• Basic Research •

Evaluation of nintedanib as a new postoperative antiscarring agent in experimental extraocular muscle surgery

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Abstract

● **AIM:** To investigate the efficacy of nintedanib on reducing postoperative inflammation, fibrosis and adhesion formation following extraocular muscle surgery in rabbits in comparison with triamcinolone acetonide (TA).

● **METHODS:** Reinsertion of superior rectus muscle in right eyes of 30 New Zealand white rabbits were performed. They were randomized to receive one of the following treatments: 0.9% normal saline, one of 1-, 5-, and 10 μmol doses of nintedanib subconjunctivally immediately after surgery and on postoperative day 1, 2, 3, 5, and 7, and TA immediately after surgery. As a control group, unoperated left eyes (n=6) were used. On the 28th day, six eyes from each group were enucleated and histopathologically and immunohistochemically analyzed to assess the postoperative inflammatory changes, fibrosis and adhesion. Transforming growth factor beta, matrix metalloproteinase-2 and alpha smooth muscle actin expressions were evaluated.

● **RESULTS:** Conjunctival and scleral inflammation in TA and nintedanib groups were significantly reduced compared to saline (sham) group. Conjunctival vascularity and rectus muscle fibrosis were significantly reduced in 10 μmol nintedanib group. Nintedanib groups were the most effective groups in reduction of perimuscular fibrosis. Neither three nintedanib groups nor TA group differed statistically from sham group with regard to adhesion. The expressions of transforming growth factor beta, alpha smooth muscle actin and matrix metalloproteinase-2 were reduced in nintedanib groups compared to saline group.

● **CONCLUSION:** Nintedanib appears to attenuate postoperative inflammation and fibrosis after extraocular muscle surgery. Nintedanib may be a safer and stronger alternative agent in extraocular muscle surgery when compared to steroids. Further investigation is needed to prove antiadhesive effect of nintedanib.

● **KEYWORDS:** extraocular muscle surgery; fibrosis; inflammation; nintedanib; triamcinolone acetonide

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INTRODUCTION

Main factors that affect success of extraocular muscle (EOM) surgery are inflammation, fibrosis and adhesion formation. Fibrosis, resulting from chronic inflammation, is the replacement of normal tissues by connective tissue and leads to contracture of muscle. Adhesion, on the other hand, is the attachment between muscle and surrounding tissues that leads to restriction of the muscle[1]. Therefore, wound repair with minimal scar formation is important for obtaining normal function and structure of injured tissues.

It is often difficult to deal with adhesions, once formed. Today, the most common medical therapy used for antiinflammatory and antifibrotic effect is steroids. But their adequacy is questionable. Various treatment options, such as polyglactin 910 mesh[2-3], silicone sheet[4], mitomycin C[5-7], 5-fluorouracil[8-9], seprafilm (Genzyme, Cambridge,

Massachusetts)[10], bevacizumab[11], amniotic membrane[12-14], all-trans-retinoic acid[15], and pirfenidon[16] have been used to provide the least amount of adhesion formation after EOM surgery. However, these methods have not been yet in use because of their unavailability and associated complications. Thus a new antiscar agent which leads minimal side effects is needed.

Nintedanib, a multiple tyrosine kinase inhibitor, selectively binds to and inhibits vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR). Nintedanib also inhibits members of the Src family of tyrosine kinases including Lyn, Lck and Flt-3[17]. It is clinically used for idiopathic pulmonary fibrosis (IPF) treatment[18]. In pulmonary fibrosis animal model studies, nintedanib's anti-fibrotic and anti-inflammatory activities by affecting fibroblast proliferation, migration, differentiation, extracellular matrix (ECM) protein secretion and fibrotic gene expression have been shown[19-22]. These data made us hypothesize that nintedanib may also show these antifibrotic effects in EOM surgery.

