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Evaluation of effect of nilotinib in an experimental corneal neovascularization model

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ABSTRACT

Aim: This study aims to investigate the neovascularization-inhibiting effect of topical nilotinib and to determine the effective dose of nilotinib.

Material and Method: In this study, 42 healthy Wistar albino rats were randomly divided into six groups. The left corneas of all rats except group 1 were cauterized with silver nitrate. Group 1 was the healthy control, with no corneal vascularization, which did not receive any treatment; Group 2 (sham) did not receive treatment, only topical DMSO; Groups 3, 4, and 5 received topical nilotinib at doses of 10, 20, and 40 µM three times a day, respectively; Group 6 received 5 mg/dL topical bevacizumab three times for a day for seven days. On the 8th day, photographs of the corneas were taken, and the percentage of corneal neovascularization area was calculated. Following all rats being killed via anesthesia, the corneas were removed to determine the levels of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) ELISA and corneal immune staining.

Results: Other than Group 3, the percentage of neovascular corneal area was lower in the treatment groups compared to Group 2 ($p<0.05$). The intensity of VEGF and PDGF immune staining was also lower in the treatment groups. The treatment groups showed no significant differences compared to Group 1, except Group 3. The VEGF ELISA levels were statistically significantly lower in the treatment groups compared to Group 2 ($p<0.05$), with the exception of Group 3. The PDGF ELISA levels were statistically significantly lower in the treatment groups compared to Group 2 ($p<0.05$), and the Group 4 PDGF levels were statistically the lowest among the treatment groups.

Conclusion: Nilotinib was as effective as bevacizumab in the regression of corneal neovascularization. We observed that nilotinib was effective at doses of 20 µM and more.

Keywords: Corneal neovascularization, nilotinib, bevacizumab, VEGF, PDGF

INTRODUCTION

The cornea is optically clear, which is necessary to maintain visual acuity; defects in any of its layers due to infection, chemical or traumatic injury, or autoimmune disease can impair corneal clarity and therefore reduce visual acuity. Corneal neovascularization (CNV) has been reported in different corneal pathologies, and vessels sometimes appear to play different roles in the pathology (1). The cornea maintains avascularity by maintaining homeostasis, in which proangiogenic stimuli are balanced by antiangiogenic factors. Vascular endothelial growth factor (VEGF) is a member of the platelet-derived growth factor (PDGF) supergene family. VEGF has five sub-members, namely VEGF-A, -B, -C, -D, and placental growth factor (PGF), which can bind to three separate tyrosine kinase cell surface VEGF receptors (2).

PDGFs are growth factors released by vascular endothelial cells (VECs) in sprouting vessels and, like VEGFs, act through binding to the tyrosine kinase receptor (3). Antiangiogenic efficacy can be significantly affected by the inhibition of both VEGFRs and PDGFRs by a tyrosine kinase inhibitor (TKI) (4).

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Bevacizumab is a monoclonal antibody that binds to VEGF, thereby inhibiting VEGF-mediated signaling pathways and blocking angiogenesis (5). Bevacizumab, originally approved for the treatment of metastatic colorectal cancer, has been used in ophthalmology (off-label) with promising results in the treatment of exudative age-related macular degeneration, proliferative diabetic retinopathy, retinal vein occlusion, and iris rubeosis (6-8). Recently, studies have reported topical and subconjunctival bevacizumab for the treatment of corneal neovascularization. Although corneal neovascularization was not completely eliminated in these studies, it was reported that bevacizumab had a significant effect (9-14).

Nilotinib is a new-generation TKI that inhibits PDGF and indirectly VEGF, it has been approved for the treatment of chronic myeloid leukemia (CML) and shown to reduce fibrosis (15-18). In this study, we evaluated the clinical safety and efficacy of topical nilotinib for the treatment of CNV and compared these effects with topical bevacizumab.

MATERIAL AND METHOD

All procedures were carried out in accordance with the ethical rules and the principles. This study was carried out with the permission of The Fırat University Experimental Animal Studies Ethics Committee (Date: 02.09.2020, Decision No: 2020/05).

This study used 42 Wistar albino male rats aged 8–10 weeks and weighing 250–300 g. A power analysis was conducted to determine the number of animals to be used in the experiments, whether the animals were to be divided into groups (and if so, each group would have at least eight animals), and with 8% deviation, type 1 error (α)=0.05, and type 2 error (β) (power=0.80) In order for the rats to continue feeding, chemical burns were created only on the left cornea with the use of silver nitrate.

