Original Article

Evaluation of a New Potential Bevacizumab Biosimilar in Corneal Neovascularization

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Abstract

Objectives: To compare the antiangiogenic effects of bevacizumab and a new potential bevacizumab biosimilar (anti-human VEGF GU01) on suture-induced corneal neovascularization (CNV) in rabbits.

Materials and Methods: CNV was induced in the right eyes of 15 rabbits by placing 7-0 black silk suture in the corneal stroma (3 mm wide and 1-1.5 mm from the superior limbus). All sutures were removed on day 7, and the rabbits were randomly divided into three groups. An injection of 0.1 mL balanced salt solution (control group), 2.5 mg/0.1 mL bevacizumab (bevacizumab group), or 2.5 mg/0.1 mL of anti-human VEGF GU01 (potential bevacizumab biosimilar group) was administered subconjunctivally. After suturing, standard corneal images were obtained with a surgical microscope on day 7 (pre-injection) and day 14 (7 days post-injection) to analyze the CNV area, which was calculated in square millimeters using the ImageJ program. On day 14, all animals were sacrificed and corneal specimens were analyzed histopathologically by hematoxylin-eosin staining.

Results: On day 14, the percent reduction in CNV area was significantly greater in the bevacizumab and bevacizumab biosimilar groups compared to the control group (control: 24.6%, bevacizumab: 82.2%, biosimilar: 83.4%; $p<0.05$). There was no statistically significant difference between the bevacizumab and biosimilar groups with respect to the CNV regression rates ($p>0.05$).

Conclusion: This experimental study showed that the potential bevacizumab biosimilar anti-human VEGF GU01 was as effective as subconjunctival bevacizumab in the treatment of CNV.

Keywords: Bevacizumab, bevacizumab biosimilar, anti-human VEGF GU01, corneal neovascularization

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Introduction

Maintaining corneal transparency is one of the main requisites for clear vision.[1] Stress and hypoxia associated with various causes (infection, inflammation, ischemia, degeneration, trauma, or limbal stem cell deficiency) can lead to the pathological growth of blood vessels in the cornea, causing loss of transparency. This condition is called corneal neovascularization (CNV).[2]

CNV is treated using surgical and medical methods with varying degrees of success.[3,4] These treatments aim both to prevent the formation of new vessels (focusing on the underlying etiology and pathophysiology) and to induce regression of existing pathological vessels.[5] Vascular endothelial growth factor (VEGF), a key mediator of vascular development, has been shown to play an important role in CNV formation.[6] Thus, VEGF inhibition can mitigate the condition.

There are several well-known anti-VEGF agents, such as ranibizumab (Lucentis; Genentech, USA), bevacizumab (Avastin; Genentech, USA) and aflibercept (Eylea; Bayer, Germany), that have been trialed or used in the treatment of CNV.[7] Among these agents, topical or subconjunctival bevacizumab is one of the most commonly used treatments for CNV, despite being an

off-label indication.[8] While other anti-VEGFs are also effective in the treatment of CNV, they are less frequently prescribed due to high cost. Biosimilar molecules have been developed to facilitate access to anti-VEGF agents.[9,10] To this end, the immunology laboratory of our university developed the novel anti-VEGF monoclonal antibody anti-human VEGF GU01, a potential bevacizumab biosimilar (with support from the Ministry of Science, Industry and Technology within the scope of project no: 0192-SanTez-2013-1).[11] Anti-human VEGF GU01 is a mouse-derived monoclonal immunoglobulin G antibody produced with the hybridoma method using the HiTrap Protein G HP clone and purified by fast protein liquid chromatography.[11]

The aim of this study was to evaluate the effect of anti-human VEGF GU01 in reducing CNV.

Materials and Methods

Approval for this study was obtained from Gazi University Animal Care Ethics Committee (project no: G.Ü.ET-15.025, date: 17/04/2015). The study was conducted with 15 healthy young New Zealand rabbits weighing between 2.5 and 3 kg.

Before the procedures, topical proparacaine HCl 0.5% (Alcaine; Alcon, USA) was instilled into the eyes. Under general anesthesia (intramuscular ketamine 50 mg/kg and xylazine 5 mg/kg), 7-0 black silk sutures were placed in the right eye of each rabbit under a surgical microscope. All sutures were placed through the mid-stroma, located central to the upper limbus in an area 1-1.5 mm long and 3 mm wide, by the same researcher (M.C.Ö.). Topical moxifloxacin eye drops (Vigamox, Alcon, Fort Worth, Texas, USA) were applied to the eyes twice a day for one week for infection prophylaxis. After 7 days, adequate CNV formation was observed in all eyes and the sutures were removed under general anesthesia.

