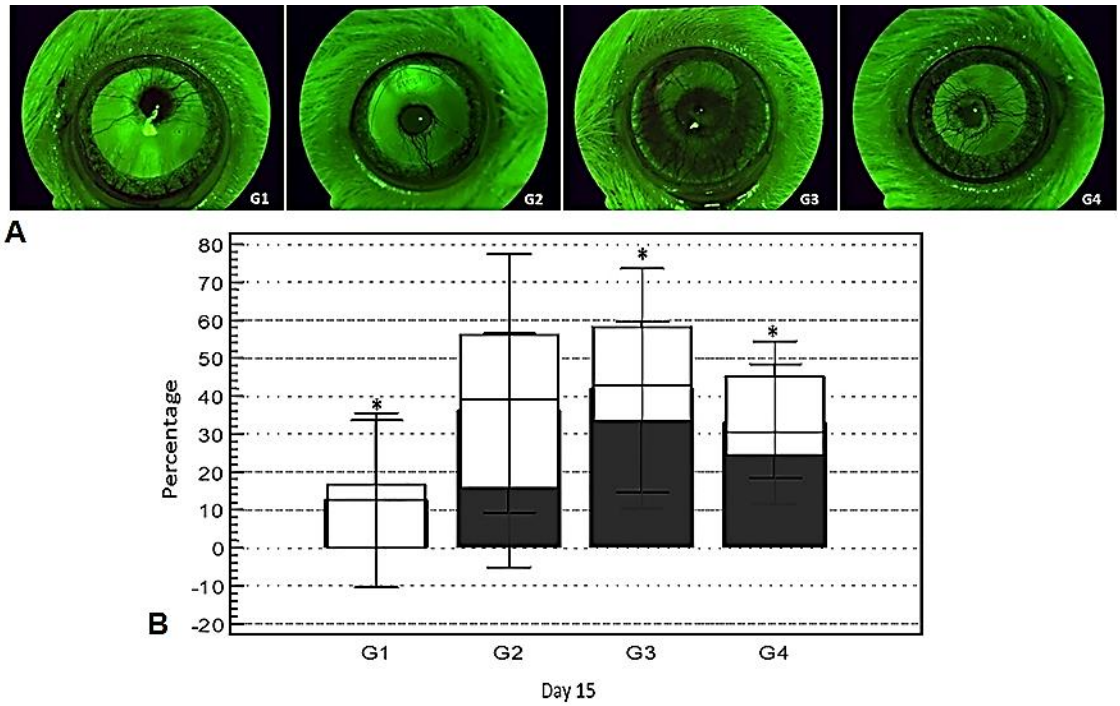

FIGURE 2. A, images of corneas with vessels, using a green filter (red free) in the G1, G2, G3, and G4 groups, on day 15 of the evaluation. B, comparison between the percentages of the corneal area containing vessels in the G1, G2, G3, and G4 groups, on day 15 of the evaluation. *$P < 0.05$; G1 versus G3 and G4. Veterinary Ophthalmology Service, Unesp/FCAV, Jaboticabal, SP, Brazil, 2017.

Table 2. Mean values with standard deviations, representing the development of neovascularization in the G1, G2, G3, and G4 groups, on days 2, 4, 6, 8, 11, 13, and 15. Veterinary Ophthalmology Service, Unesp/FCAV, Jaboticabal, SP, Brazil, 2017.

Day	G1 (n = 6)	G2 (n = 6)	G3 (n = 6)	G4 (n = 6)
2	0*	1.00*	1.00*	----
4	0	0.66 ± 0.57	1.00	1.00
6	0.50 ± 1.00	1.33 ± 0.57	2.00	1.50 ± 0.57
8	1.33 ± 1.50	2.25 ± 1.50	3.00	2.40 ± 0.54
11	0.66 ± 1.03	1.40 ± 1.51	1.80 ± 1.60	2.50 ± 1.22
13	0.50 ± 1.22	0.40 ± 0.89	1.00 ± 1.50	0
15	0	0	0	0

*P < 0.05, G1 versus G2 and G3

---- not assessed

Table 3. Mean values with standard deviations, representing the corneal neovascularization profiles in the G1, G2, G3, and G4 groups, on days 2, 4, 6, 8, 11, 13 and 15. Veterinary Ophthalmology Service, Unesp/FCAV, Jaboticabal, SP, Brazil, 2017.

Day	G1 (n = 6)	G2 (n = 6)	G3 (n = 6)	G4 (n = 6)
2	0*	1.00*	0.25 ± 0.50*	----
4	0	0.55 ± 0.73	0.50 ± 0.57	0.50 ± 0.57
6	0.31 ± 0.70**	0.87 ± 0.68	1.00 ± 1.15	1.28 ± 0.87**
8	1.21 ± 1.32♢	1.78 ± 1.30	2.60 ± 0.96♢	1.87 ± 0.99
11	1.46 ± 1.40♣	2.27 ± 1.23♣	2.78 ± 0.73♣	2.62 ± 1.01♣
13	1.50 ± 1.50♣	2.27 ± 1.23♣	2.83 ± 0.64♣	2.62 ± 1.01♣
15	1.50 ± 1.50♣	2.22 ± 1.22♣	2.83 ± 0.64♣	2.62 ± 1.01♣

*P < 0.05, G2 versus G1 and G3

**P < 0.05, G1 versus G4

♢P < 0.05, G1 versus G3

♣P < 0.05, G1 versus G2, G3, and G4

---- not assessed

DISCUSSION

In vivo models for the study of the mechanistic basis of angiogenesis and for investigating the action of alleged anti-angiogenic drugs should be affordable and non-invasive, when monitoring of the growth of new blood vessels. Rats are excellent model organisms for preclinical studies of angiogenesis, since they are small, easy to handle, incur a low cost, and have a mapped genome. The corneas of these animals have dimensions that allow rapid procedures for the induction of angiogenesis.[8,24]