Bevacizumab (Altuzan, Roche, USA) and nilotinib (Tasigna, Novartis, Switzerland) were dissolved in dimethylsulfoxide (DMSO). Topical treatment started one hour after the corneal burn procedure and continued for seven days (19). These rats were randomly divided into six equal groups: Group 1 was the healthy group, where a chemical corneal burn was produced in all the other groups. Group 2 (sham group) did not receive treatment, only topical DMSO three times a day. Groups 3–5 received 10 μM, 20 μM, 40 μM (respectively) topical nilotinib and Group 6 received 5 mg/dL topical bevacizumab three times a day (20).

Rat corneas were photographed at X40 magnification using a Sony digital camera (CCD IRIS model DXC 107 AP) mounted on a slit lamp microscope. The percentage of neovascularization area in the photograph to the entire corneal area was calculated using MATLAB R2007b version 7.5 (MathWorks, Natick, Massachusetts, USA) software.

The enucleated eyes were fixed in 10% buffered formalin solution and cut in the dorsoventral position so that the cornea, iris, lens, and optic nerve were in the same plane. Tissue samples were then routinely processed and cut to a thickness of 5 μm and stained by Hematoxylin & Eosin (H&E). Selected samples were stained by periodic acid–Schiff and Masson's trichrome. The thickness of the central cornea and corneal epithelium was measured via software. The number of leucocytes and vessels was counted in per mm^2 in corneal stroma between the limbuses. The percentage of damaged corneal epithelium was determined by measuring damaged epithelium/total epithelium.

The avidin–biotin complex (ABC) method was used for immunohistochemistry. The sections were deparaffinized in xylene and dehydrated using a series of graded alcohols. An UltraVision™ ONE Detection System: HRP Polymer/AEC Chromogen (ThermoFisher Scientific, Rockford, IL, USA) was used according to the manufacturer's protocols. Briefly, antigen retrieval was accomplished by microwaving the sections for 15 min in citrate buffer at pH=6 and then allowed to cool for 20 min. The sections were washed in PBS, and primary antibodies were applied after a hydrogen peroxide block (5 min) and an Ultra-V block (5 min). The sections were incubated in primary antibodies, including VEGF (Bioss, 1/100, bc-0279R) and PDGF (PDYN, 1/100, A5830) Then, an incubation with primary antibodies against immunodetection was performed for 60 min at 37°C with biotinylated goat anti-polyvalent, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3- amino-9-ethylcarbazol (AEC, ThermoFisher Scientific, Rockford, IL, USA) as the chromogen substrate. The sections were counterstained with Gill's hematoxylin for 30 sec, and coverslips were attached using aqueous mounting media.

The corneal tissue samples were studied immediately. The protein levels in the supernatants were determined by the Lowry method (21). The VEGF and PDGF levels in the supernatants were measured using the Enzyme-Linked Immuno Sorbent Assay (ELISA) method. All results were calculated in units of mg/protein.

The tissue VEGF levels in the supernatant were studied using the rat VEGF-ELISA kit (Sunred Biotechnology Company, reference no. 201-11-0660) in accordance with the kit procedure. Absorbances were read

spectrophotometrically at 450 nm in an EPOCH 2 (BioTek Instrument, Inc., USA) microplate reader. All results are expressed in units of ng/L. The measuring range of the kit was 11–3000 ng/L, and its sensitivity was 10.127 ng/L. The intra-assay CV was <9%, and the inter-assay CV was <11%.

The tissue PDGF levels in the supernatant were studied using the rat PDGF ELISA kit (Sunred Biotechnology Company, reference no. 201-11-0692) in accordance with the kit procedure. Absorbances were read spectrophotometrically at 450 nm in an EPOCH 2 (BioTek Instrument, Inc., USA) microplate reader. All results are expressed in units of ng/mL. The measuring range of the kit was 0,05–15 ng/mL, and its sensitivity was 0,05 ng/mL.

This study was conducted in accordance with the ARVO Ophthalmic and Vision Research Statement on Animal Use. The subjects were kept at room temperature (22–25°C) for 12 h of light (7:00 AM to 19:00 PM) and 12 h of darkness (19:00 PM to 7:00 AM) and fed ad libitum in specially built cages.

Statistical Analysis

Data obtained in the study were analysed statistically using the Statistical Package for the Social Sciences (SPSS) version 22.0 software (SPSS Inc., Chicago, IL, USA). The data obtained were stated as mean±standard deviation (SD). The One-Way ANOVA test was applied for multiple comparisons. In paired comparisons between groups, the post hoc Tukey test was applied. A value of $p<0.05$ was accepted as statistically significant.