The current study aimed to investigate histopathologically and immunohistochemically the effect of nintedanib on inflammation, fibrosis and adhesion formation after experimental EOM surgery in rabbits using triamcinolone acetonide (TA) as a reference agent. We claimed that nintedanib could be used as an adjunctive treatment to reduce postoperative inflammation, fibrosis and adhesion formation after EOM surgery.

MATERIALS AND METHODS

Ethical Approval All animal experiments were handled according to the ARVO Statement guidelines for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Experiments Ethics Committee of the Kocaeli University Medical Faculty.

Animal Treatment Thirty New Zealand rabbits, weighing 2500 to 4000 g and 15 to 20 weeks old, were purchased from Aykut Bolu Experimental Animal Production and Supply Center (Bolu, Turkey). All rabbits were acclimatized for 2wk before experiments. Rabbits were randomly allocated to one of five treatment groups: 0.9% normal saline (NS; Sham) group (n=6), TA treatment group (n=6) and three different concentrations of nintedanib-treatment groups (n=6). The right eyes were used for the experiments. As a control group, left eyes of rabbits were used (n=6). Left eyes that did not undergo any procedure were used to comparatively demonstrate the effect of surgery on histopathological and immunohistochemical parameters. Standard preoperative procedures and surgical method have been applied to all rabbits. Standard EOM surgery was performed by one surgeon. Rabbits in sham group were treated with 0.1 mL 0.9% NS by subconjunctival injection immediately after surgery and on postoperative day (POD) 1, 2, 3, 5, and 7. Rabbits in TA group were treated with 0.1 mL of 4 mg (40 mg/mL) triamcinolone (Kenacort-A, Deva, Turkey) by subconjunctival injection immediately after surgery. Rabbits in nintedanib groups received subconjunctival injection with 0.1 mL of 1, 5, or 10 micromolar (μmol) of nintedanib immediately after surgery and on POD 1, 2, 3, 5, and 7. Surgical wound appearance was recorded by photographs on the 3rd day, 1st, 2nd and 4th weeks after surgery.

Preparation of Nintedanib Solution Nintedanib 50 mg powder obtained from MedChemExpress local distributor company (Suarge, Turkey) was used. The required amount of nintedanib was weighed with precision balances (Shimadzu, Japan) and dissolved in DMSO (Dimethylsulfoxide; SantaCruz, USA) to prepare a 1 mmol stock nintedanib solution. Working solutions of 1, 5 and 10 μmol were prepared from the obtained stock solution and placed in sterile vials and carried to the surgical field.

Anesthesia Technique Both 5 mg/kg xylazine hydrochloride (Rompun, Bayer, Turkey) and 40 mg/kg ketamine hydrochloride (Keta Control, Doga Pharma, Turkey) were administered intramuscularly for general anesthesia. For topical anesthesia, 0.5% proparacaine hydrochloride drops were used (Alcaine, Novartis, Switzerland).

Surgical Technique Surgery was performed by using an operating microscope (Leica, Germany). Before starting surgery, one drop of 2.5% phenylephrine was applied to minimize intraoperative bleeding. After surgical antisepsis with povidone iodine, a traction suture was placed on the superior limbal border to deviate the eye inferiorly. So that superior rectus muscle (SRM) was easily visible. A limbal peritomy and sharp dissection of Tenon's capsule were performed respectively. SRM was fixed with two 6.0 vicryl sutures (polyglactin) and detached from its insertion. In De Carvalho *et al*'s[23] study minimal inflammatory response was reported after EOM surgery in rabbits. So, 1 cm of the underlying scleral bed was cauterized to control bleeding and to exacerbate inflammatory response[2]. The muscle was sutured to its original scleral insertion site and 8-0 vicryl sutures were used to close the peritomy. After the surgery was completed, subconjunctival drug treatment was performed. Agents were injected through 30-gauge needle slowly at surgical sites. Moxifloxacin eye drop was applied for one week postoperatively.

