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ORIGINAL PAPER

Efficacy of fumagillin bicyclohexylamine on experimental corneal neovascularization in rat model

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Abstract

Purpose Fumagillin has been previously used to treat corneal microsporidial keratitis and also identified as an angiogenesis inhibitor. This study aimed to evaluate efficacy of fumagillin bicyclohexylamine on the rat model of corneal neovascularization induced by silver nitrate cauterization.

Methods Twenty-four Albino Wistar rats (n = 24) were divided into three groups. Following silver nitrate-induced corneal injury, eyes in Group 1 received one drop of 5 mg/mL topical fumagillin bicyclohexylamine four times daily for 10 days. Group 2 received subconjunctival injection of 0.1 mL fumagillin bicyclohexylamine (2.5 mg/mL) on day 1 and day 5. Group 3 received artificial tears and lubricants four times daily for 10 days as control. On day 10, animals were sacrificed. Corneal specimens were obtained and prepared to assess vascular endothelial growth factor (VEGF-C) levels and corneal angiogenic microvessel density.

Results There was no significant difference in VEGF-C levels between the groups (P = 0.994). Assessment of angiogenic microvessel density for peripheral corneal zone also did not reveal significant difference between the groups (P = 0.113). However, mean vascular density in Group 1 and Group 2 was significantly higher for both midperipheral and central corneal zones in comparison with Group 3 (P = 0.003, P = 0.015).

Conclusions Previously proved to be effective for treatment of microsporidial keratitis in humans, topical and subconjunctival concentration or dosing of fumagillin bicyclohexylamine failed to reduce corneal neovascularization induced by silver nitrate in this study. Further studies comparing different concentrations and dosing may detect inhibitory effects of fumagillin on corneal neovascularization without inducing toxicity.

Keywords Fumagillin bicyclohexylamine · Corneal neovascularization · VEGF-C · Silver nitrate · Rat cornea

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Introduction

Angiogenesis is a key process both in physiological and pathophysiological situations [1]. Anterior segment inflammation may result in corneal

neovascularization (CNV), which leads to corneal opacity, undesired bleeding during flap creation in laser in situ keratomileusis (LASIK) and graft rejection after penetrating keratoplasty [2]. CNV comprises both upregulation of angiogenic factors and downregulation of anti-angiogenic factors [2]. In addition, corneal lymphangiogenesis has been shown to be associated with the degree of corneal angiogenesis on human-vascularized corneas [3–5]. However, previous clinical and experimental studies showed that many therapeutic agents including steroids [2, 6], cyclosporine [7], indomethacin [2], methotrexate [8], rapamycin [9], low molecular weight heparin sulfate [10] and thalidomide [2] have potential to inhibit CNV. Doxycycline and minocycline have also been shown to inhibit CNV in rats with topical or systemic administration [11–14]. Focusing on pro-angiogenic vascular endothelial growth factor (VEGF) activity with a neutralizing anti-VEGF antibody has also drawn much attention recently [15].

Fumagillin is a naturally secreted, water-insoluble antibiotic produced by *Aspergillus fumigatus* shown to be effective for inhibiting intestinal protozoa including *Entamoeba histolytica* [16, 17]. Fumagillin bicyclohexylamine, the water-soluble form, is used commercially for the treatment of nosematosis, a microsporidal disease of honey bees caused by *Nosema apis* [18]. Moreover, topical administration of fumagillin bicyclohexylamine has been previously used to treat corneal microsporidial infection, which clinically presents as epithelial or stromal keratitis in immunocompromised subjects including AIDS patients [19–22].

Fumagillin was also shown to inhibit endothelial cell proliferation in cultures contaminated with *A. fumigatus* and identified as an angiogenesis inhibitor [23]. A synthetic analogue of fumagillin, TNP-470, has been previously reported to be effective as an inhibitor of inflammatory corneal angiogenesis [1]. In this study, we evaluated the efficacy of topical and subconjunctival application of water-soluble form of the original molecule—fumagillin bicyclohexylamine on the rat model of CNV induced by silver nitrate cauterization.

Methods

Twenty-four Albino Wistar rats with a weighing between 250 and 300 gr were used according to a protocol approved by Istanbul University Experimental animals Local Ethics Committee (No: 2013/110). All procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. The rats were kept in individual cages under standardized controlled room temperature of 22.0 °C with a 12:12-h light–dark illumination cycle.

