• Clinical Research •

Effect of single subconjunctival injection of bevacizumab on primary pterygium: clinical, histopathological and immunohistochemical study

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Abstract

• AIM: To evaluate the effect (clinically, histopathologically and immunohistochemically) and safety of a single intra-pterygium injection of bevacizumab.

• METHODS: Prospective interventional study comprised 40 eyes of 40 patients with primary fleshy pterygia who attended the Outpatient Clinic of Department of Ophthalmology, Assiut University Hospitals, Egypt from May 2015 to May 2016. Patients were randomly classified into 2 groups: the first group received a single intralesional injection of bevacizumab (Avastin; Genentech, San Francisco, CA, USA); the second group comprised patients who did not receive subconjunctival bevacizumab. Excision of pterygium and conjunctival auto graft was done in both groups. The excised pterygium tissues were subjected to histopathological and immunohistochemical evaluation.

• RESULTS: The study comprised 40 eyes of 40 patients (33 men, 7 women) of age range from 31-58y. The study group included 22 eyes. The control group included 18 eyes. A decrease in the vascularity of the pterygium was noted in all injected cases. The mean vessel count was higher in non-injected pterygia than that in injected pterygia and the difference was statistically significant (*P*=0.001). Also, the mean vessel count in both groups was significantly higher than normal conjunctive (*P*=0.005 and 0.001). A statistically significant difference in vascular endothelial growth factor (VEGF) expression between injected and non-injected cases was detected in the epithelial, stromal and endothelial cells (*P*=0.0001, 0.016, 0.014). No serious intraoperative complications occurred in both groups.

• CONCLUSION: The use of single intra lesional injection of Avastin in pterygium decreased vascularity and decreased VEGF expression in injected pterygium after one month. Our study proved the effect of single intra lesional injection of Avastin on pterygium. Further studies may enable limiting the need for surgery and improve quality of life for patients with pterygia.

• KEYWORDS: pterygium; vascularity; bevacizumab; vessel count
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INTRODUCTION

Pterygia are characterized by the encroachment of a fleshy fibrovascular tissue from the bulbar conjunctiva onto the cornea. The pathogenesis of pterygia is presently uncertain and it has also been postulated that the development of pterygia depends on a changed angiogenic stimulator-to-inhibitor ratio[1]. One of the most important known mediators of angiogenesis in pterygia is vascular endothelial growth factor (VEGF)[2]. Bevacizumab (Avastin®) is a full-length, humanized, monoclonal antibody against all types of VEGF. It binds to and neutralizes the biologic activity of all types of human VEGF, so it prevents interaction with its receptors on the surface of endothelial cells[3].

Bevacizumab has recently been used for the treatment of neovascular eye diseases, particularly choroidal neovascular membrane in age-related macular degeneration (AMD)[4]. Bevacizumab and other anti-VEGF have been used either as a primary treatment or as an adjunctive therapy after pterygium excision. It has also been used by both topical and subconjunctival routes[5-11].

Anti-VEGF agents have been used as primary treatment, as peri-operative adjuvant, and as treatment for early recurrent pterygium[12]. Anti-VEGF was proved to be a well-tolerated therapy for exudative AMD with no major safety issues[13]. In this study we evaluate the effect (clinically, histopathologically and immunohistochemically) of a single intra-pterygium injection of bevacizumab after one month.

SUBJECTS AND METHODS

This prospective interventional study comprised 40 patients

with primary pterygium who attended the Outpatient Clinic of Department of Ophthalmology, Assiut University Hospitals from May 2015 to May 2016. The study was conducted according to the principles of the Declaration of Helsinki and was approved by the Human Research and Ethics Committee of Faculty of Medicine, Assiut University. Written informed consent was obtained from participants before their enrollment. Exclusion criteria included previous intraocular surgery or trauma, pregnant and lactating women, previous stroke or myocardial infarction.

