• Basic Research •

The effect on wound healing of pazopanib and bevacizumab compared with corticosteroid in experimental glaucoma filtration surgery

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Abstract

• AIM: To compare the effects of bevacizumab and pazopanib with corticosteroids on wound healing after trabeculectomy.

• METHODS: In the study, 35 New Zealand white rabbits were randomly divided into five groups. Apart from the first group, limbus-based trabeculectomy was performed for the eyes of rabbits. No postoperative treatment was administered for group I. Topically administered saline, prednisolone acetate (1%), bevacizumab 5 mg/mL, pazopanib 5 mg/mL for group II, III, IV and V respectively were applied for groups 6h daily for 28d. On day 28 of the experiment, eyes were enucleated and histologically and immunohistochemically analyzed.

• RESULTS: The fibroblast counts of groups IV and V were determined to be lower than those of groups II and III (*P*<0.05). In the mononuclear cell (MNC) count evaluation, no statistically significant difference was determined between the treatment groups (*P*>0.05). The immunohistochemical staining intensity of fibroblast growth factor β (FGF-β) and vascular endothelial growth factor (VEGF) was determined to be lower in groups IV and V than in groups II and III (*P*<0.05). No statistically significant difference was determined between groups IV and V in respect of fibroblast count, MNC count, FGF-β and VEGF staining intensity (*P*>0.05). The platelet derived growth factor β (PDGF-β) intensity was lower in group V than in groups II, III and IV (*P*<0.05). While the PDGF-β staining intensity was significantly lower in group IV than in group II, the difference compared with group III was not statistically significant (*P*>0.05).

• CONCLUSION: Bevacizumab and pazopanib might be good alternatives of corticosteroid treatment on delaying wound healing in glaucoma surgery.

• KEYWORDS: trabeculectomy; wound healing; corticosteroid; bevacizumab; pazopanib

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INTRODUCTION

Glaucoma is a chronic and progressive optic neuropathy caused by various pathological processes, which is typically seen with visual field defects, causes degeneration of retina ganglion cells and is usually accompanied by elevated intraocular pressure (IOP)[1]. It is the 2nd most preventable cause of blindness worldwide[2]. Glaucoma treatment: with changing according the type of glaucoma, treatment is frequently medical. Laser treatment and surgical treatment can be applied on patient who do not tolerate or in situations that treatment is insufficent. Currently, the most frequently used surgical treatment is trabeculectomy[3]. Following trabeculectomy surgery, rapid wound healing causes failure of the surgical procedure[4-5]. Scar tissue (subconjuctival fibrosis) which will form as a result of the progression of the wound healing process is the most important cause of failure in trabeculectomy[6].

The use of anti-fibrotic agents is generally accepted in the treatment following glaucoma surgery[7-8]. Topical corticosteroids are the drug groups most often used as anti-fibrotics. In the vast majority of cases, this drug groups alone is sufficient to obtain surgical success. However, in cases where the chance of success is low, such as patients of a younger age, African ethnicity, with a history of glaucoma surgery or with uveitis, steroids remain insufficient. Mitomycin C (MMC) and 5-fluorouracil (5-FU) are widely used to prevent fibrosis in the wound site in glaucoma filtration surgery (GFS) in cases where corticosteroids are inadequate[9]. However, both these drugs

which can be classically accepted and other cytotoxic agents with similar effects can lead to hypotonia, increased infection risk, the development of staphyloma and other serious side-effects, especially in the long-term and this has therefore led to research into new agents[10-15].

Bevacizumab (Avastin, F.Hoffmann-La Roche Ltd., Basel, Switzerland) is a long-term effect recombinant monoclonal mouse antibody which is synthesised against the vascular endothelial growth factor (VEGF) molecule and binds to all isoforms of VEGF-A. FDA approval was obtained in 2004 for its use in the treatment of metastatic colorectal cancers[16]. It is used without licence in age-related macular degeneration (AMD), choroid neovascularisations which develop associated with high myopia, premature retinopathy, diabetes mellitus (DM) and retina and iris neovascularisations which develop associated with retinal vascular obstructions[17-20]. In a study by Ozgonul *et al*[21] of 48 rabbits, subconjuctival and intravitreal injections to the bleb region of 0.1 mL (1.25 mg) bevacizumab were compared with 5-FU and histopathological evaluation was made of inflammation, vascularisation and fibrosis formation. It was concluded that the effect on the bleb of subconjunctival administered bevacizumab was more successful in respect of these 3 parameters compared to the other groups[21]. In another study of 20 patients, the postoperative topical use of a corticosteroid+bevacizumab combination was seen to have a positive effect on bleb formation in high-risk patients and a preventative effect on vascularisation[22].