RESULTS

Neovascular Area

The neovascularization area percentage was significantly smaller in Groups 4, 5, and 6 compared to Group 2 ($p<0.05$). Although the area of neovascularization was less in Group 3 compared to Group 2, no statistically significant difference was observed ($p>0.05$). Further, there was no statistically significant difference when Groups 4, 5, and 6 compared to each others ($p>0.05$) (**Figure 1**).

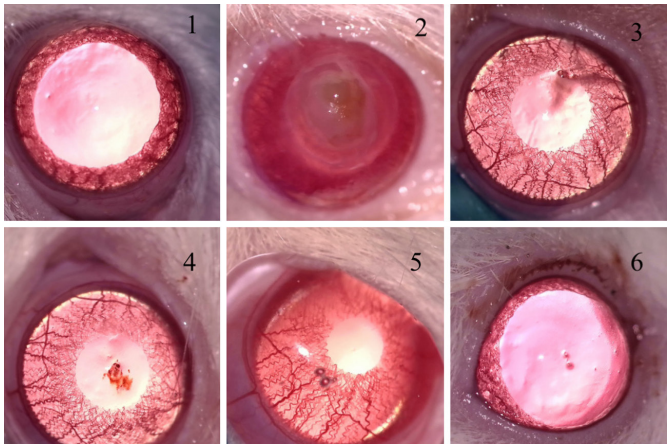

Figure 1. Comparison of corneas in control and treatment groups,.1: Normal cornea in Group 1. 2: Diffuse keratitis and severe neovascularization in Group 2. 3: Moderate neovascularization in Group-3. 4-6: Minimal epithelial keratinization and mild neovascularization in Group 4,5 and 6.

Histopathological Changes

In Group 1, corneas were well organized with the epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium. No inflammatory cell infiltration was present. However, all animals in Group 2 and some rats in Group 3 showed corneal neovascularization, multifocal moderate polymorphonuclear cell (PMNC) activity, macrophage infiltration, corneal hemorrhage, corneal rupture, and anterior uveitis (**Figure 2**). Uveitis lesions were characterized by neutrophilic infiltrations in the iris leaflet and processus ciliaris.

These changes were not noted in other treatment groups, including Groups 4, 5, and 6. However, these animal groups showed corneal stromal scar formation and thinning of the epithelial layer (**Figure 2**). The subepithelial scars consisted of either fibroblasts or collagen fibers.

The number of corneal vessels and inflammatory cells were higher in Group 2 than the treatment groups ($p<0.05$). In fact, almost no inflammatory cell infiltration or neovascularization was detected in Groups 4, 5, and 6 (**Table 1**). Groups 4, 5, and 6 prevented inflammatory infiltration and vascularization. Corneal thickness and central corneal epithelial thickness were altered in the treatment groups as compared to the control group. However, this increase was not statistically significant ($p>0.05$) (**Table 1**).

Table 1. Histological measurements in control and treatment groups

Groups	Number of vessels/ corneal stroma (mm²)	Number of PMN cells/ corneal stroma (mm²)	Central corneal thickness	Central corneal epithelial thickness	Epithelial damage (as % of corneal epithelium)
Group 1	0.00 ^{b*c*}	0.00 ^{b*c*}	106.32±21.94 ^{b*c*}	28.55±4.78	0.00 $^{b*c*\ d*e*f*}$
Group 2	4.42±3.06^{a*c*d*e*f*}	5.99±7.81^{a*c*d*e*f*}	229.24±106.87 ^{a*d*e*f*}	26.52±8.80	12.13±4.95 ^{a*d*e*f*}
Group 3	1.15±1.62^{a*b*d*e*f*}	1.90±2.44 ^{a*b*d*e*f}	205.34±49.73 ^{a*d*e*}	28.95±19.50	9.68±3.95 $^{a*d*\ e*}$
Group 4	0.00 ^{b*c*}	0.00 ^{b*c*}	150.34±17.19 ^{b*c*}	28.55±11.53	2.87±1.17 ^{a*b*c*f*}
Group 5	0.00 ^{b*c*}	0.00 ^{b*c*}	139.23±13.18 ^{b*c*}	34.45±10.94	1.20±1.57 $^{a*b*c*\ f*}$
Group 6	0.00 ^{b*c*}	0.00 ^{b*c*}	174.66±12.79 ^{a*b*}	26.73±12.01	7.79±1.88 $^{a*b*d*\ e*}$

a: According to the group 1, b: According to the group 2, c: According to the group 3, d: According to the group 4, e: According to the group 5, f: According to the group 6 shows significant difference (*=p<0.05)

