

Drug Modification of Angiogenesis in a Rat Cornea Model

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PURPOSE. To evaluate the influence of some widely used antiglaucoma agents on angiogenesis in a novel rat cornea model.

METHODS. Angiogenesis was induced in 32 rats by slow-release polymer pellets containing basic fibroblast growth factor (bFGF) placed in a corneal micropocket. Angiogenesis was later measured and compared in groups of rats given one of four antiglaucoma drug therapies and one control group. The drugs were commercially available preparations of prostaglandins, β -blockers, α -2 agonists, and carbonic anhydrase inhibitors given for 7 days in a manner similar to that used in humans. Growth was measured by calculating the maximum linear vessel growth divided by pellet-limbus distance.

RESULTS. Biomicroscopic observation disclosed that all tested animals showed an induction of neovascular reactions in their corneal stroma. The growth index results for the control, latanoprost, dorzolamide, brimonidine, and timolol malate groups were 1.65 ± 0.16 , 1.98 ± 0.18 , 1.85 ± 0.19 , 2.03 ± 0.38 , and 1.65 ± 0.14 , respectively, confirming the hypothesis that topically delivered antiglaucoma drugs modify the normal angiogenic response. Of them, the prostaglandins showed the most prominent angiogenic stimulatory effect ($P = 0.03$).

CONCLUSIONS. This modified micropocket assay of corneal angiogenesis in rats demonstrated the stimulatory effect of several widely used topically delivered antiglaucoma medications on the angiogenic process. The results indicate that the selection of drugs for treating different ophthalmic diseases should take into account their influence on angiogenic processes. (*Invest Ophthalmol Vis Sci.* 2008;48:250–254) DOI:10.1167/iov.06-1337

Corneal angiogenesis is a major cause of blindness worldwide.¹ Several corneal disorders, including chemical burns and other trauma-related injuries, infections, immunologic diseases, and degenerative disorders can induce corneal angiogenesis, thereby altering corneal function and causing blurred vision or even blindness.

Angiogenesis, the formation of new blood vessels derived as extensions from existing vasculature, is a highly complex pathophysiologic process.^{2–4} Its function is to provide the tissues' metabolic needs in conditions in which simple oxygen diffusion is not enough. Under physiological conditions, angiogenesis occurs during wound healing and inflammation, in the female reproductive organs (periodically), and during embryonic development. It is also present in numerous diseases, including tumor growth and metastases, myocardial ischemia, systemic inflammatory conditions (e.g., psoriasis and rheumatic arthritis), and ocular diseases (e.g., proliferative diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration).⁵ The pathologic angiogenic process is initiated when the homeostatic balance is broken. Normal homeostasis relies on a fine-tuned counterbalance of endogenous inhibitory and excitatory mediators of angiogenesis. Down-regulation of inhibitors of angiogenesis or upregulation of the expression of stimulating growth factors will lead to loss of balance and the initiation of angiogenesis in different pathologic states. At least 15 angiogenic growth factors have been identified, including tumor growth factor (TGF), fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF).^{6,7}

Topical ophthalmic medications, especially those used in glaucoma therapy, contain a variety of substances that mimic the activity of natural humoral and local neural transmitters, such as prostaglandins, α - and β -blockers, and carbonic anhydrase inhibitors. They bind to and alter the function of regulatory molecules, usually neurotransmitter receptors or enzymes. Several of the compounds used for treating glaucoma have been shown to alter the angiogenic processes.^{8–11}

In this study, we examined the effect of antiglaucoma medications on the angiogenic process by means of an *in vivo* assay system. We searched for compounds that can alter the normal angiogenic balance and that may have an influence on specific ophthalmic angiogenic processes, such as corneal neovascularization and neovascular glaucoma.