To date, there is no consensus on the optimal protocol for the induction of corneal angiogenesis in rats.[10] The implantation of pellets containing drugs or pro-angiogenic molecules are emerging in the literature as reliable strategies. However, the vascular response associated with these procedures is very intense and unpredictable.[25] In addition, the investment required for pellet production is high. Alkaline cauterization remains the most widely used method for the induction of corneal angiogenesis, but its effectiveness may vary according to the protocol that is employed.[6,7] The lack of reproducibility represents a common problem, among studies involving models of angiogenesis induced by alkaline cauterization.[10]

The aim of this study was to establish a reproducible and inexpensive protocol for the induction of corneal neovascularization using alkali in rats, that is easy to perform, and presents a low risk of undesirable complications, such as the perforation of the eye. The alkaline cauterization produced in corneas in this study leads to a timely inflammatory response,[8,11–16] where the release of chemokines recruits leukocytes that produce angiogenic factors, such as the vascular endothelial growth factor, the fibroblast growth factor 2 and the tumor necrosis factor, among others. These growth factors attract endothelial cells, smooth muscle cells, fibroblasts, leukocytes, and platelets, triggering the process of neovascularization.[26]

In the USA, some biomedical laboratories sell disposable sticks coated with silver nitrate and potassium nitrate, suitable for corneal cauterization.[14,16] In Brazil, these sticks are not easily found, and need to be imported. Thus, we opted for preparing an aqueous solution containing 75% silver and 25% potassium nitrate, which was stored in an amber vial (protected from light) to avoid the oxidation of the compounds. For cauterization, filter papers were embedded in this alkaline solution

and pressed onto the corneas of rats. The filter paper embedded with alkali used in this study, proved to be as effective, but more economical than commercial sticks.

The vascularization parameters were studied at different times for up to 15 days. In agreement with the literature, vessel growth tends to stabilize by the end of this period of time.[8]

The corneal cauterization observed in this study was similar to that observed in previous reports.[8] Most of the eyes in the current study presented burn intensities of grades 2 and 3. This result is similar to that reported by Hurmeric et al.[27] and Habot-Wilner et al.[13] who used protocols in which the cauterization times were 8 and 10 seconds, respectively. These authors used commercial sticks to induce angiogenesis. We hypothesized that these commercially available coated sticks might be more concentrated. Therefore, this may explain the similarity between the values obtained in these studies and ours, as they used a shorter exposure time.

Groups whose corneas were exposed to alkali for a longer period of time presented higher values for the neovascularization profiles, that were either moderate or intense, as previously described by Yu et al.[20] Interestingly, in our study, the vessels developed in a progressive manner until the eighth day post-cauterization in all study groups. In previous studies, vessel development ceased on the third day post-cauterization.[2]

In this study, larger corneal burns (3 mm) were observed compared to those obtained in previous studies using sticks coated with silver and potassium nitrate.[8,15] Thus, we expected the corneal fractions containing vessels to be much larger than those reported in previous studies. Interestingly, however, they were smaller. Sella et al.[15] induced 2-mm burns and observed that the vascularized percentage of the corneal area was 42.6 ± 19.59% on the 7th day after the procedure. Manzano et al.[12] and Hepsen et al.[11] induced cauterizations of 1.8 mm and 5 mm, and observed vascularization in 63.5% and 56.9% of the corneal areas, respectively. We hypothesized that if these commercially coated sticks were more concentrated they could induce a greater inflammation, thus obtaining larger corneal fractions containing vessels.

The time intervals during which the filter papers were in touch with the corneas changed the vascular response. On day 15 of the evaluation, these results differed

significantly between the groups. The G3 and G4 groups showed intense neovascularization when compared with the G1 group. This suggested that the exposure time and maintaining the filter paper on the cornea at the time of washing can modify the result, when corneas are compared at a later time.

CONCLUSIONS

In this study, we demonstrated the efficacy of an aqueous solution composed of 75% silver and 25% potassium nitrate, for the induction of corneal angiogenesis in rats. Four protocols were studied, and three of these (G2, G3 and G4) provided consistent results, with good reproducibility. As well, we recommend the protocol G3, as a result of this group had the larger corneal neovascularization profiles and corneal fractions containing vessels.

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CAPÍTULO 3 – Artigo Científico[1]

Effect of Metronidazole Ophthalmic Solution on Corneal Neovascularization in Rats

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ABSTRACT

Purpose: To evaluate the effect of metronidazole ophthalmic solutions on corneal neovascularization (CNV) in a rat model.

Methods: A chemical burn was created in the right central cornea of 40 rats. Animals were randomized and distributed into four study groups (n = 10 rats per group) designated Met_0.1%, Met_0.5%, sham, and untreated groups. Chemical-burned corneas in the Met_0.1% and Met_0.5% groups received ophthalmic solutions of 0.1% and 0.5% metronidazole, respectively. Corneas in the sham group received phosphate-buffered saline (metronidazole diluent). All treated eyes received ophthalmic solution at intervals of 6 hours for 30 days. Untreated corneas received no treatment. CNV was evaluated postinjury using corneal photographs at different evaluation time points. The main CNV outcome measures were: burn intensity, index of CNV, and percentage of vascularized corneal area. Five rats from each group were euthanized, on days 15 and 30; the samples were collected for histological analyses. Differences with $p < 0.05$ were considered significant.

Results: CNV was observed in the eyes from day 7 postinjury. However, the indices of CNV for the Met_0.1% and Met_0.5% groups were smaller than those for the sham and untreated groups ($p < 0.05$). Furthermore, corneas treated with 0.1% or 0.5% metronidazole had smaller vascularized areas compared to control corneas. On histological study, the presence of blood vessels confirmed clinical outcomes.

Conclusions: Regular instillation of 0.1% or 0.5% metronidazole had a significant inhibitory effect for CNV on chemical burns-induced in a rat model.

Keywords*:* rats, metronidazole, cornea, angiogenesis, neovascularization

INTRODUCTION

Under normal homeostatic conditions, the cornea is transparent and avascular. However, the corneal angiogenic privilege is not absolute, in part because it results from the balance in the expression of anti-angiogenic and pro-angiogenic molecules [1, 2]. When the expression of anti-angiogenic and pro-angiogenic molecules is altered, a pathological condition referred as corneal neovascularization (CNV) may occur [2].