All procedures were performed with animals under general anesthesia induced by intramuscular injection of ketamine hydrochloride (50 mg/kg, Ketalar, Eczacibasi, Turkey) and xylazine (3 mg/kg, Rompun, Bayer, Germany). A topical anaesthesic, proparacaine hydrochloride (Alcaine, Fort Worth, Texas), was also applied to the corneal surface. A modified silver nitrate cauterization technique described by Mahoney and Waterbury was the choice to induce corneal neovascularization [24]. Accordingly, central right cornea of each animal was cauterized by an applicator coated with 75% silver nitrate and 25% potassium nitrate (Hemostop, Hizmet Medikal, Istanbul, Turkey) for 10 s under slit lamp. Residual silver nitrate was then rinsed off with 5 mL of saline. For the sake of standardization of the lesions, a single investigator (FA) executed the procedure. Following the cauterization, topical 0.3% tobramycin ointment was applied and animals were randomized into three groups. Eyes in Group 1 ($n = 8$) received one drop of topical fumagillin bicyclohexylamine (5 mg/mL, Fumidil B, Ceva, Turkey) prepared in normal saline four times daily for 10 days. Group 2 ($n = 8$) received subconjunctival injection of 0.1 mL of 2.5 mg/mL fumagillin bicyclohexylamine diluted in saline with 27-G needle on day 1 and day 5. Group 3 ($n = 8$) as a control received artificial tears and lubricants containing carboxymethylcellulose sodium four times daily for 10 days. All corneas were macroscopically observed with a magnifying lens for evolving CNV daily. On day 10, animals were sacrificed by inducing cardiopulmonary arrest with intraperitoneal administration of 1 cc xylazine. Corneas were excised beyond 2-mm limbus–sclera line. After dividing corneas in two halves through the center of the lesion, one half

was used to prepare sections for VEGF assessment and the other half for light microscopy.

Corneal tissues for VEGF-C assessment were homogenized in lysis buffer containing 2.5 mM tris hydrochloride (HCl), 25 mM ethylene diamine tetra acetic acid (EDTA), 1% Triton-X 100 and a protease inhibitor (Complete Mini Tablets, Roche, Germany). Samples were centrifuged at 13,000 rpm at 4 °C for 10 min, and the supernatant was extracted and stored at − 80 °C. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Plates were developed using a color reaction, and the absorbance was measured at 570 nm using an Elx800 Universal Microplate Reader (BIO-TEK, Winooski, VT, US). Rat VEGF-C levels were quantified by enzyme-linked immunosorbent assay (ELISA) with commercially available kit according to manufacturer's protocol (Diaclone SAS, France). Rat VEGF-C standard solution was diluted as to be of minimum 47 pg/mL and maximum 3000 pg/mL according to the manufacturer's recommendations. Absorbance was measured at 450 nm in an Elx800 Universal Microplate Reader (BIO-TEK, Winooski, VT, US). Detection limit was set at 47 pg/mL of VEGF-C.

Corneal tissues for histolopathological examination were fixed in 10% buffered formalin for 24 h. Then, tissues were dehydrated through graded concentrations of ethanol and embedded in paraffin wax. Two sections were cut from the paraffin block. One was stained with hematoxylin and eosin (HE) and the other underwent immunohistochemical staining (IHC), performed automatically with IHC staining device (Ventana Benchmark XT, Ventana Medical Systems, Tucson, AZ). Relevant consumable kit containing chromogens based on horseradish peroxidase (HRP) and 3′,3′-diaminobenzidine tetrahydrochloride (DAB) was used along with CD34 antibody (Clone QBEnd-10, Thermo Scientific-Lab Vision, US) diluted at 1/400. Counter staining was completed with hemotoxylin and bluing reagent. The procedure was finalized after xylene dehydration.

Corneal sections (halves of the corneas) subject to evaluation under light microscopy were measured in mm from limbus to limbus at cut edge. Then, they were marked concentrically with the corresponding ends of segments of equal distance on the side of cut edge as to be central, paracentral and peripheral (limbal) zones, which were the region of interest (ROI). CD34-stained slides were examined for assessment of angiogenic microvessel density under ×200 magnification. The number of microvessels was counted per viewed area. The mean microvessel count of five different viewed areas belonging to each particular zone mentioned above was recorded as the angiogenic microvessel density. Light microscopy was performed by an examiner (OA) blinded to the treatment groups.