MATERIALS AND METHODS

Experiment Design

A controlled corneal neovascularization model was designed that involved a rat corneal micropocket assay. We modified some previously published corneal assay techniques^{12–16} and implanted pellets containing a slow-release polymer and bFGF. The same corneal micropocket surgical procedure was performed on one eye of each of 32 Wistar rats (weighing 200–250 g). The animals were then divided into five groups according to experimental drug therapy. The protocol of a 7-day postoperative observation and treatment period was based on previous evidence in the literature that the neovascular response reaches the pellet at approximately day 5 and stabilizes at days 7 to 8.¹²

The study was approved by the Tel Aviv Sourasky Medical Center Review Board for Animal Trials. All experiments were conducted in accordance with the Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research,

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according to which simultaneously performed bilateral visual disabling procedures should be avoided. Thus, one eye of the experimental animal was left untreated.

Pellet Preparation

Pellets were made of a slow-release ethylene vinyl acetate polymer (Elvax 40; Aldrich Chemical, Milwaukee, WI) and contained recombinant bFGF. The Elvax polymer was dissolved in an ethylene chloride solution (Bio-Laboratory, Jerusalem, Israel) at a concentration of 100 mg/1 mL (10%). The solution was rotated in a water bath at 37°C for 30 minutes, vortexed vigorously for another 5 minutes, and then dried in a laminar airflow hood for 24 hours under sterile conditions. The polymer was cut with a scalpel blade into uniformly sized pellets of $1 \times 1 \times 0.1$ mm, under a microscope.

Recombinant bFGF (donated by a generous grant of the United States Air Force) at a concentration of 100 ng/2 μ L was prepared by dissolving the lyophilized product with sterile phosphate buffer solution (PBS) containing 0.1% BSA (Sigma-Aldrich, St. Louis, MO). This mixture was put in a vial together with the pellet and allowed to incubate while being rotated for 40 minutes in a slow ELISA plate rotator (Titramax 1000; Heidolph, Schwabach, Germany). The pellets were then placed with the rest of the solution for further deposition and drying in a laminar airflow hood under sterile conditions for another 10 minutes, after which they were stored at -20°C. Each uniform pellet contained approximately 100 ng bFGF. The pellets were used 24 hours later, after being sterilized with 100% alcohol solution.

Pellet Implantation

The animals were anesthetized with intraperitoneal injection of xylazine base 10 mg/kg (XYL-M2 Veterinary; VMD, Arendonk, Belgium) and ketamine HCl 90 mg/kg (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA). The cornea was prepared with a drop of povidone iodine 0.4% (Polydine; Fischer Pharmaceuticals, Tel Aviv, Israel) and locally anesthetized with oxybuprocaine hydrochloride 0.4% (Localin; Fischer). The eye was proptosed with the use of forceps. With the guidance of an operating microscope (Carl Zeiss Meditec, GmbH, Oberkochen, Germany), a linear keratotomy was made with a surgical blade located 1 to 1.5 mm central to the temporal limbus. The average depth of a rat cornea is 200 μ m. A stromal micropocket measuring approximately 1.2×1.2 mm was carefully dissected with a stiletto, and a single pellet was incorporated into it with forceps. One drop of an antibiotic preparation (ciprofloxacin base 0.3%; Ciloxan; Alcon, Puurs, Belgium) was instilled once in the surgical eye to prevent infection.

Drug Application

The animals were separated into five groups according to drug therapy: (1) none (control), (2) β -blocker (timolol maleate 0.1%, Nyolol gel; Novartis, Basel, Switzerland) once daily, (3) adrenergic agonist (brimonidine titrate 0.15%, Alphagan P; Allergan, Irvine, CA) twice daily, (4) prostaglandin (latanoprost 0.005%, Xalatan; Pharmacia, Puurs, Belgium) once daily, and (5) carbonic anhydrase inhibitor (CAI; dorzolamide HCl 2%, Trusopt; Merck Sharp & Dohme-Chibret, Clermont-Ferrand, France) twice daily.

Each drug therapy was applied for 7 days in a manner similar to that used for humans, and consisted of commercially available preparations, including preservatives used as designed by the manufacturing companies.