Following suture removal, the 15 rabbits were randomly divided into three groups. The control group received 0.1 mL of balanced salt solution; the bevacizumab group received 2.5 mg/0.1 mL bevacizumab (Avastin 100 mg/4 mL; Genentech, USA); and the biosimilar group received 2.5 mg/0.1 mL anti-human VEGF GU01 (10 mg/0.4 mL; Gazi University Immunology Laboratory; Ankara, Türkiye) administered subconjunctivally (Table 1). The injections were administered using a 30-gauge needle, from the upper quadrant, 1 mm from the limbus (M.C.Ö.).

A surgical microscope (Möller-Wedel FS 3000, Haag Streit, Germany) with mounted recording system (Avermedia software, Taiwan) were used for imaging. The area of CNV was displayed at a magnification of 16x and quantified in square millimeters using the ImageJ program (Wayne Rasband, Research Services Branch, National Institutes of Health, Bethesda, MD, USA) on day 7 (pre-injection) and 14 (7 days after injection). To standardize area calculations, an image of a ruler at 16x magnification was obtained with the surgical microscope. A 1-mm line marked on the standard ruler image was loaded into the ImageJ program and recorded as 1 mm for numerical analyses. On each photograph, the neovascularized region was demarcated using the program's cursor. CNV areas calculated before treatment were accepted as 100%, and the percent reduction in this area was determined 1 week after treatment. These measurements were performed by an investigator blinded to the treatment groups (A.Ö.). Afterwards, all animals were sacrificed and the right eyes were enucleated. The corneal regions where CNV was located were excised, keeping the limbal region intact. Half of each tissue was stored in formaldehyde for 24 hours and embedded in paraffin, then stained with hematoxylin-eosin for histopathological evaluation. The other tissue halves were sent to the immunology laboratory for immunohistochemical examination with CD31 stain, which is an endothelial cell marker. Slides with 4-µm sections were photographed and evaluated under a microscope at 200x magnification.

Statistical Analysis

The sample size of the study was determined based on the number of animals used in similar experimental models in the field. An a priori power analysis was not performed. The SPSS program (version 22, IBM, USA) was used for statistical analysis. As the data distribution and sample number were not sufficient for parametric analyses, the Kruskal-Wallis test was used for comparisons of pre-treatment and post-treatment CNV areas, and the Mann-Whitney U test was used to compare percent regression in CNV area post-treatment. p values <0.05 were considered significant.

Results

On day 7 after suturing, sufficient CNV induction was observed in all eyes. The mean CNV area in the control, bevacizumab, and biosimilar groups was 2.59 ± 0.34 mm^2, 2.37 ± 0.40 mm^2, and 2.36 ± 0.44 mm^2, respectively. There was no significant difference in initial CNV area between the groups ($p>0.05$) (Figures 1 and 2). No keratitis or other suture-related complications were observed.

After treatment, the mean CNV regression rate was significantly higher in the bevacizumab ($82.2\%\pm3.0\%$) and biosimilar ($83.4\%\pm3.8\%$) groups compared to the control group ($24.6\%\pm3.9\%$) ($p<0.05$). There was no statistically significant difference in CNV regression rate between the bevacizumab and biosimilar groups ($p>0.05$) (Figures 1 and 3). In hematoxylin-eosin staining, a large number of vessels were observed in the control group, while almost no vessels were observed in the bevacizumab and biosimilar groups (Figure 4). Immunohistochemical evaluation could not be performed due to insufficient CD31 staining.

Table 1. The study groups and treatments administered

Control group (n=5)	0.1 mL balanced salt solution
Bevacizumab group (n=5)	2.5 mg/0.1 mL bevacizumab
Biosimilar group (n=5)	2.5 mg/0.1 mL anti-human VEGF GU01

