Original Article

Intravitreal Topotecan Inhibits Laser-induced Choroidal Neovascularization in a Rat Model

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Abstract

Purpose: A two-phase preclinical study was designed to determine the safe dose of intravitreal topotecan and its inhibitory effect on experimental choroidal neovascularization (CNV) in a rat model.
Methods: In phase I, 42 rats were categorized into 6 groups, 5 of which received intravitreal topotecan injections of 0.125 μg, 0.25 μg, 0.5 μg, 0.75 μg, and 1.0 μg/5 μl, respectively; the control group received an injection of normal saline. Ophthalmic examination and electroretinography (ERG) were performed on days 7 and 28, and enucleated globes were processed for histopathology and immunostaining for glial fibrillary acidic protein. In phase II, CNV was induced via laser burns in 20 rats and the animals were divided into 2 groups. One group received topotecan and the other received normal saline intravitreally. Four weeks later, mean scores of fluorescein leakage on fluorescein angiography as well as mean CNV areas on histology sections were compared.
Results: In phase I, clinical, ERG and histopathologic results were unremarkable in terms of retinal toxicity in all groups. Based on the results of phase I, a dose of 1 μg/5 μl topotecan was chosen for phase II. Leakage scores obtained from late-phase fluorescein angiography were significantly lower in topotecan-treated than control eyes ($P < 0.01$) four weeks after induction of CNV. Compared to control eyes, topotecan-treated eyes showed a significantly lower incidence of fibrovascular proliferation (8.7% vs. 96.2%) and significantly smaller areas of CNV ($P < 0.01$).
Conclusion: Intravitreal injection of topotecan at a dose of 1 μg/5 μl is safe and may be a promising treatment for CNV.

Keywords: Choroidal Neovascularization; Electroretinography; Intravitreal Injection; Topotecan

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INTRODUCTION

Choroidal neovascularization (CNV) is the main cause of substantial vision loss in wet-type age-related macular degeneration (AMD), affecting millions of elderly individuals worldwide.[1-4] This aberrant

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neovascularization, similar to other retinal ischemic diseases such as diabetic retinopathy or retinopathy of prematurity, develops and progresses with hypoxia signaling and consequent pathological activation of the hypoxia inducible factor (HIF) pathway. The activation of this cascade leads to expression of pro-angiogenic genes such as vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), stromal derived growth factor-1 (SDF-1), placental growth factor (PlGF), and platelet-derived growth factor (PDGF). Pathological neovascularization is initiated due to the coordinated functions of these factors.[5-10] Currently, intravitreal injections of anti-VEGF medications are the standard treatment for CNV.[2,11-14] However, limitations such as a high treatment cost and the risk of endophthalmitis associated with repeated intravitreal injections are concerns.[15-17] Moreover, multiple intravitreal injections of anti-VEGF agents increase the risk of retinal pigment epithelial atrophy.[18] Hence, seeking new treatment strategies for CNV are necessary. Since pathological activation of the HIF pathway is considered as the master regulator of ocular neovascularization,[9] targeting this pathway may be a potentially effective therapeutic strategy for CNV.

Amongst the chemical compounds that inhibit HIF activity, topotecan, a semisynthetic water-soluble analog of the plant alkaloid camptothecin, inhibits HIF-1α transcription and its angiogenic properties.[19-22] As a topoisomerase I inhibitor, topotecan also exhibits antitumor effects in a variety of pediatric tumors, such as retinoblastoma, by generating a double-stranded DNA break and consequent cellular apoptosis.[23-29] In order to reduce the side effects of systemic administration of topotecan for treatment of retinoblastoma, periocular and intraocular administration routes were evaluated in a series of studies.[30-35] Intravitreal injection of 5 μg topotecan resulted in high concentrations and substantially improved bioavailability in rabbit vitreous for up to 48 h, with the advantages of significant reduction of intravitreal dose and lack of systemic or retinal toxicity.[34,35]

To the best of our knowledge, the anti-angiogenic activity of topotecan for treatment of neovascular AMD has not yet been reported. This study was conducted in two phases in order to determine the safe dose of intravitreal topotecan and to investigate its inhibitory effect in an experimental CNV model in rats.

METHODS

Study Design

A two-phase study was designed to determine (i) the safety of intravitreal injection of topotecan and (ii) the inhibitory effect of intravitreal topotecan in a rat model of laser-induced CNV.