Biomicroscopic Examination

On postoperative days 3 and 7, while the rats were under short-term anesthesia, the experimental eyes were examined with an operating microscope. Two measurements were obtained for each eye: the linear response representing the maximum vessel length (an average of the five longer vessels) extending from the limbus toward the micropocket (measured in millimeters), and the circumferential response representing the neovascularization zone (measured in clock hours).

Histologic Examination

On postoperative day 7, the rats were killed in a CO₂ chamber, and the experimental eyes were enucleated. After fixation of the eyes in 10% formaldehyde, the corneas were excised and embedded in paraffin blocks. The hematoxylin and eosin-stained sections were evaluated by a light microscope for vessel growth and stromal inflammatory reaction. Histologic corneal images were taken by microscope (BH-2; Olympus, Tokyo, Japan) and a zoom digital camera (Camedia c40; Olympus).

Statistics

The results are presented as the mean \pm SEM. Group differences were evaluated by one-way analysis of variance (ANOVA) followed by the Kruskal-Wallis test.

RESULTS

Biomicroscopic observation disclosed that all pellets with bFGF induced a neovascular reaction (Fig. 1). Pellets without a growth factor did not elicit a response. All 32 corneas were transparent and free of gross inflammation. Limbal vessels were seen to sprout into the cornea toward the pellet by postoperative day 3. Their length at that time did not exceed 0.3 to 0.5 mm, but their circumferential growth exceeded 4 to 5 clock hours. The extent of the circumferential growth decreased as the vessels increased in length toward day 7. The vascular response was intense, well-localized, and reproducible. Light microscopic examination demonstrated intrastromal capillaries between the limbus and the pellet, encircling the pellet (Fig. 2). This neovascular process was surrounded by a mild degree of inflammatory cell infiltration.

The maximum linear vessel length and the circumferential growth were measured in each eye. Circumferential growth did not show any significant difference among any of the groups either at day 3 or day 7. A growth index (the maximum linear vessel growth divided by the pellet-limbus distance) was used to compensate for the pellet-limbus distance differences. The normal response elicited in the control group showed a mean linear vessel length of 2.29 mm and 1.5 clock hours. Compared to the nonmedicated control eyes, latanoprost, dorzolamide, brimonidine, and timolol maleate yielded a growth index of (mean \pm SD) 1.65 ± 0.16 , 1.98 ± 0.18 , 1.85 ± 0.19 , 2.03 ± 0.38 , and 1.65 ± 0.14 , respectively. Prostaglandins, CAIs, and α -agonists demonstrated an excitatory effect on angiogenesis compared with the control eyes, whereas β -blockers failed to show any effect (Fig. 3). Latanoprost caused the most prominent excitatory effect, demonstrating not only a higher growth index ($P = 0.03$, growth index, 1.977; lower 95% confidence interval of the mean, 1.784; upper 95% confidence interval of the mean, 2.169; Fig. 4), but also a gross and bulky reaction, as demonstrated biomicroscopically.

DISCUSSION

In this study, we evaluated the influence of some widely used antiglaucoma agents on angiogenesis in a novel rat cornea assay. We determined the angiogenic effect of latanoprost, dorzolamide, brimonidine, and timolol in a bFGF-stimulated corneal angiogenesis model. We believe this to be the first study to explore the effect on angiogenesis of topically administered modulators given as ophthalmic preparations. Our results demonstrated a stimulatory effect of these medications, which included prostaglandins, CAIs, and α -agonists. We used commercially available preparations designed for humans in an experimental setting that mimicked the manner in which these preparations might induce angiogenesis in the clinical setting. The most prominent effect was demonstrated by prostaglandin. The clinical implications of our observations can only be