VEGF: Vascular endothelial growth factor

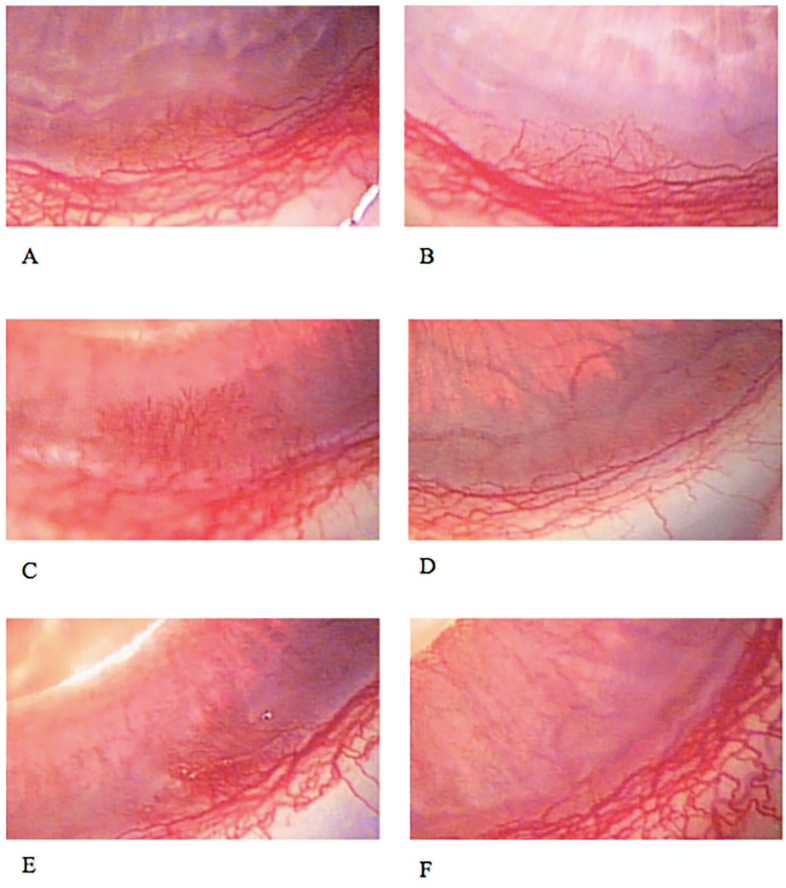

Figure 1. Appearance of corneal neovascularization before treatment (day 7, immediately after suture removal) and after treatment (day 14). A) Pre-treatment control group. B) Post-treatment control group. C) Pre-treatment bevacizumab group. D) Post-treatment bevacizumab group. E) Pre-treatment biosimilar group. F) Post-treatment biosimilar group

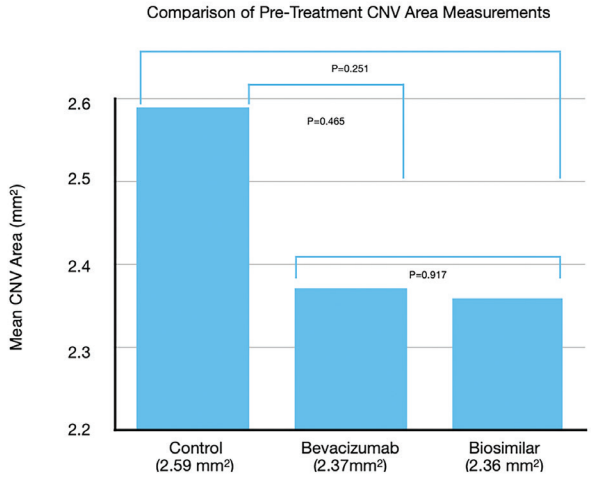

Figure 2. Corneal neovascularization (CNV) areas on day 7 after suturing. p values were calculated using the Kruskal-Wallis test. There was no statistically significant difference between the groups

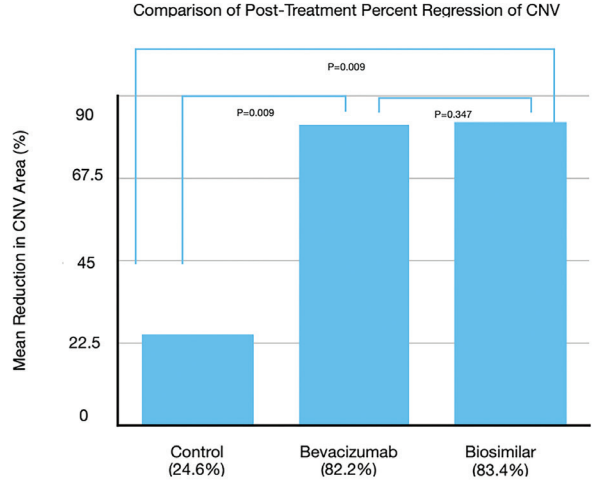

Figure 3. Percent regression of corneal neovascularization (CNV) areas 7 days after treatment. p values were calculated using the Mann-Whitney U test. CNV area decreased significantly more in the bevacizumab and biosimilar groups than in the control group. There was no statistically significant difference in CNV regression between the bevacizumab and biosimilar groups