Animal Models, Preparation, and Grouping

Sixty-two Lister Hooded pigmented rats (Razi Institute for Vaccine and Serum Research, Hessarak, Karaj, Iran) aged 5-6 months and weighing 250-300 g were included. The animals were maintained in plastic cages under a 12/12 hour dark-light cycle with access to water and commercial rodent food *ad libitum*. All experimental procedures were conducted with adherence to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic research and were approved by the University of Tehran School of Veterinary Medicine animal care and utilization committee. Before starting the experiments, all animals were examined using a slit lamp biomicroscope and an indirect ophthalmoscope. Animals with any ocular abnormality were excluded from the study. For all procedures, the rats were anesthetized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Pupillary mydriasis was induced using 1% tropicamide (Mydrax, Sina Daru, Tehran, Iran).

Phase I

Forty-two rats were randomized into 6 groups, 5 of which received intravitreal topotecan injections of 0.125 μg (A), 0.25 μg (B), 0.5 μg (C), 0.75 μg (D), and 1.0 μg (E) per 5 μl of normal saline, and the control group (F) received 5 μl of normal saline in the right eyes. The contralateral eyes were not injected and served as non-injected controls. Injections were performed under sterile conditions using a surgical microscope by an expert ophthalmologist (RN) who was unaware of the doses. Ophthalmic and electroretinography (ERG) examinations were performed at baseline and on days 7 and 28. Clinical signs such as ocular inflammation, cataract formation and retinal damage were investigated after the injections. After the last examination, the animals were sacrificed and enucleated globes were processed for histopathological examination and immunohistochemical studies for glial fibrillary acidic protein (GFAP). Based on clinical and paraclinical data, the maximum safe dose was chosen for phase II.

Electroretinography

Baseline electroretinograms were obtained just before injections. ERG was performed as described before.[36] Briefly, the rats were adapted to darkness for 12 h. All of the following procedures were performed under deep red illumination. The animals were anesthetized with ketamine and xylazine. The rats were then placed on a heated platform (temperature, 38°C) to keep their body temperature constant during the measurements. Their pupils were dilated using a single drop of 1% tropicamide (Mydrax, Sina Daru, Tehran, Iran). Two reference electrodes were inserted into the subcutaneous tissue behind the ears and one ground electrode

was inserted subcutaneously at the base of the tail, and electrode impedance was verified. Thereafter, a gold-wire electrode (Roland Consult, Brandenburg, Germany) internally covered with a small amount of 2% methylcellulose gel (EyeGel, Eyeol, UK) was positioned to touch the central cornea. Again, the rats were adapted to darkness for 10 min. The rats were exposed to standardized flashes of light in a Ganzfeld bowl. Responses were recorded using a Retiport/scan electrophysiological unit (RETIanimal Roland Consult, Brandenburg an der Havel, Germany) with scotopic flash ERG at light intensities of 0.01, 3, and 10 cd.s/m^2, and an additional run for photopic flash ERG after light adaptation for 10 min. The light intensity used for flashes in the photopic ERG measurements was 3 cd.s/m^2. Amplitudes of the a-wave were measured from baseline to the trough of the a-wave, and b-wave amplitudes were measured from the trough of the a-wave to the peak of the b-wave. In the absence of an a-wave, b-wave amplitude was calculated from baseline to the peak of the b-wave. ERG measurements were repeated in the same manner described above, 7 and 28 days after the injections. ERG measurements of the topotecan-injected (right eye) and non-injected control eyes (left eye) were recorded simultaneously. To reduce individual and daily variations in ERG amplitudes, mean values of right to left ratios for a- and b-wave amplitudes were calculated and compared between the study groups. ERG results in each group were also compared at different time points of the study.

Histopathologic and immunohistochemical examinations

After the last ERG assessment, the animals were euthanized by a rapid intracardial injection of an overdose bolus of ketamine and xylazine. Both eyes were enucleated immediately and the globes were fixed in 10% formalin for 24 h. Each globe was bisected axially and after tissue processing and embedding into paraffin blocks, thin tissue sections were prepared at 5 different tissue planes (200 µm apart). Hematoxylin and eosin staining and immunohistochemical staining for GFAP were performed. For immunohistochemistry, 5-µm tissue sections were deparaffinized and hydrated by immersion in xylene and graded alcohol solutions. Following treatment with proteinase K (S 3020, Dako, Denmark) for 5 min, the slides were incubated overnight at 4°C with a 1:100 dilution of polyclonal rabbit anti-GFAP protein (S 0334, Dako, Denmark). All the stained slides were evaluated under light microscopy (BX41, Olympus, Japan) by two masked ophthalmic pathologists (MRK, SBH) for the presence of retinal hemorrhage, inflammation, necrosis and atrophy.