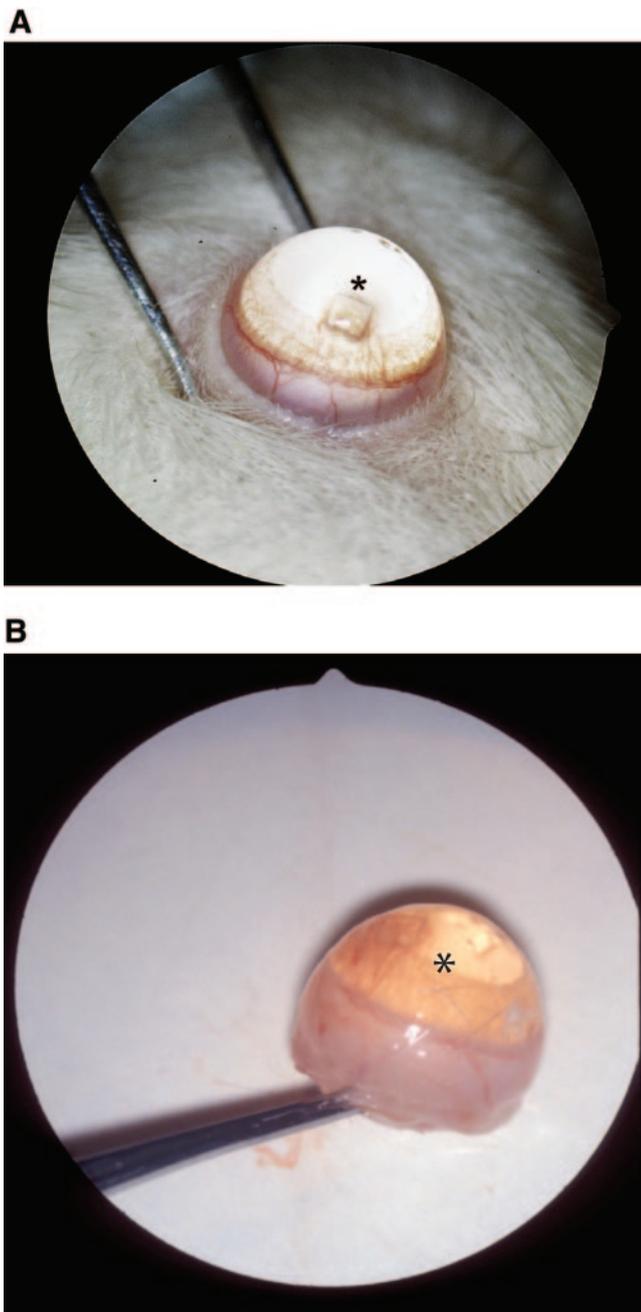


FIGURE 1. bFGF induced angiogenesis in the rat cornea. Photographs obtained before (A) and after (B) enucleation, taken 7 days after pellet implantation with incorporated bFGF (*). The cornea is avascular; thus, any vessels seen are new. Vessels formation can be observed, growing from the limbal vasculature toward the pellet.

speculated on, but it is clear that the excitatory angiogenic effect of each compound must be taken into account when the drug is considered for patients with active ocular angiogenic processes, such as in neovascular glaucoma or corneal neovascularization,¹⁷ and in bleb failure, where an excessive angiogenic process may also be responsible.¹⁸

Our demonstration of the stimulatory effects of prostaglandins and α -agonists on angiogenesis supports some indirect observations by others. Wilkinson-Berka⁸ suggested that prostaglandins might have a tumor-promoting effect through stimulation of angiogenesis. Although this is mechanistically possible, no cases have been reported to date. Emerging evidence in

experimental models indicates that cyclooxygenase (COX)-2 inhibitors modulate angiogenesis through interaction with the VEGF and nitric oxide (NO) systems.⁹ Adrenergic agents up-regulate myocardial angiogenesis by a paracrine mechanism involving VEGF in rat myocytes.¹⁰ Xiang et al.¹¹ recently reported the effect of systemically administered CAIs on angiogenesis. The number of capillaries and postcapillary venules was significantly decreased in tumor tissue after treatment with acetazolamide, showing a significant inhibitory effect of CAIs on endothelial cell proliferation and angiogenesis. Our findings on carbonic anhydrase inhibitors (using dorzolamide HCl) contradict the reported effect of acetazolamide. This discrepancy may be attributable to the different compounds that were used. We did not find any publications on the effect of β -blockers on angiogenesis.