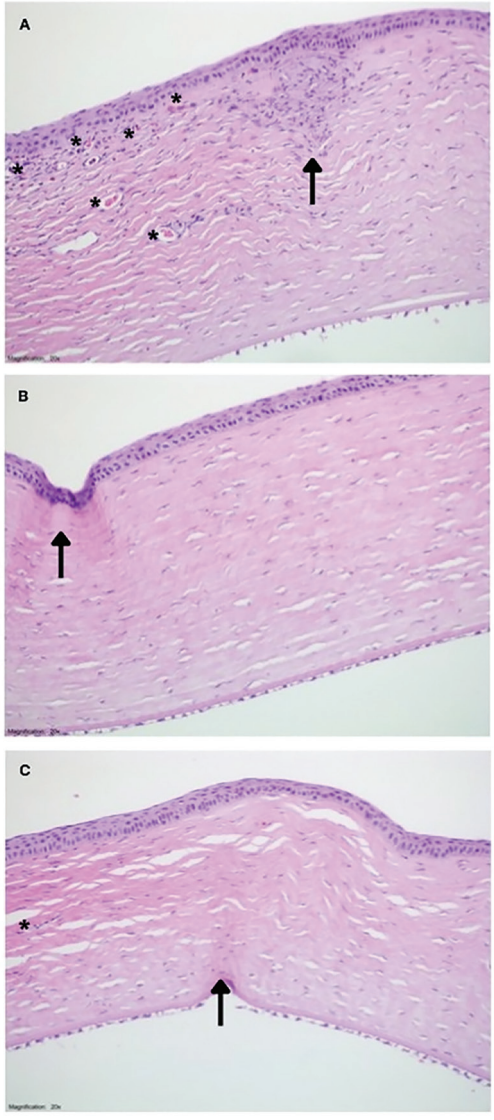

Figure 4. Histopathology images: A) Control group: signs of inflammation in the suture area (arrow) and erythrocyte-containing vessels (asterisks) in the stroma. B) Bevacizumab group: signs of inflammation in the suture area (arrow) but no erythrocyte-containing vessels in the stroma. C) Biosimilar group: signs of inflammation in the suture area (arrow) and minor erythrocyte-containing vessels (asterisk) in the stroma

Discussion

The term biosimilar is used for substances that are very similar to biological agents but not identical in terms of efficacy, safety, and purity. The increasing use of biosimilar drugs reduces drug costs and facilitates patient access to medication. However, the production of biosimilars is a complex process. Minor structural differences in the manufacturing process can significantly alter effectiveness and safety.[9] In our study, we investigated the effectiveness of anti-human VEGF GU01, a potential bevacizumab biosimilar produced in our immunology laboratory, in an experimental CNV model.[11]

There are currently no commercially available ophthalmic preparations of bevacizumab, and the preparation process for ocular use varies from country to country. While the drug is manufactured in single bottles containing 4 mL or 16 mL, ocular use requires tiny doses prepared in syringes. This user-dependent drug preparation introduces the risk of contamination. Cases of severe endophthalmitis and vision loss following bevacizumab injection have been reported in different parts of the world.[12,13,14]

In 1998, Yatoh et al.[15] demonstrated that VEGF inhibition prolonged graft survival in mice, leading to many experimental and clinical studies on the effect of bevacizumab on CNV. Experimental studies were generally conducted with suture-induced CNV models. The CNV created in these models is more compatible with the CNV that occurs in humans, both pathophysiologically and in appearance.[16,17] In the light of this information, we used the suture-induced CNV model in our study.

The duration of CNV is an important factor in treatment selection. While medical treatment can be more effective during active vessel formation (i.e., stages of new CNV development), surgical interventions such as fine needle diathermy have been shown to be more effective in stages where mature vessels develop (i.e., when CNV becomes chronic).[18,19] An important factor in this difference is thought to be pericytes that cover the vessels within the first two weeks of CNV development, thereby reducing their susceptibility to pharmacological agents.[5] Lin et al.[18] examined the timing of anti-VEGF therapy in the treatment of CNV and observed that early anti-VEGF treatment was beneficial, whereas late anti-VEGF treatment had no regressive effect on CNV. In our study, treatment was applied in the early period. Our results demonstrated more than 80% regression in CNV area on day 14 both in the bevacizumab- and anti-human VEGF GU01-treated groups.