All data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, US). Data were described with mean and standard deviation (Mean ± SD) and median, interquartile range [Median ($Q1$–$Q3$)]. Distribution of data was evaluated with Kolmogorov–Smirnov test. Quantitative variables were analyzed by ANOVA, Kruskal–Wallis or Mann–Whitney U tests according to distribution of variables.

Results

Following induction of corneal vascularization, corneal neovascularization became macroscopically visible within 2–3 days in a similar pattern for all groups. The neovascularizations emerged from 360° adjacent limbus and extended up to central cornea by 7–10 days. Table 1 summarizes assessment of angiogenic microvessel density on CD 34 stained slides for peripheral, midperipheral and central corneal zones. Accordingly, there was no significant difference in mean microvascular density between the groups in peripheral corneal zone ($P = 0.113$). However, mean vascular density in Group 1 and Group 2 were significantly higher for both midperipheral and central corneal zones in comparison with Group 3 ($P = 0.003$, $P = 0.015$).

Regarding lymphangiogenesis and hemangiogenesis, Table 2 shows VEGF-C levels in groups. No significant difference in VEGF-C levels was detected between the groups ($P = 0.994$).

Discussion

Owing to a balance between pro-angiogenic and anti-angiogenic factors, the healthy cornea is avascular and alymphatic. Pro-angiogenic elements in the cornea, which have been investigated in previous reports,

Table 1 Corneal angiogenic microvessel density

	Mean ± SD	Median	(*Q*1–*Q*3)	*P
Peripheral corneal angiogenic microvessel density				
Group 1	2.6 ± 0.9	2.0	2.0–3.8	0.113
Group 2	6.4 ± 4.2	6.0	2.0–12.0	
Group 3	3.0 ± 2.3	3.0	1.0–5.0	
Midperipheral corneal angiogenic microvessel density				
Group 1**	7.8 ± 1.3	8.0	7.3–8.8	***0.003***
Group 2**	9.4 ± 6.5	6.0	4.0–14.0	
Group 3	2.4 ± 1.7	2.0	1.0–3.0	
Central corneal angiogenic microvessel density				
Group 1**	4.0 ± 2.1	3.5	3.0–6.3	***0.015***
Group 2**	7.4 ± 8.7	5.0	3.0–5.0	
Group 3	2.0 ± 0.8	2.0	1.0–3.0	

$P < 0.05$ is statistically significant

Group 1: topical fumagillin bicyclohexylamine, Group 2: subconjunctival injection of fumagillin bicyclohexylamine, Group 3: control (healthy eyes)

(Q1–Q3): interquartile range

SD standard deviation

*Kruskal–Wallis

**Significant difference between Group 3 (Mann–Whitney $P < 0.01$)

Table 2 VEGF-C levels in groups

	VEGF-C levels in groups (pg/mL)			*P
	Mean ± SD	Median	(*Q*1–*Q*3)	
Group 1	468.0 ± 205.5	447.8	275.6–677.9	0.994
Group 2	462.8 ± 167.3	413.6	330.1–655.9	
Group 3	473.6 ± 191.3	527.2	315.1–687.8	

Group 1: topical fumagillin bicyclohexylamine, Group 2: subconjunctival injection of fumagillin bicyclohexylamine, Group 3: control (healthy eyes)

SD standard deviation

(*Q*1–*Q*3): interquartile range

*ANOVA

include VEGF, basic fibroblast factor (bFGF) and matrix metalloproteinase 2 (MMP-2), whereas anti-angiogenic factors comprise angiostatin, restin, endostatin and soluble vascular endothelial growth factor receptor 1 (VEGFR-1), which acts as an endogenous VEGF trap [2]. VEGF-C was previously described as a predominant lymphangiogenic factor and weak angiogenic factor [25, 26]. Following alkaline burns in particular, VEGF-C levels were shown to increase significantly during 3rd to 14th posttraumatic days, despite absence of lymphatic or blood vascular structures in the normal rat cornea. Moreover, the same study also showed a strong correlation between lymphangiogenesis and hemangiogenesis in alkali-burned corneas [27]. Therefore in this study, we preferred to examine VEGF-C levels as a direct biological marker of corneal lymphangiogenesis and an indirect one of corneal hemangiogenesis along with CD34 staining for assessment of microvessel density.