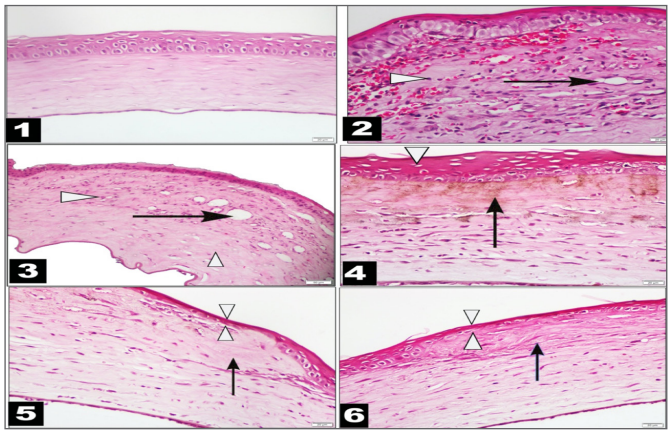

Figure 2. Comparison of histological analysis of the corneas in control and treatment groups, H&E, x40. 1: Normal corneal histology in Group 1. 2: Epithelial necrosis (arrow head), diffuse neutrophilic infiltration in corneal stroma and anterior chamber in Group 2. 3: Moderate neutrophilic infiltrate (arrow head) and neovascularization (arrow) in Group-3. 4: Epithelial keratinization and subepithelial fibrosis in Group-4. 5: Thinning in corneal epithelium and subepithelial fibrosis in Group-5. 6: Thinning in corneal epithelium (arrow heads) and subepithelial fibrosis in Group-6.

VEGF ELISA Levels

The mean VEGF levels in Groups 1, 2, 3, 4, 5, and 6 were 597.78±41.65, 1204.69±242.99, 863.57±72.17, 611.54±72.35, 643.48±124.30, and 674.69±96.61 pg/mg protein, respectively. A comparison of Groups 1 and 2 revealed that VEGF levels were significantly increased in the sham group ($p<0.05$). A comparison of Groups 2 and 6 revealed that VEGF levels were significantly decreased in the bevacizumab group ($p<0.05$). While group 3 and bevacizumab groups were compared, VEGF levels significantly decreased ($p<0.05$), it was statistically insignificant when compared with Groups 4 and 5 ($p=0.96$ and 1.00, respectively) (**Table 2**).

Table 2. VEGF and PDGF ELISA levels in control and treatment groups

Groups	VEGF (pg/mg protein)	PDGF (pg/mg protein)
Group 1	597.78±41.65^{b*c*}	4.79±1.06 ^{b*c*}
Group 2	1204.69±242.99^{a*d*e*f*}	10.62±2.02 ^{a*d*e*f*}
Group 3	863.57±72.17 ^{a*d*e*f*}	8.02±0.68^{a*d*}
Group 4	611.54±72.35 ^{b*c*}	5.13±0.68^{b*c*e*f*}
Group 5	643.48±124.30 ^{b*c*}	7.31±1.11^{b*d*}
Group 6	674.69±96.61 ^{b*c*}	7.83±1.55^{b*d*}

a: According to the group 1, b: According to the group 2, c: According to the group 3, d: According to the group 4, e: According to the group 5, f: According to the group 6 shows significant difference (*:$p<0.05$)

PDGF ELISA Levels

The mean PDGF levels in Groups 1, 2, 3, 4, 5, and 6 were 4.79±1.06, 10.62±2.02, 8.02±0.68, 5.13±0.68, 7.31±1.11, and 7.83±1.55 pg/mg protein, respectively. A comparison of Groups 1 and 2 revealed a significant increase in PDGF levels in the sham group ($p<0.05$). PDGF levels signigficantly decreased while bevacizumab group compared with Group 2 ($p<0.05$), it was statistically insignificant when compared with Groups 3 and 5 ($p=1.00$ and 0.97, respectively). Among the nilotinib groups, Group 4 PDGF levels significantly decreased when compared with Groups 3 and 5 ($p<0.05$) (**Table 2**).

Immunohistochemical Findings: The positive immunoreaction of VEGF was characterized by red granules in the cytoplasm of infiltrated PMNC, macrophages, neovascular endothelium, and limbal vasculature. Positive staining for VEGF was detected in Groups 2 and 3 in the corneal inflammatory infiltrate. In other treatment groups, no positive immunostaining was detected (**Figure 3**).