Pazopanib (Glaxo Smith Kline, King of Prussia, PA) is a small molecule multi-tyrosine kinase inhibitor. It provides VEGF, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) receptor inhibition. It is used in the treatment of renal cell carcinoma (RCC), ovarian cancer, metastatic melanoma and soft tissue sarcoma[23-26]. Topical pazopanib application in humans has been shown to provide a regression in cornea neovascularisation. There have also been experimental animal studies showing that topical and intravitreal application of pazopanib regressed retinal and choroid neovascularisation[27-29]. However, to the best of our knowledge, there is no study in literature which has evaluated the effect of pazopanib on wound healing in GFS.

The aim of the current study was to histopathologically and immunohistochemically investigate the effect on postoperative inflammation, fibrosis and wound healing using bevacizumab and pazopanib in trabeculectomy surgery and to compare the results with the use of corticosteroids.

MATERIALS AND METHODS

The study was conducted in the Ophthalmology Department and the Pathology Department of Firat University Medical Faculty. Approval for the study was granted by the Animal Experiments Ethics Committee of Firat University (dated 08.01.2014). A single eye was used from each of 35 male, albino New Zealand rabbits, each weighing approximately 2500-3000 g. Throughout the study the animals were kept in special cages with suitable nutrition in the Experimental Animal Centre of Firat University.

With the exception of group I (control group), limbus-based trabeculectomy was applied to a single eye of each rabbit. In group I, no postoperative treatment was applied. To the animals in group II (sham group), physiological saline was administered, to group III, prednisolone acetate, to group IV, 5 mg/mL bevacizumab and to group V, 5 mg/mL pazopanib as 4×1 topically for 28d to the eye on which trabeculectomy had been performed.

Anaesthesia Technique In the application of anaesthesia and analgesia, a combination of intramuscular 50 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Turkey) and 6 mg/kg xylazine hydrochloride (Rompun, Bayer, Turkey) was used. Before the procedure, eyedrops of 0.5% proparacaine hydrochloride (Alcaine, Alcon, Turkey) were administered to the eyes of the animals.

Surgical Technique Following cleaning of the surgical site, by passing an 8/0 vicryl suture from the level of 12 o'clock with corneal traction, inferior and nasal rotation of the globe was provided. By cutting the conjunctiva and Tenon capsule from the limbus with blunt-tipped Westcott scissors at the level of 10 o'clock, fornix -based conjunctiva was opened. The conjunctiva and Tenon capsule were dissected with blunt dissection. A 3×3 mm^2 square scleral flap was marked using a 30° curved knife. Using the crescent knife, a half thickness scleral flap was raised until the trabeculum was seen. With a 15° curved knife, the trabeculum was excised so that trabeculectomy borders were formed of approximately 2×1 mm^2. Peripheral iridectomy was applied to the trabeculectomy site formed. The scleral flap was sutured to the sclera with 2×10/0 nylon sutures from the corners. The conjunctiva and the Tenon capsule were closed with 8/0 vicryl suture. After closure, by applying irrigation fluid to the anterior chamber , the bleb was inflated and it was checked that there was no leakage.

Histopathological Preparation and Evaluation of the Findings Following the enucleation of the operated eyes by applying intramuscular thiopental sodium at the end of the 28th postoperative day, the animals were returned to the Experimental Animal Centre. The bleb regions were excised including the conjunctiva, Tenon and sclera from the enucleated eyes and were sent to the pathology laboratory for histopathological examination. The tissue samples were fixed in 10% formalin then routine procedures were applied. Slices of 5 μm thickness were obtained from the paraffin blocks and stained with hematoxylin-eosin. The slices obtained were also stained with Masson-Trichrome and examined under a light microscope (Olymus BX-50) at ×400 magnification. On each slice, the number of fibroblasts and mononuclear cells in a