CNV alters visual acuity by inducing stromal edema, cellular infiltration, lipid deposition and scarring [2, 3]. It is estimated that up to 1.4 million people in the USA may have CNV [4]. CNV is a sight-threatening condition that may occur secondary to chemical burn, ischemia, inflammation, infection, limbal stem cell deficiency, degenerative disorders, post-corneal transplantation, and traumatic injuries, among others [5, 6]. It is also a potential complication of bacterial, parasitic, and viral infection [1].

Although the clinical relevance of CNV has been long recognized, its treatment is still challenging and associated with varying degrees of success [2, 6-8]. Current options for CNV include pharmacological approaches such as corticosteroids and non-steroidal anti-inflammatory agents, both of which may be associated with side effects. Other treatments include metalloproteinase inhibitors and monoclonal antibodies targeting vascular endothelial growth factors (VEGF), among others [6, 7]. Anti-VEGF antibodies, such as bevacizumab, ranibizumab and aflibercept, have a significant cost disadvantage despite the good results reported by different authors [9-11]. For patients in resource-poor countries, it is important to seek alternative, effective, and economically viable treatments for CNV.

Metronidazole is a low-cost drug used to treat infections caused by anaerobic bacteria and protozoans [12], to control odors in wounds [13], and to treat skin burns [14]. In ophthalmology, 0.1% and 0.5% metronidazole solutions have been used to treat keratitis [15-17]. Metronidazole also appears to have chemotherapeutic activity [18] and antioxidant effects [14]. More recently, there has been some evidence that can control inflammation [19-20] and modulate angiogenesis in non-ocular tissues [21,

22]. The aim of this study was to evaluate the angiogenic effect of metronidazole ophthalmic solutions on CNV in a rat model.

MATERIALS AND METHODS

Animals

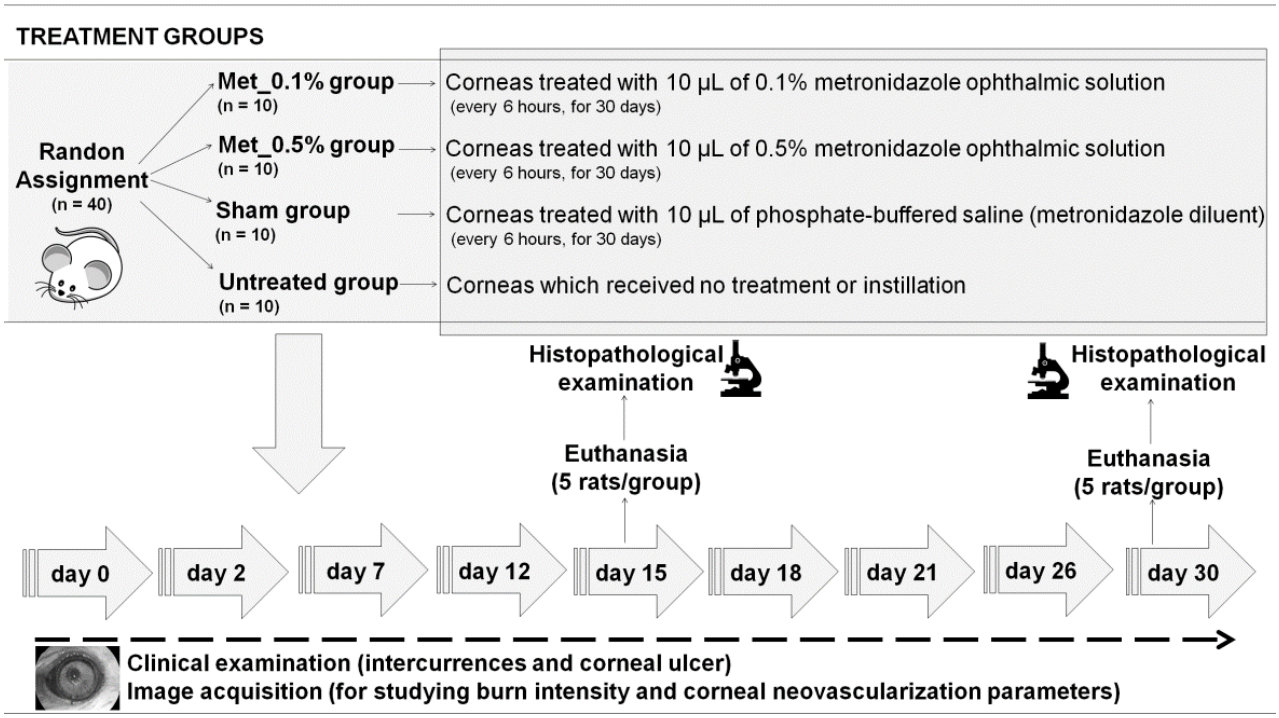
Forty male Wistar rats (Rattus norvegicus), aged between 3 and 4 months, weighing 428.40 ± 63.60 g (mean ± standard deviation), were selected for the study. All the subjects were free of signs of corneal disorders [23]. The animals were kept in appropriate cages with an alternating light/dark cycle (12 h). They were fed a commercial pellet diet (65.82% carbohydrate, 5.36% fiber, 21.0% proteins, and 4.96% fat) and received water ad libitum.

Corneal Alkali Burn Models and Treatment Groups

Corneal inflammatory angiogenesis was induced through a protocol of punctual alkaline cauterization in the right eye [24]. All procedures were performed with animals under general [25] and topical anesthesia with 0.5% proxymetacaine eye drops (Alcon, São Paulo, Brazil). A circular disc of filter paper with a diameter of 3 mm, saturated with a solution of 75% silver nitrate (Synth, Diadema, Brazil) and 25% potassium nitrate (Dinâmica Contemporary Chemistry Ltda., Diadema, Brazil), was pressed onto the axial region of the cornea, for 20 seconds [24]. Excess silver and potassium nitrate was removed by rinsing the eye. To increase reproducibility, one investigator induced the chemical burn in all animals.