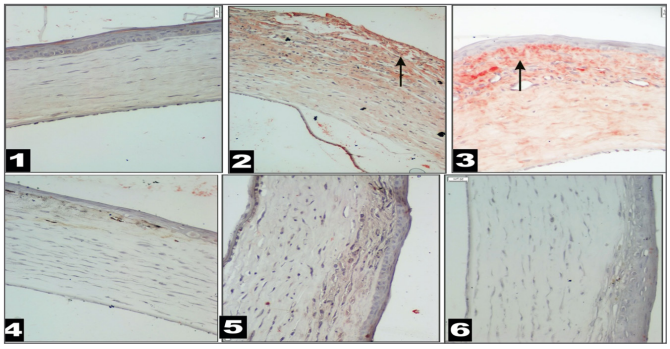

Figure 3. Comparison of immunohistochemical analysis of the VEGF in corneas in control and treatment groups, ABC Method, x40. 1: No positive immunoreactivity to VEGF in Group-1. 2: Diffuse immunoreaction in in neutrophiles (arrow), macrophages, and keratocytes in Group-2. 3: Diffuse staining in inflammatory infiltrate in neutrophiles (arrow), macrophages, and keratocytes in Group-3. 4-6: No immunostaining in other treatment groups, including Groups 4, 5, and 6.

The positive expression of PDGF was detected by red granules in the cytoplasm of fibroblasts, vascular endothelium, and keratocytes in subepithelial scar tissue. No positive expression of PDFG was seen in Group 1 (**Figure 4**).

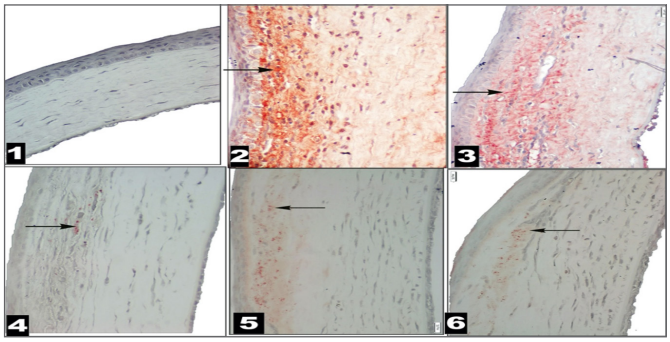

Figure 4. Comparison of immunohistochemical analysis of the PDGF in corneas in control and treatment groups, ABC Method, x40. 1: No positive immunoreactivity to PDGF in Group-1. 2: Diffuse immunoreaction in fibroblasts (arrow), vascular endothelial cells and keratocytes in Group-2. 3: Diffuse immunoreaction in fibroblasts (arrow), keratocytes, and vascular endothelial cells in Group-3. 4: Weak immunostaining in fibroblast in subepithelial scar tissue in Group-4. 5-6: Mild to moderate immunostaining in fibroblast in subepithelial scar tissue in Groups 5 and 6.

DISCUSSION

In our study, we investigated the effect of nilotinib on experimental corneal neovascularization, tried to determine the most effective dose, and compared these treatments with those of bevacizumab. We observed that the effect of 20 μM and 40 μM nilotinib doses on VEGF was similar to that of bevacizumab, but the 20 μM nilotinib dose was more effective on PDGF than bevacizumab and other nilotinib doses.

CNV can occur due to chemical burns, ischemia, infection, trauma, and inflammation, and it affects approximately 1.4 million people per year (22). The main causes of corneal neovascularization are infectious diseases, inappropriate contact lens use, and the vascular response to corneal transplantation (23). Neovascularization is created by many cellular factors (24).

VEGF expression is elevated in both animal models and human corneas with CNV and is secreted by multiple cell types, such as epithelial cells, vascular endothelial cells, macrophages, and fibroblasts (25,26). VEGF is a family of proteins with five members (VEGF-A, -B, -C, -D, and PGF) that bind to three distinct tyrosine kinase cell surface VEGF receptors and are key mediators in the development of neovessels (24-27). VEGF-A is the target of several drugs and mediates pathological neovascularization through VEGFR2 activation. VEGF-A receptors, VEGFR1 and VEGFR2, are secreted by VECs. VEGF-induced effects enhance the growth, migration, and survival of endothelial cells (26-28).