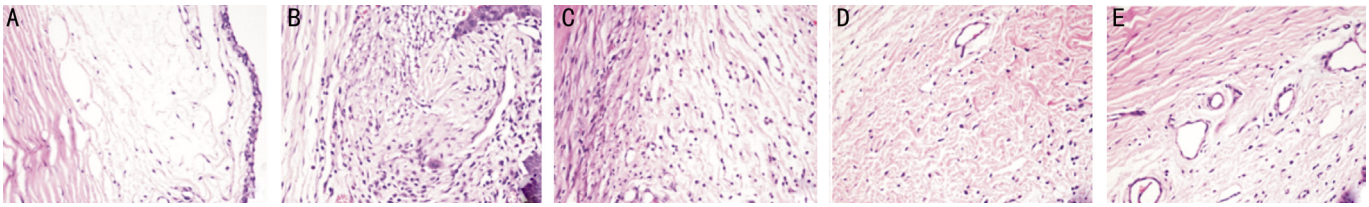

Figure 1 Images of the fibroblasts and fibrous tissue in the study groups A: Control group; B: Sham group; C: Steroid group; D: Bevacizumab group; E: Pazopanib group.

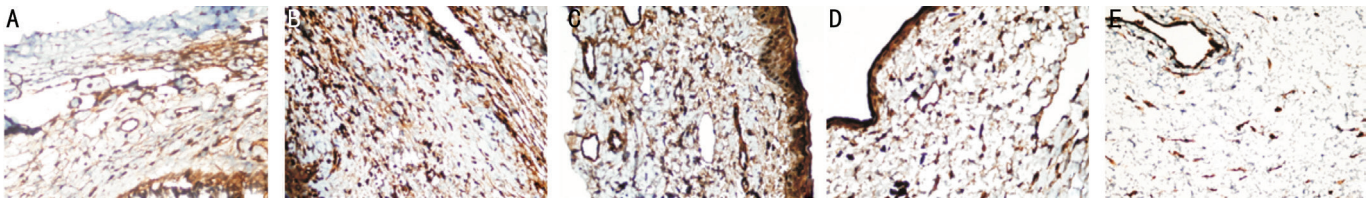

Figure 2 Immunohistochemical images of the FGF-β staining intensity in the bleb regions of the study groups A: Control group; B: Sham group; C: Steroid group; D: Bevacizumab group; E: Pazopanib group.

25 μm^2 area was determined with the aid of a specially marked micrometer placed on the microscope.

Immunohistochemical Staining of VEGF, FGF-β, and PDGF-β For immunohistochemical staining, slices were prepared of 5 μm thickness passing from the centre of the bleb. The slices were stained using an automatic immunohistochemical staining device (Ventana Benchmark XT) and VEGF, FGF-β, PDGF-β kit (Biorbyt, Cambridge, UK). The preparates were covered with a special covering substance and examined randomly under a light microscope (Olympus BX-50). With a camera attachment on the same microscope, digital photographs were taken of the tissues at ×40 magnification. The intensity of nuclear positive stained cells was evaluated as weak (+), moderate (++) and strong (+++)[30-31].

Statistical Analysis Statistical analyses of the data obtained were made using Windows Vista operating system and SPSS version 15 software. 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

In the evaluation of fibroblast count, the values of groups II and III were statistically significantly higher than those of the control group (both P<0.05; Figure 1A-1C). The fibroblast count values of groups IV and V were statistically significantly lower than those of groups II and III (All P<0.05). No statistically significant difference was determined between groups IV and V (P>0.05; Figure 1D, 1E).

In respect of the evaluation of mononuclear cell count, the group II cell count was higher than that of group I (P<0.05; Figure 1A, 1B). In the comparison between the groups applied with treatment drugs, no significant difference was determined in respect of mononuclear cell count (P>0.05; Figure 1C-1E). The mean and standard deviation values of the fibroblast and mononuclear cell counts found in the operation area of the study groups are shown in Table 1.