The results of GFAP immunoreactivity were scored by the same two ocular pathologists blinded to treated regimens on a scale from 0 to 5: 0, no staining; 1, staining limited to the internal limiting membrane and nerve fiber layer; 2, focal staining of Muller cells involving partial length of the cells; 3, diffuse staining of Muller cells involving partial length of the cells; 4, focal staining of Muller cells involving full length of the cells; 5, diffuse staining of Muller cells involving full length of the cells. Mean score of >2.5 in each study group was considered to be significant. Moreover, mean scores were compared between the study groups.

Phase II

CNV was induced in the right eye of 20 rats and the animals were randomized into treatment (10 animals) and control (10 animals) groups. Immediately after laser exposure, the treatment and control groups intravitreally received the maximum safe dose of topotecan and 5 µl of normal saline, respectively. Four weeks later, the animals were euthanized after fluorescein angiography (FAG) and the CNV areas were assessed by histopathology.

Laser-induced CNV

Anesthetized animals were positioned in front of a slit lamp laser delivery system (Topcon, Tokyo, Japan). An infrared diode laser (Microlase, Keeler Instruments Inc., USA) was used to photocoagulate 8 sites between the major blood vessels in each eye (wavelength, 810 nm; spot size, 100 µm; duration, 0.1 s; and power, 150 mW). Development of an acute vapor bubble indicated rupture of Bruch's membrane.

Fluorescein angiography

FAG was performed after intraperitoneal injection of 1 ml of 10% fluorescein sodium using the HRA2 system (Heidelberg Engineering, Dossenheim, Germany) at a 50° angle of view. Images were recorded 3 to 240 seconds after injections. Lesions observed on late-phase angiography (90-180 seconds after fluorescein injection) were graded in a blinded manner as follows: Grade 0, no leakage; grade 1, mild leakage; grade 2, moderate leakage; and grade 3, extensive leakage.

Measurement of CNV area on histopathologial sections

As described above, formalin-fixed eyes were processed, blocked in paraffin, sectioned at multiple tissue planes, and stained with hematoxylin and eosin. An attempt was made to serially section the entire CNV lesion. Sections were examined under light microscopy (BX41, Olympus, Japan, Tokyo) by two masked ophthalmic pathologists (MRK, SBH). Evaluation and quantification of a CNV lesion in its entirety on histopathological sections was performed as previously described.[37] For each lesion, the section that was estimated to have the largest CNV was chosen and photographed using a digital camera (DP12, Olympus, Japan, Tokyo).