PDGF is an important growth factor released by VECs for neovascularization. Newly formed vessels will regress spontaneously unless they are surrounded by pericytes, an event promoted by PDGF-β. PDGF stimulates VEGF transcription via the tyrosine kinase PDGF receptors (a and β). Sprouting endothelial cells secrete PDGF, and pericytes express PDGFR-β. Therefore, inhibition of the PDGF signaling pathway impairs pericyte recruitment. PDGF plays a stabilizing role on newly formed blood vessels. In one study, inhibition of PDGFR-β not only had a longer efficacy but also resensitized CNV to VEGF blockade. PDGF is effective in the progression from VEGF-dependent nascent neovascular sprouts to stable, mature vessels (24,29,30).

Antiangiogenic therapy holds great promise for the treatment of corneal neovascularization. Bevacizumab, an anti-VEGF monoclonal antibody, has been applied in the treatment of a variety of systemic malignancies (31). The effects of topical bevacizumab on the inhibition of corneal neovascularization have been demonstrated in many studies (11,20,32-34). Although anti-VEGF agents are effective, VEGF is not the only molecule involved in angiogenesis. PDGF is a molecule that also affects angiogenesis, and inhibiting this pathway could be more effective. It was determined that simultaneously blocking both the PDGF and VEGF pathway was more effective than blocking each pathway alone (29). Yet another study found that combination therapy with sunitinib resulted in greater inhibition of neovascularization in animals treated with bevacizumab alone (35).

Nilotinib is a TKI that is very potent in the treatment of CML and targets several TKs (15-17); although it has been used in eye surgery before due to its antifibrotic effects (36), it also has anti-angiogenic properties by acting on VEC and inhibiting PDGF (15,37,38). In a study by van Steensel et al. (39), in which the effectiveness of imatinib and nilotinib on orbital fibroblasts was evaluated in vitro, it was shown that imatinib and nilotinib inhibited PDGF.

Liu et al. (18) reported that the suppressive effects of nilotinib on VEGF and VEGFR in liver fibrosis were effective at 20 μM in their animal experiment. In the in vitro study of Hadzijusufovic et al. (15), the growth-inhibitory effects of nilotinib were observed in the presence and absence of VEGF; they also reported that, unlike imatinib, nilotinib suppressed endothelial cell migration in VEGF-induced tube formation. In addition, the effects of 10–20 μM doses of nilotinib and imatinib on endothelium were compared, where 20 μM nilotinib was more effective than the other doses. Based on this study, we designed our topical dose by adding 40 μM to see whether it would be more effective.

To our knowledge, there is no study on the effect of nilotinib on corneal angiogenesis so far. In our study, we chose three treatment groups to receive nilotinib doses of 10 μM, 20 μM, and 40 μM three times a day. Among the treatment groups, the nilotinib 10 μM dose was less effective in reducing neovascularization areas than the other treatment groups. This indicated that the 10 μM dose of nilotinib may be ineffective compared to the higher doses.

Baek et al. (40) stated that imatinib, a TKI, reduces the recruitment of immune cells in the corneal epithelium, and its therapeutic efficacy is similar to or better than cyclosporine treatment. Onder et al. (41) found that regorafenib, a multiple-TKI, when compared with bevacizumab and dexamethasone, had inhibitory effects on alkali-induced CNV in rats. In a study comparing rats with corneal burns receiving a nintedanib thermo-sensitive hydrogel, nintedanib, and dexamethasone, it was reported that the CNV area of the subjects receiving the nintedanib thermo-sensitive hydrogel was lower than the other groups (42).

We included bevacizumab in our study, which has been shown to be effective in experimental CNV in most studies and has been compared with different TKIs, and compared it with nilotinib (43,44).

Our study had some limitations. First, the pharmacokinetic and pharmacodynamic effects of the drug were not evaluated. Second, only short-term effects were evaluated; long-term effects were not evaluated. Third, the effect of the drug on other neovascularization pathway markers and other administration methods were not considered. The final limitation of our study is the lack of in vitro studies such as human umbilical vein endothelial cells culture to demonstrate cellular toxicity.

CONCLUSION

Our study shows that nilotinib may be dose-dependently effective in preventing corneal neovascularization. In addition, auxiliary materials such as thermo-sensitive hydrogel can be used, which can increase the topical effect or prolong the duration of the drug effect. Further studies are needed to evaluate the effect of nilotinib on corneal neovascularization.

ETHICAL DECLARATIONS

Ethics Committee Approval: This study was carried out with the permission of The Fırat University Experimental Animal Studies Ethics Committee (Date: 02.09.2020, Decision No: 2020/05).

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

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