Table 1 Fibroblast and mononuclear cell counts in the operation area of the groups mean±SD

Groups	Mononuclear cells (n=7)	Fibroblasts (n=7)
Control (group I)	0.00±0.00	15.14±2.54
Sham (group II)	37.71±12.71[a]	48.00±10.19[a]
Corticosteroid (group III)	23.14±9.82[b]	39.14±10.95[a]
Bevacizumab (group IV)	22.57±11.29[b]	22.57±5.25[b,c]
Pazopanib (group V)	16.14±5.01[b]	16.71±2.56[b,c]

[a]P<0.05 *vs* control group; [b]P<0.05 *vs* sham group; [c]P<0.05 *vs* corticosteroid group.

The FGF-β, VEGF, PDGF-β, immunohistochemical staining was determined to be statistically significantly lower in group I than in group II (P<0.05; Figures 2A-2B, 3A-3B, 4A-4B). The decrease in group III was not statistically significant in comparison with group II (P>0.05). The FGF-β and VEGF staining intensity in groups IV and V was significantly lower than that of groups II and III (all P<0.05; Figures 2B-2E, 3B-3E). No statistically significant difference was determined between groups IV and V (P>0.05). The PDGF-β immunohistochemical staining intensity in group V was lower than that of groups II, III and IV (all P<0.05; Figure 4B-4E). The PDGF-β, FGF-β, VEGF immunohistochemical staining intensity values are shown in Table 2.

DISCUSSION

Topical corticosteroids are used to inhibit wound healing following GFS[32]. It is known that topical corticosteroids increase the success of GFS by reducing bleb scarring[33]. The drugs primarily used for this purpose are corticosteroids, dexamethasone, prednisolone acetate and triamnisolone acetone. In the current study, topical corticosteroid (prednisolone acetate) was used, as it is conventionally used to delay wound healing after trabeculectomy. Although the fibroblast count

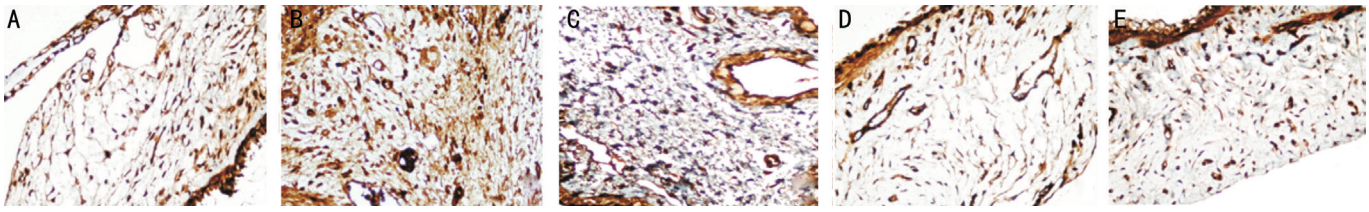

Figure 3 Immunohistochemical images of the VEGF staining intensity in the bleb regions of the study groups A: Control group; B: Sham group; C: Steroid group; D: Bevacizumab group; E: Pazopanib group.

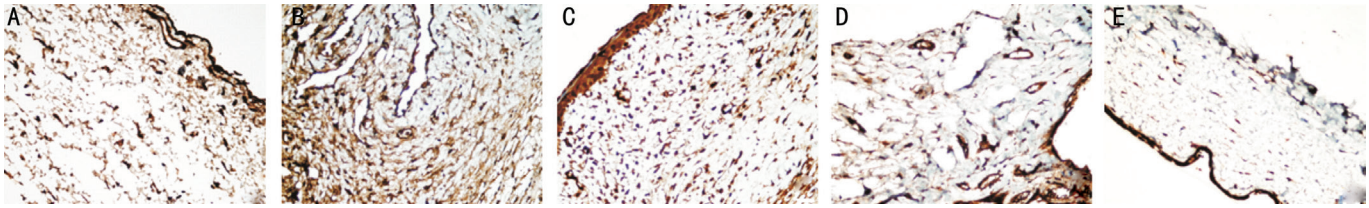

Figure 4 Immunohistochemical images of the PDGF-β staining intensity in the bleb regions of the study groups A: Control group; B: Sham group; C: Steroid group; D: Bevacizumab group; E: Pazopanib group.