Patients were randomly classified into 2 groups: the first group (22 eyes of 22 patients) received a single intralesional injection of bevacizumab (Avastin; Genentech, San Francisco, CA, USA); the second group comprised 18 eyes of 18 patients who did not receive subconjunctival bevacizumab. The two groups were of the same type of pterygium which was the fleshy type[14]. All patients were subjected to a full ophthalmic evaluation including visual acuity, tonometry and slit lamp examination.

In the first group, 0.1 mL of a 2.5 mg/0.1 mL concentration of bevacizumab was injected subconjunctivally into the body of the pterygium in operating theater following the instillation of topical anesthetic, 5% povidone iodine and antibiotic drops into conjunctival sac. Topical antibiotics, artificial tears and steroid were used for 1wk post injection. The non-injection group also received topical antibiotics, artificial tears, and steroids for 1wk. Patients were followed up at 1st, 2nd and 4th week. One month after Avastin injection excision of pterygium and conjunctival auto graft was done in both groups. The excised pterygium tissues were subjected to histopathological and immunohistochemical evaluation. Post operative evaluation stressed on degree of conjunctival injection (vascularity) and any ocular complications such as corneal abrasion, persistent epithelial defect, corneal edema, infection, subconjunctival hemorrhage or iritis. Patients in both groups were revaluated at 1mo post-excision and conjunctival auto graft. One of the authors (blinded to the patient groups) carried out the task of clinical assessment and follow-up of pterygium vascularity pre and post injection of Avastin.

As a control, six samples of normal conjunctiva were obtained during routine extracapsular cataract surgery from similar age-matched subjects who did not have pterygium or a history of pterygium excision. These control conjunctival specimens were subjected to examination as the excised pterygium tissues as follow: 1) histopathology. All specimens were formalin fixed, paraffin embedded, routinely processed and stained with heamatoxylin and eosin (HE). Blood vessels count was done on HE stained sections as described by Makhija *et al*[15]. Briefly by counting the number of vessels seen in 10 non overlapping high power fields (HPFs) and then the average was calculated. Large vessels with thick muscular walls were excluded from the count. The number of vessels count was expressed as average or mean blood vessels count/HPF; 2) immunohistochemistry. Immunohistochemistry were performed on 4 micron paraffin embedded sections using labeled streptavidin biotin method for VEGF according to manufacturer's protocol. After deparaffenization and rehydration, endogenous peroxidase activity were blocked using 3% hydrogen peroxide in methanol for 10min. Antigen retrieval was done for VEGF by boiling sections in 1 mmol/L EDTA (pH 8) for 10min. The slides were incubated overnight with primary antibody for VEGF (mouse monoclonal antibody, Ab-7 Cat No.MS-1467-P0, Thermo Fisher scientific Fremont, CA, USA) at a dilution of 1:50. The slides were then rinsed in phosphate buffered saline (PBS) for three times and incubated for 10min with biotinylated anti-polyvalent (Ultravision plus detection system anti-polyvalent HRP, ready to use, Thermo Fisher scientific, Fermont, CA, USA). After further rinsing with PBS, the slides were then incubated for other 10min with sterptavidin peroxidase (Thermo Scientific) at room temperature. The slides were rinsed 3 times in PBS and diaminobenzidine was applied for 5min at room temperature. Finally the slides were rinsed in distilled water, counterstained, dehydrated and mounted. Positive control for VEGF was angiosarcoma. Negative control slides were done by omitting primary antibody.

Assessment method: staining assessment was done on light microscope using Olympus (BH2 microscope). VEGF immunoreactivity was detected in stromal, endothelial and epithelial cells and as previously described as brown cytoplasmic staining[16]. To evaluate expression in epithelial cells both intensity and extent of staining were evaluated. Then combined score calculated by summation of both. Staining intensity was graded as follow: 0=negative, 1=weak, 2=moderate and 3=strong staining. The percentage of positive cells was also calculated (0=0, 1=1%-25%, 2=26%-50%, 3≥50%). The sum reaches maximum score of 6. For estimation of VEGF in stromal and endothelial cells, four scale system was used with 0=no expression, 1=mild, 2=moderate, 3=strong.