Following the chemical corneal burn, animals were randomized into four groups (Met_0.1%, Met_0.5%, sham, and untreated groups; n = 10 rats per group), as shown in Fig. 1. All ophthalmic solutions used in the study, including the phosphate-buffered saline (PBS), a metronidazole diluent, were from single production lots manufactured by Ophthalmos S.A. (São Paulo, Brazil). Treatments started immediately postinjury and all instillations (10 µL of 0.1% metronidazole, 0.5% metronidazole, or PBS) were performed at regular intervals of 6 hours, for 30 days. Postinjury, the animals received tramadol hydrochloride (5 mg/kg; Cristália, Itapira, Brazil) by the subcutaneous route every 8 hours, for 10 days [24].

FIGURE 1. Flowchart of the treatment groups compared in the study, and simplified experimental design.



Clinical Examination of Chemically-Burned Corneas and Image Acquisition

Clinical examinations were performed after topical anesthesia (0.5% proparacaine; Alcon) and pupillary dilatation (1% tropicamide; Alcon). In addition, fluorescein eye stain (fluorescein sodium ophthalmic strips; Ophthalmos S.A., São Paulo, Brazil) was performed. Corneas were laterally and frontally photographed using digital equipment (TRC-50DX, Topcon, Japan) on days 0, 2, 7, 12, 15, 18, 21, 26, and 30 [8, 24] and the images of CNV were evaluated by a blinded investigator.

Burn intensity and neovascularization parameters

Burn intensity were studied from lateral images [24]. They were scored based on the appearance of blisters and on the extent of their elevation from the corneal surface: 0 (no blister, not raised above corneal surface), 1 (a small blister that is slightly raised above the surface), 2 (a medium blister that is moderately raised above the surface), and 3 (a large blister) [26].

The CNV index and percentage of vascularized corneal area were studied from frontal profile images [24]. "The CNV index was scored between 0 and 3, with increments of 0.5, per corneal quadrant using a grid system based on the centripetal extent of neovascular branch outgrowth from the corneoscleral limbus. Scores for each quadrant were then summed to obtain the CNV index (range from 0 to 12) for each eye at a given time point [27]".

The percentage of vascularized corneal area was calculated using a grid map containing 110 points, which were projected by the ImageJ software (National Institutes of Health, Bethesda, MD, USA) as previously described [24, 28]. Calculations were performed using the (PV x 100)/PC formula, where PV represents the total number of points that touch the vessels, and PC represents the total number of points that touch the non-vascularized corneal areas [24, 28].

To determine whether regression of CNV occurred during the follow-up time, we calculated the percentage differences between the final and the initial CNV area.

Histopathological Examination

Five rats from each group, randomly selected, were euthanized on days 15 and 30 with intraperitoneal sodium thiopental (60 mg/kg; Cristália). The corneas were harvested, fixed in 10% buffered formaldehyde (Synth) and processed for routine inclusion in paraffin (Merck, Darmstadt, Germany). Sagittal sections of 4-μm-thickness were stained with Masson trichrome staining and examined under a light microscope (Olympus BX53F microscope, Tokyo, Japan). The number of vessels per cornea section was manually counted at 40x magnification. The vascularization was histologically scored as 1 (minimal or close to negative vascularization), 2 (limited or focal vascularization in the sub-epithelial areas), 3 (intermediate between scores 2 and 4), or 4 (intense vascularization) [29].

Data Analysis

All data were tested for statistical normality. Comparisons were performed using the chi-square test, the Kruskal-Wallis (KW) non-parametric test with Dunn's post hoc test, and the one-way analysis of variance (ANOVA) with Student-Newman-Keuls post hoc test. The odds ratio and Spearman's correlation were used as measures of

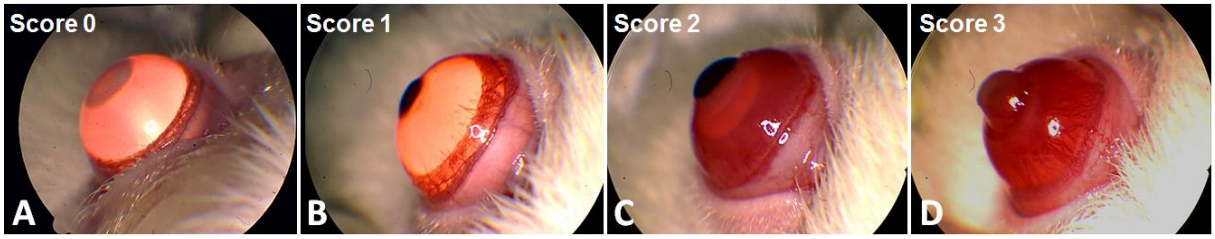
association between the study variables. Differences with $P < 0.05$ were considered significant (95% confidence interval, 95%CI). All statistical analyses were performed using the MedCalc software (version 9.3.6.0, MedCalc, Mariakerke, Belgium).

RESULTS

All corneas were fluorescein negative on day 0 postinjury. However, by day 2, 1 cornea in the Met_0.1% group (10%), 4 in the Met_0.5% group (40%), 6 in the sham group (60%), and 7 corneas in the untreated group (70%) were fluorescein positive. Corneal wound healing times were 7 days for the Met_0.1% group, 12 days for the Met_0.5% and untreated groups, and 18 days for the sham group (chi square test contingency coefficient = 0.356, $P < 0.001$).

All burn injury sites were opaque with a defined border (Fig. 2A), from days 2 to 12, corneas in all study groups presented blisters with varying degrees of elevation from the corneal surface (Fig. 2B–D). Corneas in the Met_0.1% group showed less intense burns (maximum score of 1) than corneas in the sham and untreated groups (scores ranged from 0 to 3) ($P < 0.05$, KW test with Dunn's post hoc test) (Table 1). The results in burn intensity observed between the Met_0.1% and Met_0.5% groups on day 12 of examination were not significantly different ($P > 0.05$). The results of the sham group were significantly different from those of the untreated group ($P < 0.05$) in all evaluation time points.