Fumagillin is used for the treatment of microsporidium infections in immunodeficient states including acquired immunodeficiency syndrome (AIDS) and posttransplantation subjects [28–30]. Microsporidium infection of the cornea was first reported in 1990 in a patient with AIDS in the USA, and a second case was reported in 1992 [31]. Fumagillin was found to be effective against *Encephalitozoon cuniculi* in vitro and against *Encephalitozoon hellem* when used topically on patients with keratitis [21]. Successful topical use of water-soluble fumagillin bicyclohexylamine on microsporidian keratitis series indicates that the drug in the preferred concentration and dosage is able to penetrate the inflamed cornea at least to some extent. To our knowledge, there are no studies reporting optimal dosing or concentration of topical fumagillin relevant to corneal penetration.

In addition, fumagillin, extracted from *A. fumigatus*, has also been reported to be the most potent natural angiogenesis inhibitor [23]. This activity is possibly related to fumagillin's inhibitory effect on methionine aminopeptidase-2 (MetAP-2), an enzyme which cleaves the N-terminal methionine of already translated proteins. Fumagillin forms a complex with MetAP-2 and inactivates the enzyme [32]. MetAP-2 is found ubiquitously in all organisms necessary for protein maturation and posttranslation processes, which may also cause severe toxicity in case of dysfunction [33]. Along with MetAP-2 inactivation, elevated p53 activity is also shown to inhibit angiogenesis in vivo by downregulating VEGF expression [34].

In the light of the reports mentioned above, we investigated fumagillin's anti-angiogenic efficacy on CNV. In this study, we preferred arbitrarily about 1.5 times more concentrated topical solution of fumagillin bicyclohexylamin—5 mg/mL—to test the anti-angiogenic efficacy versus original 3 mg/mL used for the

treatment of microsporidial keratitis [21]. Furthermore, we also tested 0.1 mL of 2.5 mg/mL subconjunctival injection of fumagillin bicyclohexylamine diluted in saline. However, topical and subconjunctival administration of water-soluble fumagillin bicyclohexylamine on the rat model of CNV induced by chemical burns did not produce a positive outcome as an anti-angiogenic inhibitor in the present study. Rather, we observed increased vascularization to some extent, which was not supported with significant difference in VEGF-C levels compared to controls. We acknowledge that trial of only one concentration of both topical and subconjunctival fumagillin bicyclohexylamin decreases likelihood to observe a positive outcome.

Only one study on CNV highlighted TNP-470, a synthetic analogue of fumagillin, as an inhibitor of inflammatory corneal angiogenesis on C57BL6 mice. Joussen et al. [1] administered TNP-470 topically at a concentration of 5 mg/mL three times daily and 30 mg/kg systemically once every other day and detected reduced VEGF protein content and reduced endothelial cell proliferation in murine corneas, which contradicts with our findings in rats. Fumagillin was also shown to inhibit the expression of VEGF in uterine cancer cells in vitro [35]. Kanno et al. [36] then investigated the molecule in Kaposi sarcoma-associated herpes virus replication in primary effusion lymphoma cells (PEL) in vivo. However, contrary to the expectation, they could not display an upregulation of p53 promoter activity, which is presumably a hallmark of MetAP-2-related VEGF inhibition in PEL cells treated with fumagillin [36]. In vivo efficacy of fumagillin still remains controversial.

There are still doubts concerning the systemic administration of fumagillin due to its toxic profile. A relevant study tried fumagillin orally up to 60 mg daily for 2 weeks for treatment of microsporidiosis in patients with HIV infection [28]. Significant bone marrow toxicity was reported at the highest dose of 60 mg of fumagillin, which resolved within days after termination of the treatment. Common side effects observed with oral administration of fumagillin in human trials include gastrointestinal-related cramping, diarrhea and significant loss of body weight [37]. The severe side effects associated with fumagillin may preclude the systemic administration of the agent, leaving topical and subconjunctival routes as alternative options. However, the results of our study may not confirm or disprove corneal toxicity due to limitations in design.

The limited number of experimental animals (rats) and the lack of data on optimal topical dosing of fumagillin and subconjunctival concentration and for penetration into inflamed cornea are the major limitations of this study. Yet, we exceeded the concentration used in treatment for microsporidial keratitis in humans by nearly 50%. We also acknowledge the lack of assessment for total VEGF levels other than VEGF-C as a limitation. Further studies comparing different concentrations and dosing may detect inhibitory effects on CNV.

In conclusion, our study was the first to evaluate efficacy of fumagillin bicyclohexylamine on the rat model of CNV induced by burn with validated techniques. Further studies will be required to confirm or disprove the efficacy of fumagillin as an inhibitor on CNV also in other inflammatory cornea models.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal rights statement All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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