RESULTS

This study comprised 40 eyes of 40 patients (33 men, 7 women) of age range from 31-58y. The study group included 22 eyes. The control group included 18 eyes. A decrease in the vascularity of the pterygium was noted in all injected cases (Figure 1).

The decreased vascularity was noted on first day after injection except those cases complicated by subconjunctival hemorrhage in which vascularization was masked by subconjunctival blood. Marked pterygium pallor was noted 1-month post injection (Figure 2).

Apart from subconjunctival hemorrhage (Figure 1B) which was seen in 7 cases following Avastin injection no other local complications were reported. Subconjunctival hemorrhage resolved within 2wk and did not affect further evaluation.

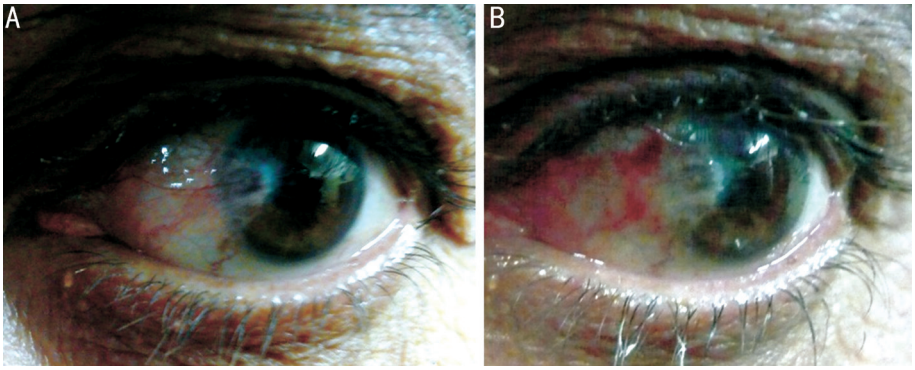

Figure 1 Decrease in the vascularity of the pterygium before and after single subconjunctival injection of bevacizumab (Avastin®) A: Pre-injected pterygium; B: A decrease in the vascularity of the pterygium in an injected case with subconjunctival hemorrhage.

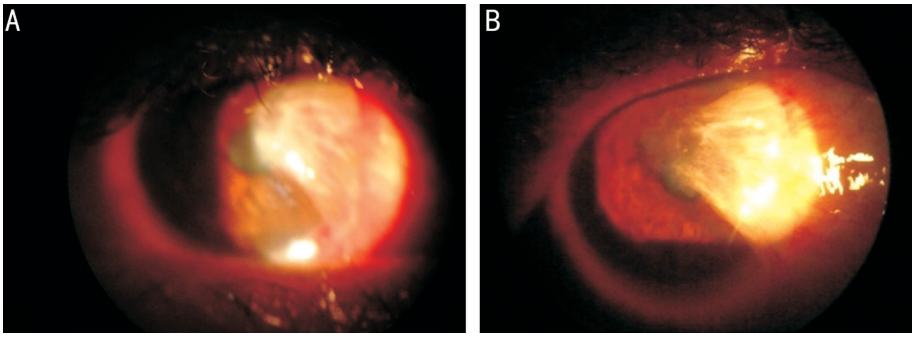

Figure 2 Pallor of pterygium first day after injection (A) and 1mo after injection (B).