FIGURE 2. Representative images of corneas that according to the intensity of the burn, i.e., the appearance of blisters and their elevation from the ocular surface, were scored as 0 (no blister, no raised above corneal surface) (A), 1 (a small blister that is slightly raised above the surface) (B), 2 (a medium blister that is moderately raised above the surface) (C), and 3 (a large blister) (D).



Formation of burn blister was not a univariate predictive factor for the development of corneal ulcer (odds ratio, 0.38; 95% CI, 0.21 to 0.69). Only 14% of corneas that presented with burns with an intensity score above 1 developed ulceration.

CNV was observed in all groups on day 7. No association between the index of initial CNV (day 7) and the burn intensity was observed (Spearman's correlation, 0.021; P = 0.89; 95% CI, 0.33 to 0.29).

Table 1. Burn intensity scores for the study groups

Evaluation	Groups																
	Met_0.1%				**Met_0.5%**				**Sham**				**Untreated**				**KW**
	Median	Max	Min	AVG rank	Median	Max	Min	AVG rank	Median	Max	Min	AVG rank	Median	Max	Min	AVG rank	*P*-value
Day 2	0	1	0	8.50[a]	1	3	0	16.00[b]	0	1	0	11.36[c]	1	3	0	17.79[b]	0.04
Day 7	0	0	0	8.00[a]	0	1	0	9.92[b]	0	3	0	13.71[c]	1	3	1	21.07[d]	0.00
Day 12	0	0	0	10.50[a]	0	0	0	10.50[a]	0	3	0	12.57[b]	1	3	1	19.57[c]	0.00

AVG, average; KW, Kruskal-Wallis non-parametric test; Max, Maximum value; Min, Minimum value.

The ***AVG*** rank is the ***average*** of the ranks for all observations within each sample.

Different letters in the same line represent the statistical differences between the groups. Such differences were considered significant when $P < 0.05$ (Dunn's post hoc test).

Burn blister was not observed in any of the study groups from the day 15 of evaluation.

Statistical results related to the index of CNV are presented in Table 2. Both one-way ANOVA and KW tests were used to identify whether there was any significant difference in CNV between the groups, since the distributional assumptions required for parametric testing were not satisfied in all cases. ANOVA revealed that the mean indices of CNV for the Met_0.1% and Met_0.5% groups were significantly lower than those for the sham or untreated groups at all evaluation time points ($P < 0.05$, ANOVA with Student-Newman-Keuls post hoc test), except on day 7. The KW test detected differences ($P < 0.05$) between groups only on days 12 and 15.

Fig. 3A shows images of CNV observed in each study group. The Met_0.1% and Met_0.5% groups had a smaller area of CNV than sham and untreated groups, (Fig. 3B). The CNV area was significantly smaller for the Met_0.1% group than for the untreated and sham groups at all evaluation time points ($P < 0.05$, KW with Dunn's post hoc test), except on day 7 ($P > 0.05$) (Fig. 3C–I). The Met_0.5% group results were significantly smaller than those of the sham and untreated groups on 18 to 30 days ($P < 0.05$). Boxplot charts revealed homogeneity in the variance of the data related to extensions in areas of CNV treated with 0.1% or 0.5% metronidazole.

Table 2. Indices of corneal neovascularization obtained for the study groups

	Groups																	
	Met_0.1%				**Met_0.5%**				**Sham**				**Untreated**				**ANOVA**	**KW**
Evaluation	Mean	SD	Median	AVG rank	Mean	SD	Median	AVG rank	Mean	SD	Median	AVG Rank	Mean	SD	Median	AVG rank	*P*-value	*P*-value
Day 7	4.50[a]	1.71	5.00	9.71	6.08[a]	3.61	6.75	14.42	6.93[a]	3.37	6.50	16.93	6.07[a]	2.62	3.00	15.00	0.47	0.36
Day 12	4.42[a]	2.15	6.00	7.36	6.33[a]	3.39	7.00	12.83	10.00[b]	2.96	12.00	19.21	8.64[a]	3.50	6.50	16.43	0.04	0.02
Day 15	3.57[a]	1.90	3.00	7.71	6.33[a,b]	3.39	7.00	13.00	9.71[b]	3.59	11.00	18.79	8.57[b]	3.55	6.00	16.36	0.02	0.04
Day 18	3.50[a]	3.54	3.50	1.50	7.00[a]	---	7.00	3.50	11.00[b]	---	12.00	7.50	11.33[b]	1.15	10.00	6.67	0.01	0.06
Day 21	3.50[a]	3.54	3.50	1.50	7.50[a]	0.71	7.50	3.50	11.00[b]	---	12.00	7.50	11.33[b]	1.15	10.00	6.67	0.01	0.06
Day 26	3.50[a]	3.54	3.50	1.50	7.50[a]	0.71	7.50	3.50	11.00[b]	---	12.00	7.50	11.33[b]	1.15	10.00	6.67	0.01	0.06
Day 30	3.00[a]	4.24	3.00	1.50	7.00[a]	1.41	7.00	3.50	11.00[b]	---	12.00	7.50	11.33[b]	1.15	10.00	6.67	0.02	0.06

ANOVA, analysis of variance; AVG, average; KW, Kruskal-Wallis non-parametric test; SD, standard deviation.