Table 1. Comparison of ERG results between the studied groups in Phase I

Groups/ERG	Group A (0.125 μg)	Group B (0.25 μg)	Group C (0.5 μg)	Group D (0.75 μg)	Group E (1 μg)	Group F (normal saline)	*P*
Preinjection (baseline)							
Scotopic (0.01 cd.s/m^2)							
b-wave	0.98±0.07	0.99±0.14	0.94±0.19	1.0±0.05	1.02±0.05	1.01±0.04	0.908
Scotopic (3 cd.s/m^2)							
a-wave	0.90±0.11	1.07±0.22	0.95±0.15	0.99±0.12	1.01±0.11	1.0±0.14	0.522
b-wave	1.0±0.19	1.07±0.22	0.95±0.15	0.99±0.12	1.01±0.11	1.0±0.14	0.965
Scotopic (10 cd.s/m^2)							
a-wave	0.94±0.04	1.01±0.08	0.97±0.15	1.01±0.1	1.09±0.13	1.0±0.14	0.302
b-wave	1.02±0.2	1.09±0.19	1.03±0.1	1.0±0.08	1.03±0.07	1.01±0.07	0.889
Photopic (3 cd.s/m^2)							
b-wave	1.07±0.13	1.09±0.09	1.06±0.16	1.09±0.16	1.0±0.09	1.04±0.16	0.732
Post-injection (week 1)							
Scotopic (0.01 cd.s/m^2)							
b-wave	0.99±0.02	1.09±0.31	1.0±0.19	1.07±0.38	1.04±0.1	1.05±0.17	0.968
Scotopic (3 cd.s/m^2)							
a-wave	0.98±0.22	0.97±0.12	1.07±0.09	1.02±0.07	0.96±0.1	0.92±0.12	0.323
b-wave	0.94±0.04	1.05±0.12	1.04±0.15	0.99±0.08	0.99±0.14	1.1±0.3	0.265
Scotopic (10 cd.s/m^2)							
a-wave	1.01±0.11	1.0±0.03	1.03±0.06	1.1±0.2	1.01±0.07	0.96±0.11	0.491
b-wave	1.01±0.15	0.96±0.13	1.08±0.08	1.0±0.02	1.01±0.06	1.04±0.09	0.355
Photopic (3 cd.s/m^2)							
b-wave	0.94±0.04	0.98±0.09	0.98±0.12	1.0±0.05	0.98±0.08	0.99±0.07	0.376
Post-injection (week 4)							
Scotopic (0.01 cd.s/m^2)							
b-wave	0.88±0.1	1.03±0.14	0.93±0.12	0.96±0.21	1.02±0.13	0.99±0.14	0.408
Scotopic (3 cd.s/m^2)							
a-wave	0.95±0.18	1.02±0.09	1.02±0.22	1.0±0.08	0.97±0.07	0.99±0.13	0.809
b-wave	0.91±0.14	1.15±0.14	1.06±0.33	1.0±0.06	1.08±0.09	1.02±0.05	0.149
Scotopic (10 cd.s/m^2)							
a-wave	0.96±0.06	0.94±0.12	1.03±0.07	1.0±0.04	1.01±0.05	1.02±0.08	0.823
b-wave	1.0±0.08	1.07±0.2	0.95±0.08	1.0±0.11	1.01±0.05	1.01±0.04	0.751
Photopic (3 cd.s/m^2)							
b-wave	0.95±0.05	1.01±0.12	0.98±0.08	0.96±0.11	1.01±0.12	1.0±0.09	0.985

Mean right-to-left ratio for scotopic ERG a- and b-wave amplitudes and photopic ERG b-wave amplitude in Groups A to F at baseline and days 7 and 28. *P* values for comparison between the groups at each time point are also shown in travitreal injections of topotecan with doses of 0.125 μg (Group A), 0.25 μg (Group B), 0.5 μg (Group C), 0.75 μg (Group D), and 1 μg (Group E) were performed. Group F received 5 μl of normal saline. ERG, electroretinography

Images were transferred to a computer and the area of CNV was outlined and measured using ImageJ software (version 1.48; U.S. National Institute of Health, Bethesda, MD, USA).

Statistical Analysis

One-way analysis of variance (ANOVA) was employed to test the differences between ERG findings in treated and control eyes. This test was also used to compare mean scores of GFAP immunoreactivity between the study groups. The significance of differences between the two time schedules for each group was tested using the paired *t*-test. *P* values less than 0.05 were considered as statistically significant. Mean CNV areas and mean angiographic scores in the treatment and control groups were analyzed using the Mann-Whitney test with significance set at $P < 0.01$. Data were analyzed using a statistical analysis software (SPSS, version 20; IBM, Chicago, IL).

RESULTS

Phase I

Follow-up examinations in phase I of the study revealed no abnormalities in eyes injected with topotecan and balanced salt solution (BSS) except for cataract formation

in three eyes and retinal detachment in one eye. Slit lamp examinations on day 7 revealed a posterior subcapsular cataract in one eye in the injected control group, one eye in Group A, and one eye in Group C, which progressed to mature cataract based on examinations on day 28. These eyes were excluded from ERG studies. Retinal detachment was diagnosed in one eye in Group E just after injection; this eye was also excluded from the study.

ERG

Table 1 shows the mean right-to-left ratios for scotopic ERG a- and b-wave amplitudes and photopic ERG b-wave amplitudes in Groups A to G at baseline, and at days 7 and 28. The ERG pattern and implicit times were almost identical in all groups. No significant differences in the amplitude ratios were observed between the groups at the specified time-points. Moreover, within each group, the differences between the records at different time points of the study were not significant.