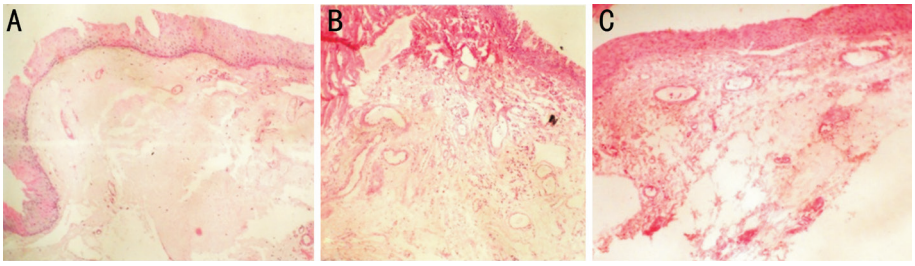

Figure 3 HE stained sections of normal conjunctiva, non-injected and injected pterygium A: Normal conjunctiva with non-keratinized stratified squamous epithelium and connective tissue stroma (HE×100); B: Non-injected pterygium with prominent vascularity and inflammatory cellular infiltrate in the connective tissue stroma (HE×100); C: Injected pterygium showing also vascularized stroma but to lesser extent than the previous image (HE×100).

There were no systemic adverse events after Avastin injection during the follow up period. One month after pterygium excision and conjunctival auto-graft surgery we noted no cases of pterygium recurrence in either group. The mean vessel count was higher in non-injected pterygia (12.65±1.246) than in injected pterygia (4.487±0.5475) and the difference was statistically significant (P=0.001). Also, the mean vessel count in both groups was significantly higher than normal conjunctive (2.100±0.1095; P=0.005 and 0.001; Figure 3).

Vascular Endothelial Growth Factor VEGF expression was found in all cases of pterygium whether injected or non-injected in epithelial cells, endothelial cells, stromal fibroblasts and inflammatory cells. The expression was diffusely cytoplasmic with intensification at the superficial layers of the epithelium.

On the other hand, in normal conjunctiva VEGF expression was negative or weakly positive in epithelial cells and negative in stroma and endothelial cells. Tables 1 and 2 showed the results of VEGF protein expression in epithelial cells, stromal and endothelial cells. A statistically significant difference in VEGF expression between injected and non-injected cases was detected in the epithelial, stroma and endothelial cells (P=0.0001, 0.016, 0.014 respectively). Negative VEGF expression was reported in normal conjunctiva. Intense expression of VEGF was noted in all layers of epithelium, blood vessels and stroma of non-injected pterygium compared to less expression in injected pterygium (Figure 4).

Table 1 VEGF expression in epithelial cells *n* (%)

Combined score	No. of cases	
	Injected (n=22)	Non-injected (n=18)
1-2	6 (27.27)	0 (0)
3-4	16 (72.73)	5 (27.78)
5-6	0 (0)	13 (72.22)

VEGF: Vascular endothelial growth factor.

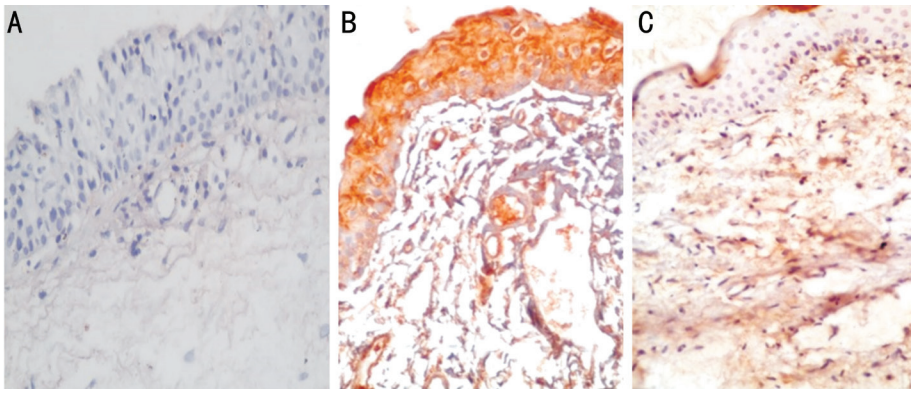

Figure 4 Demonstrated the difference between VEGF expression in normal conjunctiva, injected and non-injected pterygium A: Section from normal conjunctiva showing negative expression to VEGF (IHC×200); B: VEGF expression in non-injected pterygium showing intense expression in all layers of epithelium, blood vessels and stroma (IHC×200); C: VEGF expression in injected pterygium showing decreased expression in epithelium, blood vessels and stroma compared to non-injected pterygium (IHC×200). IHC: Immunohistochemistry.