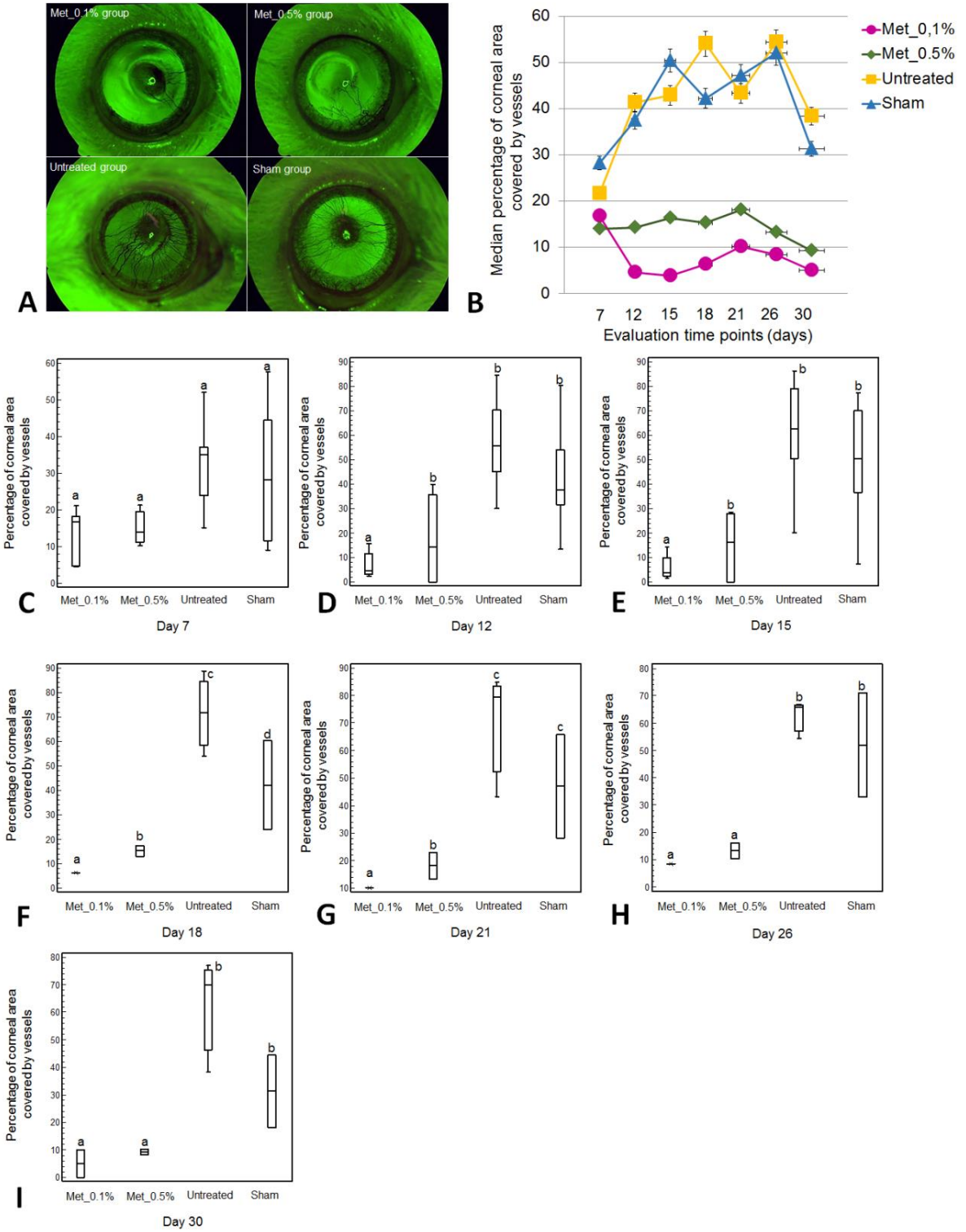
---, variance was zero because all observations were equals.

The *AVG rank is the average* of the ranks for all observations within each sample.

Different letters in the same line represent the statistical differences between the mean values. Such differences were considered significant when $P < 0.05$ (Student-Newman-Keuls post hoc test).

Corneal neovascularization was not seen on days 0 and 2 of evaluation.

While the data obtained on days 7, 12 and 15 had a normal distribution; those obtained from day 18 to 30 did not present Gaussian distribution. Therefore, all were studied with both ANOVA and KW tests.



FIURE 3. Extension of CNV. (A) corresponds to a panel containing representative images of CNV (red free filter) observed in each study group at 30 days. Visual inspection of these images shows that corneas in the Met_0.1% and Met_0.5% groups have fewer vascularized areas compared to the corneas in the untreated and Sham groups. (B) corresponds to a line chart showing the differences in percentage of vascularized corneal area between the study groups. (C–I) correspond to boxplot charts showing the extent of vascularized corneal area in each study group at days 7, 12, 15, 18, 21, 26, and 30. Different letters represent the statistical differences ($P < 0.05$, Kruskal-Wallis test with Dunn's post hoc test).

Percentage differences between the final and the initial CNV areas were calculated. The CNV decreased by 60.90% and 38.91% in the Met_0.1% and Met_0.5%groups, respectively. In the sham and untreated groups, the CNV increased by 6.35% and 87.04% respectively.

No signs of irritation or complications were observed in the chemical-burned corneas that received metronidazole for 30 days.

In the histopathological evaluation, corneas treated with 0.1% or 0.5% metronidazole exhibited fewer blood vessels than the sham or untreated corneas ($P < 0.05$, KW with Dunn's post hoc test) (Fig. 4A and B). Fig. 4C–F shows images of corneas that were histologically scored as 1, 2, 3, and 4 according to their vascularization. All corneas in the Met_0.1% and Met_0.5% groups were scored as 1 or 2, whereas more than half of the corneas in the sham and untreated groups were scored as 3 or 4 (Fig 4G and H). Approximately 30% of corneas (sham and untreated groups) that were scored as 3 or 4 had mild-to-moderate inflammatory infiltrate and focal fibroblastic activity in the central anterior stroma. In the untreated group, 20% of corneas (scored as 4) showed disorganization of stromal collagen fibers and thickening of the central epithelium.