Light microscopy

Mild inflammation at the injection site was the only observed abnormality in the topotecan-and BSS-injected eyes. No sign of intraretinal hemorrhage, retinal inflammation, necrosis or atrophy attributable to topotecan injection was observed. The overall histological appearance of topotecan- and BSS-injected eyes was identical and similar to that of non-injected eyes in terms of preserved integrity and cellular organization of retinal layers. None of the study groups developed significant GFAP immunoreactivity [Figure 1]. Average score of GFAP immunoreactivity in the topotecan-injected groups (A: Mean [SE], 1.86 [0.83]; B: Mean [SE], 1.17 [0.37]; C: Mean [SE], 2 [1.07]; D: Mean [SE], 1.57 [1.05]; and E: Mean [SE], 1 [0.0]) was not significantly different from that of the normal saline-injected group (F: Mean [SE], 1.17 [0.37]) (P = 0.148). Overall, light microscopic examination showed no signs of toxicity attributable to intravitreal injection of topotecan, correlating with ERG results, and 1 µg/5 µl was chosen as the maximum safe dose for intravitreal injection of topotecan in phase II.

Phase II

CNV scores in FAG

FAG revealed development of CNV in the laser-burnt sites 4 weeks after the application of laser. CNV areas appeared as early hyperfluorescent zones with gradual expansion and blurred margins on late phase FAG. The mean score of late-phase lesions in topotecan-injected eyes was significantly lower than that of the controls (0.125 ± 0.46 vs. 2.437 ± 0.72, $P < 0.001$) [Figure 2].

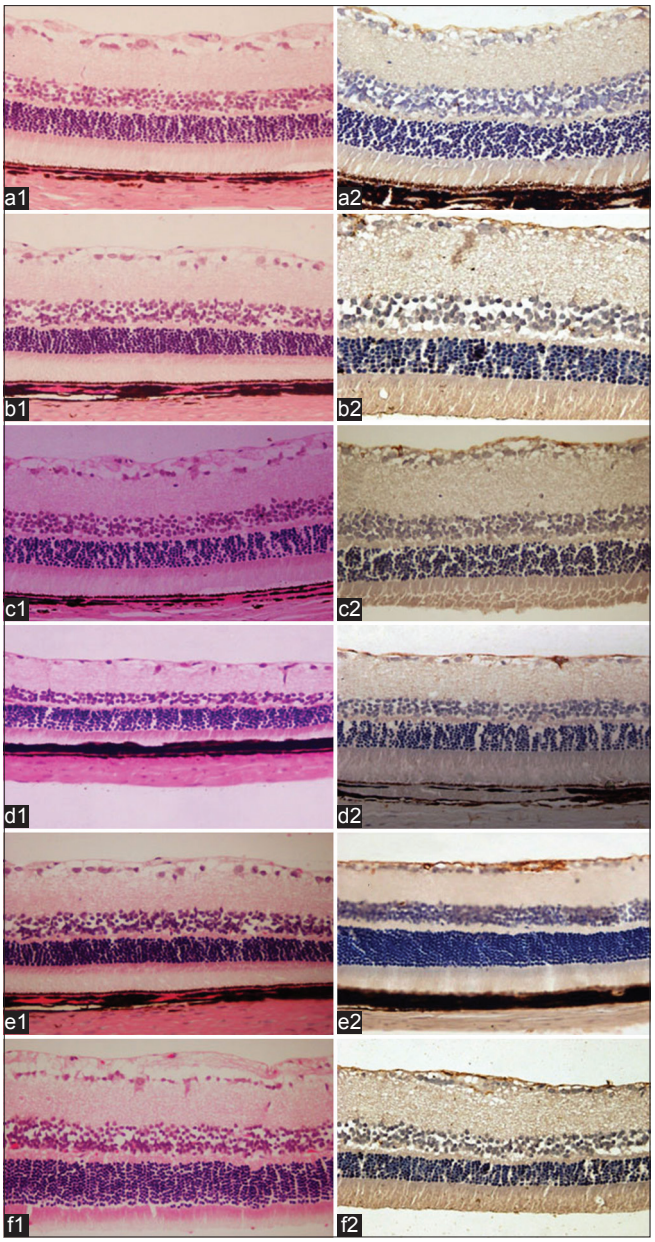

Figure 1. Intact retinal histology and unremarkable GFAP immunoreactivity in topotecan-injected eyes. Represented photomicrographs of intact rat retinas from topotecan-injected groups (a1-e1; a1: Group A, b1: Group B, c1: Group C, d1: Group D, and e1: Group E) and normal saline-injected control group (f1) (H and E, ×400). Note the non-significant GFAP immunoreactivity of the retinas in the topotecan-injected eyes (a2-e2; a2: Group A, b2: Group B, c2: Group C, d2: Group D, e2: Group E) compared to that in the control eyes (f2) (×400).