The pharmacologic basis of our findings is still undelineated. There is a distinct relationship between VEGF and the prostaglandin-cyclooxygenase system. Prostaglandins influence retinal blood flow, and they are active in inflammation as well as angiogenesis. Recent evidence reported by Wilkinson-Berka et al.¹⁹ suggests that COX-2 modulates angiogenesis by interacting with the VEGF system. Fredriksson et al.²⁰ noted that norepinephrine may stimulate expression of the vascular endothelial growth factor (VEGF) by a paracrine mechanism

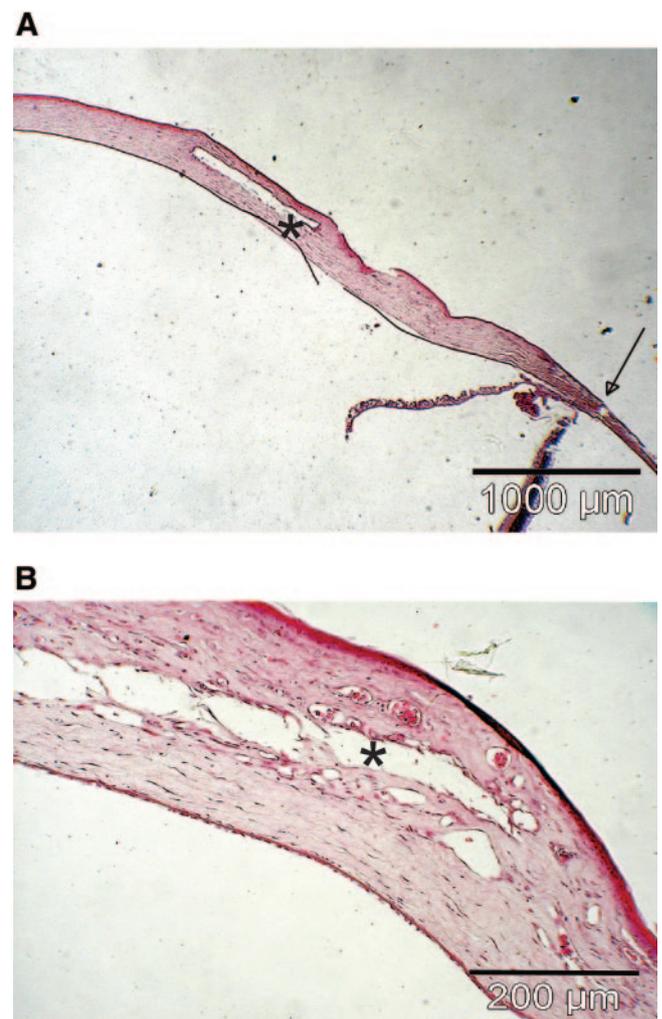


FIGURE 2. Histopathology of the rat cornea 7 days after pellet implantation, demonstrating the intrastromal pellet (*) in relation to the limbus (arrow, A) and the neovascular reaction (B). Magnification: (A) $\times 20$, (B) $\times 100$.