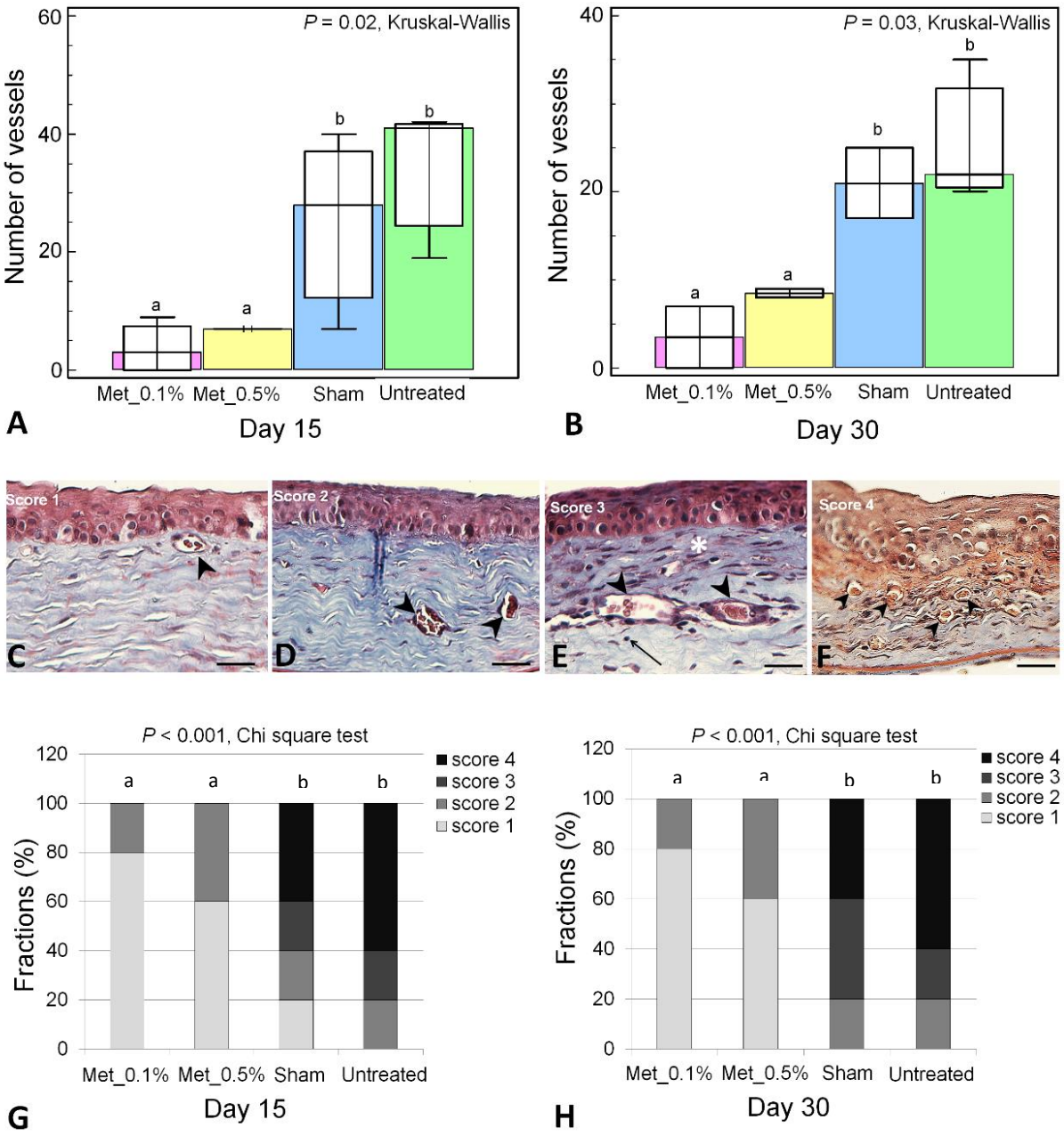


FIGURE 4. Histopathological examination of vascularized corneas. (A and B) correspond to combined boxplot and bar charts (of median values, with interquartile range error bars) showing the numbers of vessels counted in corneas from each study group on days 15 and 30 of examination. Note that corneas in the Met_0.1% and Met_0.5% groups showed fewer vessels than corneas in the untreated and Sham groups. Different letters represent the statistical differences ($P < 0.05$, Kruskal-Wallis test with Dunn's post hoc test). (C) corresponds to the photomicrography of a cornea that according to the vascularization was histologically scored as 1. The arrow heads indicate the vessels. (D) corresponds to a cornea scored as 2. The arrow heads indicate the vessels. (E) corresponds to a cornea scored as 3. The arrow heads indicate the vessels, the black arrow points to an inflammatory cell, and the asterisk indicates a focus of fibroblastic activity. (F) corresponds to a cornea scored as 4. The arrow heads indicate the vessels. Note that the corneal epithelium is thickened. Bars = 20 micrometers. (G and H) correspond to the stacked bar graphs showing percentages of corneas scored as 1, 2, 3, and 4 for each study group on days 15 and 30 of examination.

DISCUSSION

CNV is a clinical condition that, when left untreated, causes significant visual deficit [5]. Current therapies for inhibiting angiogenesis in vascularized corneas are associated with a variety of systemic side effects [10]. Moreover, some treatments have a significant cost disadvantage and are inaccessible to some part of the population in resource-poor countries. To benefit and treat patients from these countries, it is necessary to investigate an anti-angiogenic potential of more affordable drugs. Metronidazole, a nitroimidazole derivative [12, 20, 30], is inexpensive; in some countries, such as Brazil, it is available free of charge from the National Public Health System.

Nishimuta and Ito [19] reported that side effects related to topical metronidazole are lower than those of corticosteroids. In this study, no signs of irritation or ocular complications were observed after instillations of the drug. Other authors who used 0.5% metronidazole to treat keratitis also did not reported any side effects [31]. In addition, toxicological studies have shown that topical use of nitroimidazole derivatives does not cause cell mutation [19]. In contrast, systemic use of nitroimidazole derivatives has been associated with long-term side effects [20].