Table 2 PDGF-β, FGF-β and VEGF immunohistochemical staining intensity mean±SD

Groups	PDGF-β (*n*=7)	FGF-β (*n*=7)	VEGF (*n*=7)
Control (group I)	1.00±0.00	1.00±0.00	1.00±0.00
Sham (group II)	2.71±0.48[a]	2.71±0.48[a]	2.71±0.48[a]
Corticosteroid (group III)	2.28±0.48[a]	2.28±0.48[a]	2.28±0.48[a]
Bevacizumab (group IV)	1.85±0.37[b]	1.42±0.53[b,c]	1.14±0.37[b,c]
Pazopanib (group V)	1.14±0.37[b,c,d]	1.28±0.48[b,c]	1.00±0.00[b,c]

[a]*P*<0.05 *vs* control group; [b]*P*<0.05 *vs* sham group; [c]*P*<0.05 *vs* corticosteroid group; [d]*P*<0.05 *vs* bevacizumab group.

on the histology slices was reduced compared to the sham group, this had no statististically significant effect. This small reduction in fibroblast count suggested that the effect of corticosteroids on wound site healing could be limited. Corticosteroids reduce inflammatory cell migration to the wound site. In the current study the mononuclear cell count in the corticosteroid group was significantly reduced compared with the sham group. This finding suggested that the effects of corticosteroids on wound site healing could be on the anti-inflammatory activity. Therefore, despite occasional corticosteroid use in the clinic, rapid wound healing could be associated with it only being able to be one-way. There was no statistically significant difference between the corticosteroid group and the sham group in respect of PDGF-β, FGF-β and VEGF immunohistochemical staining intensity. This finding suggests that corticosteroids have no effect on growth factors in wound healing.

Corticosteroids, which are the most frequently used drug group in the clinic are sufficient alone to provide successful surgery in the vast majority of cases. However, they remain inadequate for some risk groups[34-35]. Therefore, alternative agents are being sought to increase the success of GFS. MMC and 5-FU have started to be widely used in cases where corticosteroids are insufficient[9]. However, there are known to be serious side-effects of these agents[10-15]. In the current study, the effect of corticosteroids on the parameters affecting wound healing was seen to be limited. From this starting point, groups were formed in the current study to compare the histopathological effects of bevacizumab and pazopanib with corticosteroids which are conventionally used.

VEGF is an important molecule in the response to wound healing. VEGF is not only a strong indicator for angiogenesis but is also important as a key regulator of fibroblast and inflammatory cell migration and proliferation[36]. Inflammatory cells which will migrate to the wound site with increased vascular permeability in the early stage as a result of stimulation formed with surgical trauma, will reach the wound site in fewer numbers with reduced vascular permeability of anti-VEGF. This role of anti-VEGF in the blocking of wound healing and angiogenesis can lead to a reduction in the development of scar tissue and response to wound healing.

In a study by Ozgonul *et al*[21], subconjunctival 0.1 mL (1.25 mg) bevacizumab was applied in an experimental trabeculectomy model and vascularisation, inflammation and fibrosis in the bleb region were seen to be significantly decreased compared to the other groups. In another study, a postoperative topical corticosteroid+bevacizumab combination was seen to significantly increase the formation of functional bleb and significantly decrease bleb vascularisation in high risk patients[22]. In a clinical study by Jonas *et al*[37] of 2 cases of neovascular glaucoma secondary to corticosteroid glaucoma and venous obstruction, success was reported to have been obtained with intravitreal 1.5 mg bevacizumab combined with surgery and this success was considered to be associated with angiogenesis inhibition. Previous studies have reported the rapid and marked reduction of abnormal new vessels and IOP after intraocular bevacizumab in neovascular glaucoma patients[18,38-40]. However, in a case by Kahook *et al*[41] of unsuccessful trabeculectomy because of encapsulated

filtration bleb, bleb revision was made with a needle, the bleb became more diffuse following 1 mg bevacizumab and the neovascular structures in the conjunctiva over the bleb were observed to have regressed. Akkan and Cilsim[42] concluded that subconjunctival bevacizumab administration in primary open-angle glaucoma patients who underwent primary trabeculectomy was more effective and safe than MMC.