CNV areas on histopathologic sections

Eyes treated with intravitreal topotecan showed significantly smaller neovascular choroidal outgrowths [Figure 3a and b] as compared to control eyes; this finding was concordant with FAG results. An approximately 3.3-fold decrease in average CNV area in each eye was observed in

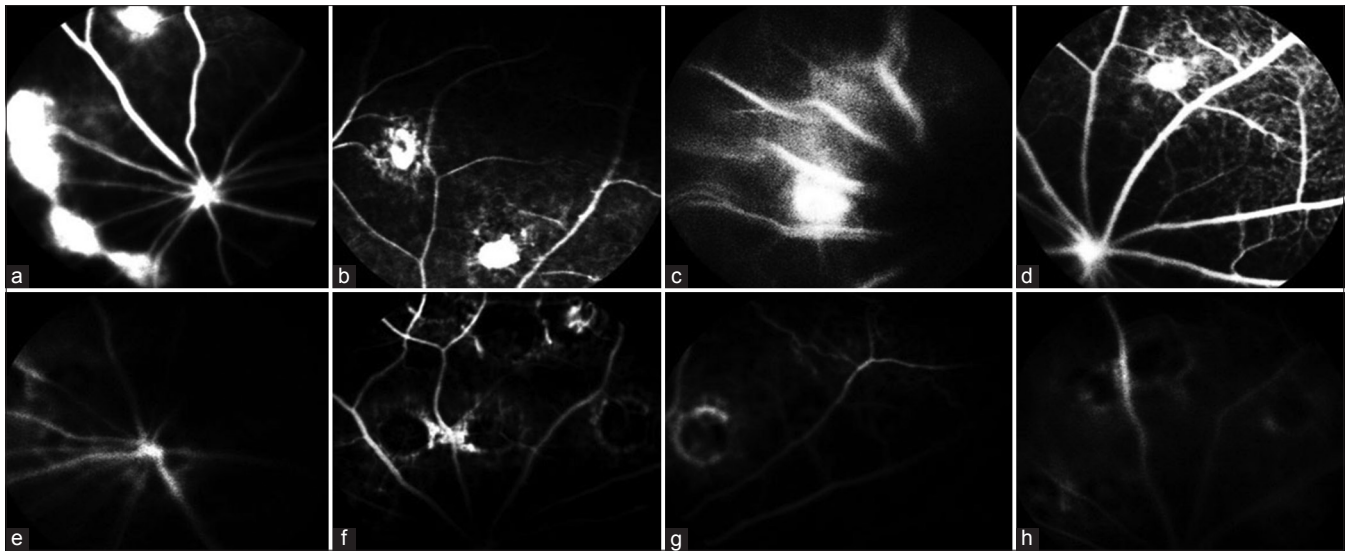

Figure 2. Attenuation of fluorescein leakage in topotecan-injected eyes. Note the significant late fluorescein leakage from laser-induced CNV in the normal saline-injected eyes (a-d) compared to the attenuated leakages in the topotecan-injected eyes (e-h) on FAG.

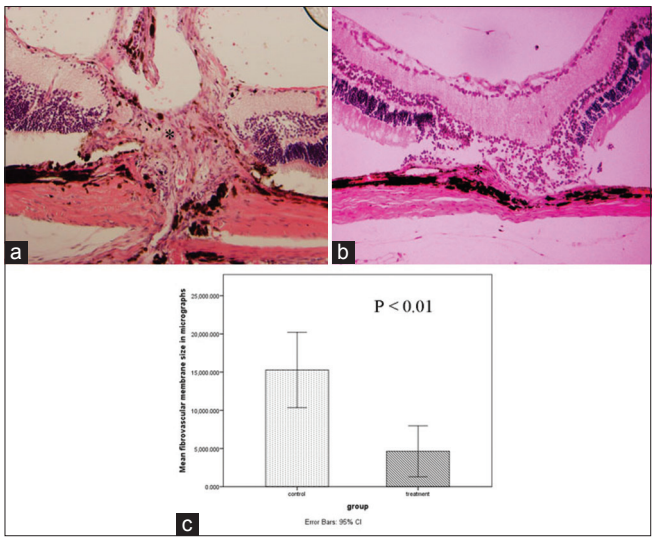

Figure 3. Attenuation of CNV following intravitreal administration of topotecan in laser-induced CNV rat model. Representative histologic sections of CNV four weeks after laser photocoagulation (a and b), CNV (asterisk) in rats that received intravitreal normal saline (a) and topotecan (b). (c) Quantification of data. A 3-fold decrease in the area of CNV was observed in rats that received intravitreal topotecan compared to that in the controls (*$P < 0.01$).