Healing properties have been attributed to metronidazole [14, 21, 32]. These properties arise from metronidazole's ability to promote contraction of myofibroblasts, stimulate the proliferation of epithelial cells, inhibit local inflammation and reduce bacterial load [14, 20, 21, 32]. In fact, our results on the occurrence of corneal ulcer and times of Corneal epithelial wound healing suggest that metronidazole may enhance healing, depending on the concentration used. Corneal ulcer in the Met_0.1% group, healed at 7 days postinjury, while corneal ulcer in the Met_0.5% group, healed at 12 days postinjury. It is important to note that in this study, corneal ulceration was not strongly influenced by the formation of burn blisters, as shown by the odds ratio calculation. A possible explanation for differences in healing between corneas in the Met_0.1% and Met_0.5% groups, is that the action of many drugs on corneal healing can be dose-dependent [33, 34]. Thus, drugs used at higher or lower concentrations than an optimal dose may be less effective or ineffective in enhancing corneal healing [34]. In the present study, 0.1% metronidazole was more effective than 0.5%

metronidazole in promoting healing. However, it is not possible to affirm that 0.1% is the optimum concentration of metronidazole, since dose-response curves were not constructed. The concentrations evaluated herein are commercially available and are widely used to treat some kinds of infectious keratitis [16, 31].

Evidence suggests that metronidazole has antioxidant and immunosuppressive effects [14, 20], and that it can modulate tissue angiogenesis [21, 22, 35]. However, in the latter respect, the results from studies by different authors are controversial. While some authors have observed that metronidazole has pro-angiogenic action [21], others have reported that it inhibits angiogenesis [22, 35]. Sampaio et al [21] observed that metronidazole contributed to healing in second intention wounds and stimulated collagen production and angiogenesis. In contrast, Pezo [35], studying the chicken chorioallantoic membrane, found that metronidazole inhibited new blood vessels formation. Michalska et al. [22] observed that metronidazole, when combined with clindamycin, inhibits neovascularization in benign gynecological tumors. The clinical and histopathological findings in this study agrees with the results of studies that associated metronidazole with inhibition of tissue angiogenesis.

The chemical-burn model used in this study is known to induce diffuse formation of vessels in a large region of the cornea [7, 24, 36]. The clinical parameters of CNV revealed that the pattern of initial vessel development was identical for all studied groups, even though, some differences in the evolution of CNV were detected only from day 12. We believe that the differences in the evolution of CNV between the study groups result from the treatment protocols used. The clinical and histopathological findings of this work strongly suggest that metronidazole inhibits growth of vessels and promotes regression of CNV in a rat model. After 30 days of treatment, there was a significant reduction of vascularization in corneas treated with 0.1% or 0.5% metronidazole. The anti-angiogenic effects of metronidazole have apparently been consistent, since the boxplots (Fig. 3C–I) revealed homogeneity (i.e., low dispersion) in the variance of the data related to extensions of vascularized areas in corneas treated with 0.1% or 0.5% metronidazole. CNV area results in this study are similar to those observed by Gal-Or et al. [11] when they studied the effects of aflibercept and bevacizumab on CNV.

To our knowledge, there is no study on whether metronidazole exacerbates the expression of anti-angiogenic molecules, reduces the production of pro-angiogenic molecules, or both. Some authors have suggested that it acts by reducing inflammation [19, 20]. Additionally, metronidazole reduces the generation of free radicals by neutrophils [37, 38], attenuating the oxidative stress associated with inflammation and CNV [39]. This hypothesis needs to be further investigated.

An interesting finding from this study was that the placebo (PBS) was not inert and can introduce bias against tested ophthalmic products. In agreement with this, the literature is replete with clinical trials where placebo procedures were assumed to be inert [40-42] but were not in this study. In this study, the corneas in the sham group developed less intense burn injuries than the corneas in the untreated group. We suspect that the placebo attenuated burn injury by providing moisture to the ocular surface (lubrication), as well as by removing pathogens, allergens, and cell degradation products [43]. Previous studies have shown that the use of ocular lubricants causes a transient increase in thickness of the tear film [44], which dilutes molecules that stimulate the appearance or progression of superficial corneal damage.

In conclusion, regular instillation of 0.1% or 0.5% metronidazole inhibits and decreases the progression of CNV on chemical burns-induced in a rat model. Additional research is required to determine the mechanism of action of metronidazole ophthalmic solutions on corneal angiogenesis, as well as to establish an optimum dose and possible side-effects.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All procedures performed in the study were in accordance with the ARVO Statement for Use of Animals in Ophthalmic Vision and Research. The ethical principles established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 2011) were followed. The research protocol was approved by the Ethics Committee on Animal Use (protocol number 06174/14) of FCAV/Unesp, Jaboticabal.

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CAPÍTULO 4 – Considerações Finais

Os resultados no Capítulo 2 deste estudo, referentes ao artigo “Corneal angiogenesis based on diferente protocols of alcaline cauterization in murine models”, mostraram que variações nos procedimentos para cauterização alcalina pontual ensejam diferentes padrões de resposta vascular, ou seja, diferentes perfis de crescimento e de invasão de vasos na córnea. Nem sempre, neste estudo, observaram-se padrões homogêneos de resposta vascular, o que, em estudos comparativos, poderia resultar em *viéses* e comprometer a reprodutibilidade dos resultados. Ademais, um de quatro protocolos de cauterização aqui avaliados, um não apresentou boa repetibilidade intralaboratorial. Melhores resultados e alta repetibilidade, em termos de homogeneidade de resposta vascular, foram conseguidos por meio de protocolo no qual um papel filtro, com 3 mm em diâmetro, saturado em solução de nitrato de prata e de potássio 3:1, vol/vol, foi gentilmente pressionado sobre a região axial da córnea, por 20 sec. Ato contínuo, o papel filtro foi removido e a córnea foi lavada por 1 min com solução de Ringer lactato.

Os resultados no Capítulo 3 deste estudo, referentes ao artigo “Effect of Metronidazole ophthalmic solution on corneal neovascularization in rats”, mostraram que a instilação regular (a cada seis horas) de colírios de metronidazol, 0,1% ou 0,5%, por 30 dias, inibiu o crescimento de vasos e promoveu regressão de neovascularização corneal induzida por cauterização alcalina, em ratos.