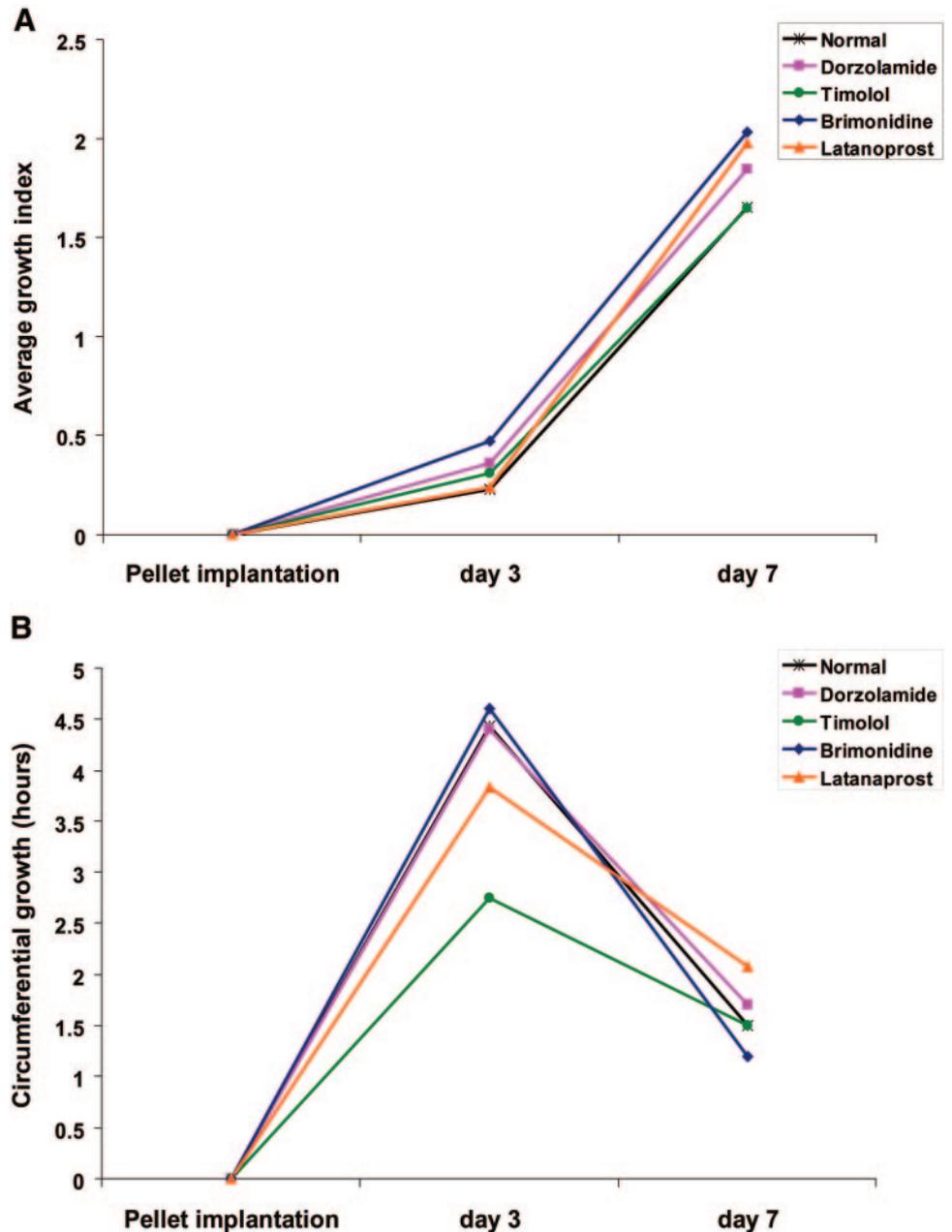


FIGURE 3. Corneal neovascularization response in all five groups at days 3 and 7 after pellet implantation with recombinant bFGF, expressed as linear growth, using the average growth index (A) and circumferential growth in clock hours (B).

that involves increased VEGF mRNA levels. Finally, Prasad et al.²¹ demonstrated that CAIs can suppress angiogenesis, at least in part, by inhibiting the expression of aquaporin (AQP)-1 water channels that are highly expressed in the cornea.