topotecan-treated eyes (mean [SE], 4635.72 [1346.07] μm^2; $n = 10$ eyes) compared to that in the control eyes (mean [SE], 15276.83 [8142.58] μm^2; $n = 10$ eyes), which was statistically significant ($P < 0.01$) [Figure 3c].

DISCUSSION

Clinical, ERG and light microscopic results of this study suggested that a single intravitreal injection of topotecan with doses of up to 1 µg/5 µl had no toxic effect on the rat retina. More interestingly, FAG and histopathologic findings revealed that topotecan, when injected intravitreally at a dose of 1 µg/5 µl, could effectively inhibit angiogenesis in a rat model of laser-induced CNV. Our results suggest that intravitreal topotecan has anti-HIF properties, and support its use as a novel approach for treatment of neovascular AMD.

In addition to VEGF as the leading cause of CNV formation, other angiogenic and growth factors such as IGF-1 have been found to be present in choroidal neovascular membranes of patients with AMD.[38-41] It has been demonstrated that IGF-I signaling, through induction of HIF-1α expression, up-regulates VEGF expression in cultivated neuroblastoma cells,[29] and induces corneal and retinal angiogenesis in rabbit models.[42] It has also been shown that topotecan, a topoisomerase I poison, inhibits HIF-1 transcriptional activity and HIF-1 protein accumulation in human glioma cells under hypoxic conditions.[28] Moreover, topotecan has been demonstrated to inhibit HIF-1 mRNA translation without affecting HIF-1 protein half-life or mRNA accumulation.[27] Therefore, topotecan, which targets HIF-1α as the principal regulator of ocular neovascularization, can theoretically inhibit CNV through its well established negative impact on angiogenic factors.[9,10]

Although not documented, the cost of topotecan is less than other current anti-VEGF drugs. However, further investigations are necessary to determine the cost effectiveness, potency and persistent anti-angiogenic effects on CNV formation of this drug as compared to current anti-CNV medications.

Despite the well-known consequences of systemic administration of topotecan, such as gastrointestinal

disturbances and myelosuppression, this drug has been reported to have a safe profile for local delivery routes such as periocular or intravitreal injections.[30-35] Moreover, by bypassing the blood-retinal barrier, a stronger effect of topotecan can be achieved when it is administered intravitreally. Buitrago et al[34,35] investigated the pharmacokinetics as well as the safety of a single intravitreal administration of 5 µg topotecan in rabbit eyes and demonstrated that an effective concentration of this drug was maintained for up to 16 h after injection in the vitreous while plasma concentrations were negligible. Moreover, after intravitreal injection of topotecan at a dose 200 times less than the periocular dose, total vitreous exposure was reported to be almost 347 times higher than that associated with the periocular route.[34] Buitrago et al did not report any sign of structural or functional ocular toxicity four weeks after intravitreal injection of topotecan, and these findings are in agreement with those of the current study.

In phase I of our study, all implemented doses of intravitreal topotecan were found to be safe, and therefore, we could not determine the maximum tolerated and/or minimum toxic dose for intravitreal administration of this agent. Further studies with administration of higher doses of intravitreal topotecan should be conducted to elucidate the maximally tolerated dose as well as the ocular pharmacokinetics of that particular dose of topotecan.

In summary, our study showed that intravitreal injection of topotecan up to a dose of 1 µg/5 µl showed no functional or structural retinal toxicity, and that such treatment significantly attenuated laser-induced CNV in a rat model, suggesting that topotecan may serve as a safe and important therapeutic agent for neovascular AMD. To the best of our knowledge, this is the first report on the inhibitory effect of topotecan on CNV. However, further studies should be conducted to investigate the potency and duration of action of this agent in comparison with current medications used for CNV.

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Conflicts of Interest

There are no conflicts of interest.

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