Table 2 VEGF expression in stromal and endothelial cells

VEGF expression	Injected cases (n=22)	Non-injected cases (n=18)
VEGF in endothelial cells		
0	0	0
+	10	0
++	12	15
+++	0	3
VEGF in stroma		
0	0	0
+	9	0
++	13	13
+++	0	5

VEGF: Vascular endothelial growth factor.

DISCUSSION

Pterygium is an elastotic degeneration of conjunctival tissue with a stromal overgrowth of fibroblasts, blood vessels accompanied by an inflammatory cell infiltrate, and abnormal extracellular matrix accumulation composed of elastin and collagen[17]. Over expression of VEGF in pterygium, tissue and ocular inflammation together with the abundance of new vessels supported the role of angiogenesis in the formation of pterygia[1,16,18]. Bevacizumab is a humanized monoclonal antibody that recognizes and blocks VEGF-A, resulting in an antiangiogenic effect[19].

There have been several reports of the use of bevacizumab with successful results in both primary and recurrent pterygia[20-21]. In the current study, a single intralesional injection of 0.1 mL of a 2.5 mg/0.1 mL of bevacizumab was used for primary pterygium and we investigated its effect after one month. No serious ocular or systemic side effects were noted during the follow-up period. The only reported adverse event was temporary subconjunctival hemorrhage which could be an incidental finding due to the injection process itself and did not impair further follow-up. Bahar *et al*[22] reported no ocular or systemic adverse effects in patients treated with subconjunctival bevacizumab for recurrent pterygium.

We reported a decrease in the vascularity of the pterygium in all injected cases, which might be of value during subsequent pterygium excision as it minimizes intraoperative bleeding. The clinically noticed decrease in pterygium vascularity was also documented histologically. The mean vessel count was lower in injected pterygium than in non-injected pterygium. The mean vessel count in both pterygium study groups was found to be significantly higher than normal conjunctive. Lekhanont *et al*[23] found an initial decrease in conjunctival hyperemia scores after subconjunctival bevacizumab injection. In addition, a significant decrease in the vascular component of the pterygia was reported by Besharati *et al*[5]. Although Bahar *et al*[22] reported the use of subconjunctival bevacizumab on corneal vessel density in recurrent pterygia, Razeghinejad *et al*[24] reported that a single, intraoperative, subconjunctival bevacizumab injection did not have an influence on recurrence or postoperative hyperemia after primary pterygium excision.

In our study we found increased VEGF expression in all cases of pterygium whether injected or non-injected in comparison to normal conjunctiva. Jin *et al*[25] found that decreased anti-angiogenic factors, together with increased stimulators, have been hypothesized in the formation and progression of pterygia. Immunohistochemical studies by Marcovich *et al*[2] and Lee *et al*[26] have shown that VEGF levels are more expressed in pterygium than in normal conjunctiva. Also, Gebhardt *et al*[27] reported that VEGF increased in pterygia in comparison with that in healthy human conjunctiva. Similarly, over expression of VEGF in pterygium tissue was described by Jin *et al*[25] and Hosseini *et al*[7].

Our study proved the effect of single intralesional injection of Avastin on pterygium vascular pattern and the decrease in VEGF expression in injected pterygium after one month. One month duration after Avastin injection could be a good interval before excising injected pterygia. Further studies with longer follow up periods, and larger number of patient is needed to evaluate the effect of injection on pterygium recurrence after

excision. This may enable limiting the need for surgery and may decrease recurrence rate which should be investigated in a future study.

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Conflicts of Interest: Mohamed TA, None; **Soliman W,** None; **Fathalla AM,** None; **El Refaie A,** None.

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