The suture-induced CNV model was preferred for this study because it is both easy to create and highly similar to the pathophysiology of CNV in humans. However, more aggressive and permanent neovascularizations can be observed in clinical practice, especially in cases such as chemical burns and limbal stem cell deficiency. Therefore, evaluating the efficacy of anti-human VEGF GU01 in such demanding models may be important in terms of revealing the true clinical potential of the drug.[17]

There is no consensus on the dose and frequency of subconjunctival bevacizumab administration for the treatment of CNV. In a 24-patient study, single doses of 2.5 and 5 mg subconjunctival bevacizumab were found to have a similar effect on CNV regression.[20] In another experimental study involving 100 rats, comparable effects were observed with 1 mg, 5 mg, and 25 mg subconjunctival bevacizumab in the treatment of CNV.[21] In our study, we administered the drug at a dose of 2.5 mg. We did not repeat injections in our study because the half-life of subconjunctival bevacizumab was shown to be between 1.8 and 2.8 weeks in rabbits.[22]

Corneal transplantation is the most successful of all organ transplants, with a success rate of 90%.[23] However, when surgery

is performed in an inflamed or vascularized recipient bed, this rate can fall to 20-40%.[24] Bevacizumab is one of the most commonly used anti-VEGF agents in the treatment of CNV. Dekaris et al.[25] administered 25 mg/0.5 mL subconjunctival and 25 mg/mL topical bevacizumab postoperatively to evaluate the benefit to graft survival after penetrating keratoplasty in 50 eyes considered high-risk for surgery. After three years of follow-up, graft transparency was preserved in 70% of the patients.

Aside from increased proangiogenic growth factors, extracellular matrix remodeling also plays an important role in CNV. Matrix metalloproteinases (MMP), especially MMP-2 and MMP-9, facilitate the invasion of new vessels by destroying the basement membrane and stromal structure. These enzymes, secreted by inflammatory cells and activated epithelial/stromal cells, may increase the effect of angiogenic factors such as VEGF and support the progression of vascularization. Although MMP levels were not directly evaluated in our study, the suppression of neovascularization by the biosimilar VEGF inhibitor indirectly suggests that MMP activity may also have decreased.[26,27]

Study Limitations

One of the main limitations of this study is the small number of animals in the experimental groups. The use of five animals per group may limit the statistical power and generalizability of the results obtained. Although post-hoc power analysis indicated sufficient study power (>95%), advanced-phase experimental and clinical studies with larger samples would allow a more robust evaluation of the effectiveness of anti-human VEGF GU01. Another limitation of our study is that staining with CD31 antibody, which is used to evaluate corneal vascularization at the immunohistochemical level, was unsuccessful. A possible reason for this is that the cold chain was disrupted during transport or storage of the CD31 antibody. Temperature-sensitive antibodies such as CD31 must be stored at a constant temperature in the range of 2-8 °C to maintain biological activity. Any disruption in the cold chain may adversely affect the staining result by reducing the antibody's ability to bind to the target antigen. Therefore, immunohistochemical analysis could not be performed in our study, and the results were obtained only by clinical and histopathological evaluation. Considering this technical problem in future studies, the use of alternative vascular endothelial markers such as CD34 or von Willebrand factor in addition to CD31 may be considered. In addition, analyzing levels of proangiogenic and angiogenic molecules would have strengthened the study.

Conclusion

The evidence suggests that the widespread use of bevacizumab in corneal surgeries will increase surgical success. Once phase studies are completed, anti-human VEGF GU01, a potential bevacizumab biosimilar, has the potential to increase surgical success rates in high-risk penetrating keratoplasties. This experimental study is the first in the literature to document subconjunctival administration of anti-human VEGF GU01.

Ethics

Ethics Committee Approval: Approval for this study was obtained from Gazi University Animal Care Ethics Committee (project no: G.Ü.ET-15.025, date: 17/04/2015).

Informed Consent: Not applicable (animal study).

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Declarations

Authorship Contributions

Surgical and Medical Practices: A.Ö., E.Ü.B., M.C.Ö., Concept: A.Ö., E.Ü.B., M.C.Ö., Design: A.Ö., E.Ü.B., M.C.Ö., Data Collection or Processing: A.Ö., M.A.İ., M.C.Ö., Analysis or Interpretation: A.Ö., M.A.İ., M.C.Ö., Literature Search: A.Ö., N.Y., M.C.Ö., Writing: A.Ö., N.Y., M.C.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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