One of the most important technical challenges in angiogenesis research is the selection of the appropriate assay. The ideal assay should be reliable, technically straightforward, easily quantifiable, and physiologically relevant. Several assays have been described in the literature.^{22,23} The corneal angiogenesis assay that we used in the present study is considered by many to be a gold standard for angiogenesis testing *in vivo*.²⁴ The neovascularization pattern observed in all our experimental groups was consistent with the pattern described by others.^{12,13} The pellets we selected were made of the slow-release polymer Elvax (Aldrich Chemical)²⁵ and contained recombinant bFGF. We believe that this is the first description of such use of the Elvax compound. Preparation of Elvax is quick, and we found it easier to work with than the more commonly used preparation (Hydron; Hydron Protective

Coatings, Wolverhampton, UK). The method of measuring angiogenesis in a corneal pocket model is not well standardized, and various methods are being used, some of which include complex data acquisition and imaging software manipulations, used to determine the net volume of vascular growth.²⁶ In the present study, we chose to adopt the simplest method, in which two measurements were obtained for each eye: the linear response representing the maximum vessel length (an average of the five longest vessels) extending from the limbus toward the micropocket (in millimeters), and the circumferential response representing the neovascularization zone (in clock hours). In the angiogenic response after administration of the proangiogenic agent, numerous small vessels emerged from the limbus. We chose to measure the five longest vessels, as we noted that this measurement gives us both good measurement of the intensity of the response. The measurement is simple and easily performed, in accordance with our initial goal of finding an easy and simplistic procedure. We also noted that despite meticulous implantation of the Elvax

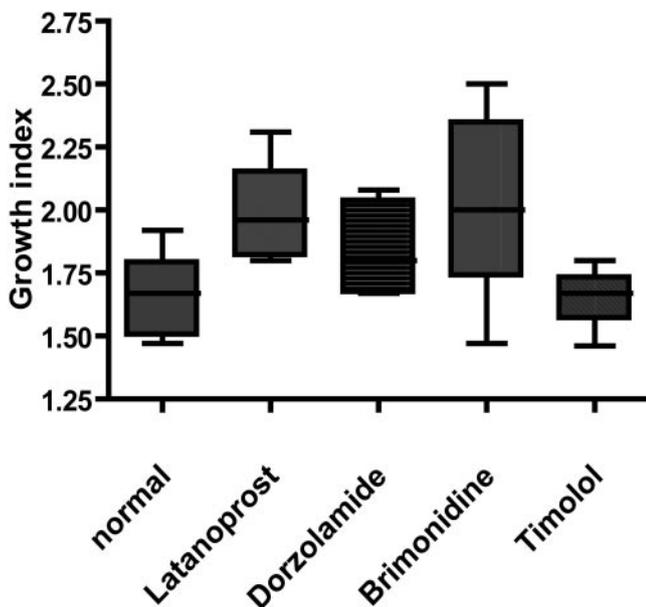


FIGURE 4. Comparison of all five groups (normal, latanoprost, dorzolamide, brimonidine, and timolol) using the growth index (the maximum linear vessel growth divided by the pellet-limbus distance). One-way ANOVA test ($P = 0.03$).

matrix, there was a slight difference (between 1 and 1.5 mm) in the pellet-limbus distances among the animals at day 7. We used a growth index to compensate for these differences.

Yet, we must emphasize that despite the advantages of usage of the growth index, a major drawback to its use is its lack of sensitivity as a parameter for measuring the angiogenic response. Therefore, the lack of statistical significance achieved with brimonidine and dorzolamide does not rule out the possibility that they can cause significant neovascularization. The effect of the prostaglandins achieved statistical significance and is therefore very likely to be real and important. The estimated calculation of type II error, performed using a type II error calculator,²⁷ determined that the observed β level was 0.08.

Research on angiogenesis modulators relies on *in vitro* or *in vivo* models using compounds that are administered systemically. Our modified model enabled us to explore the use of modulators given topically in a rat corneal micropocket assay.

This study also showed the applicability of a rat corneal micropocket assay in ophthalmic research. Novel drugs are currently being explored for modulation of neovascular diseases in the eye, such as age-related macular degeneration and diabetic retinopathy, in which VEGF plays a key role. We recommend a corneal micropocket assay using pellets containing VEGF to investigate those drugs.

Our results call for careful consideration in the use of selected drugs in patients with various ophthalmic diseases that may involve anterior segment neovascular processes, such as neovascular glaucoma, corneal keratoplasty, corneal infections, and erosions.

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