In the current study, the fibroblast count in the bevacizumab group was determined to be statistically significantly lower than that of the sham and corticosteroid groups. The reduction in mononuclear cell count was significant compared to the sham group but not to the corticosteroid group. The reduction in PDGF-β immunohistochemical staining intensity was significant compared to the sham group but not to the corticosteroid group, whereas the reduction in FGF-β and VEGF immunohistochemical staining intensity was significant compared to both the sham and corticosteroid groups. These results suggest that the inhibitor effect of bevacizumab on wound healing has antifibrotic and anti-angiogenic effects in addition to the mononuclear cell inhibition effect of corticosteroids.

Bevacizumab, which is an anti-VEGF and is used in many ocular pathologies, inhibits wound healing at several stages. However, while positive effects can be formed in the pathological angiogenesis process with the inhibition of VEGF activity with anti-VEGF drugs, thereby reversing the VEGF efficacy, it may lead to negative side-effects in the physiological angiogenesis process[43]. Even if the eye is isolated from systemic circulation with the blood-brain barrier, in neovascular eye diseases, this barrier can be destroyed and there could be systemic transfer. Several cases of acute elevation of systemic blood pressure, cerebrovascular accidents, myocardial infarction, and transient ischemic attack were also reported[44].

The application of bevacizumab is absolutely not recommended in pregnancy and childhood, acute intraocular infection, acute myocardial infarct, angina pectoris, hypertensive crisis, kidney failure, active glomerulonephritis and known allergies and relatively not recommended in cases of uncontrolled hypertension, neurodegenerative diseases and respiratory failure[45-46]. Therefore, although this study obtained successful histological results with bevacizumab, these side-effects must always be kept in mind.

Pazopanib is a small molecule, multi-tyrosine kinase inhibitor that inhibits VEGFR, PDGFR, FGFR and c-kit and is used in patients with advanced stage RCC or soft tissue sarcoma[23-26]. Previous studies have shown that oral and topical application of pazopanib in choroid and retina neovascularisations regressed the neovascularisation and improved visual acuity[27,47-48]. It has also been reported that patients with neovascularisation receiving topical 0.5% pazopanib have shown a decrease in neovascularisation area and vascular diameter. In that study, which also evaluated reliability and efficacy, no systemic or ocular side-effects were encountered[49].

In a study by Singh *et al*[50] topical 10 mg/mL pazopanib was applied 4 times per day for 12wk to a patient group with subfoveal choroid neovascularisation secondary to AMD and to a healthy group and reliability and efficacy were evaluated. Although no serious side-effects were seen, ocular irritation was reported as the most common side-effect and it was concluded that 10 mg/mL topical pazopanib was well tolerated. In a study by Csaky *et al*[51] topical 5 mg/mL pazopanib and 10 mg/mL pazopanib applied 4 times per day a patient group in neovascular AMD. As a result; pazopanib was well tolerated. Daily pazopanib eye drops in neovascular AMD subjects did not result in therapeutic benefit beyond that obtained with ranibizumab alone.

To the best of our knowledge, there is no study in literature that has evaluated the effects of pazopanib on wound healing in GFS. Therefore, in this study it was decided to make a topical application of pazopanib, which is effective on wound healing at several stages. Pazopanib was applied at a dose of 5 mg/mL, 4 times a day for 28d. A significant reduction was seen in fibroblast count in the pazopanib group compared to the sham and corticosteroid groups. In respect of mononuclear cell count, a significant reduction was observed compared to the sham group and the reduction was seen to be similar to that in the corticosteroid and bevacizumab groups. The decrease in FGF-β and VEGF immunohistochemical staining intensity was significant in comparison with the sham and corticosteroid groups. The inhibitor effect of pazopanib on mononuclear cell and fibroblast count, VEGF and FGF levels was similar to that of bevacizumab. However, the decrease in PDGF-β immunohistochemical staining intensity was significant compared to the other treatment groups. With PDGF-β, which is an effective mediator on wound healing, migration of fibroblasts to the wound site is stimulated. Therefore, PDGF-β inhibition prevents fibroblast migration to the wound site and as a result, scar formation is reduced. The reduction in fibroblast count supports this view. The fact that pazopanib creates significant PDGF-β inhibition in addition to the antifibrotic and anti-angiogenic effects of bevacizumab renders it superior to the other drugs.

The results of this study indicate that bevacizumab and pazopanib could be good alternatives for wound healing and because of the PDGF inhibition effect of pazopanib, this could be one step ahead of bevacizumab.

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