Histopathologic Evaluation All rabbits were euthanized on the postoperative 28th day. After the surgical sites were excised, SRM and surrounding tissue were put in formalin solution and fixed by immersion method for 48h. After washing process in running tap water, the tissues were passed through

Table 1 Parameters for histopathologic evaluation[10,13-14,24]

Parameters	Histopathologic evaluation
Conjunctival inflammation	0: No inflammation
	1: A few lymphocytes and plasma cells beneath epithelium
	2: Mild inflammatory infiltrate composed of lymphocytes, plasma cells and polymorphnuclear leucocytes beneath epithelium and congestion
	3: Grade two plus neutrophils in the epithelium
	4: High concentrations (collections) of lymphocytes, plasma cells, polymorphonuclear leucocytes and histiocytes (both intraepithelial and subepithelial) and ulceration
Scleral inflammation	0: Absent
	1: Present
Conjunctival vascularity	0: White avascular conjunctiva
	1: Some avascularity
	2: Normal vascularity
	3: Mildly increased vascularity suggestive of ongoing inflammation
	4: Moderately increased vascularity
	5: Severely increased vascularity
Rectus muscle fibrosis	0: Absent
	1: Present
Perimuscular fibrosis	0: No fibrosis
	1: Mild perimuscular fibrotic reaction (stained collagen is detectable only in thin bands immediately adjacent to muscle)
	2: Easily detected thick bands
	3: Well-developed, dense bands of collagen
	4: A severe fibrotic response replacing large areas
Adhesion between SRM and sclera	0: No fibrosis
	1: Fibrous tissue present between sclera and superior rectus muscle
	2: Grade one plus fibrous septa between superior rectus muscle fibers
	3: Grade one plus Grade two plus fibrous tissue expansion through tenon capsule and subconjunctival area

gradually increasing series of ethyl alcohol (70%, 90%, 96%, 100% respectively; Merck, Germany) and dehydration was performed. After 30min of transparency with toluene (Merck, Germany), the tissues were kept in pure paraffin for 2h and embedded in paraffin blocks at room temperature. Serial cuts of 4 μm thickness were taken from the paraffin blocks with a microtome (Leica SM 2000R, Germany). Staining of the sections was started from the insertion of SRM. Haematoxylin eosin (H&E) staining was used to assess the presence of conjunctival vascularity, scleral and conjunctival inflammation. Demonstration of collagen fibers and grading of SRM and perimuscular fibrosis as well as the amount of scar tissue formation between muscle and sclera (adhesion) were performed by Masson's trichrome (MT) staining. Parameters for histopathologic evaluation are listed in Table 1.

Immunohistochemical Evaluation The 4-μm sections taken from paraffin blocks on polylyzed slides were kept in a 56°C oven for 1 night to deparaffin, and then they were kept in toluene for 3 times for 5min and thoroughly cleared of paraffin. Then, it was kept in alcohol for 2×5min at 100°, 1×5min at 96°, 1×5min at 90°, 1×5min at 70° alcohol and finally in distilled water for 2×5min. The sections kept in phosphate buffered saline (PBS) solution for 5min, placed in citric acid solution for antigen retrieval, boiled for 10min in the microwave and kept for 20min to cool. Then, protein block solution (ab64264, Abcam) was applied for 10min to block non-specific antibody binding to the sections washed in PBS. Matrix metalloproteinase-2 (MMP-2; ab2462, Abcam, 1:200 dilution ratio), transforming growth factor-β (TGF-β; ab190503, Abcam, 1:200) and alpha-smooth muscle actin (α-SMA; ab7817, Abcam, 1/50 dilution rate) primary antibodies were dropped and kept at +4°C for one night/overnight. Tissue sections washed with PBS were incubated with biotiny goat antipolyvalent solution (ab64264, Abcam) for 10min. After 10min of incubation, the sections with MMP-2, TGF-β and α-SMA expressions were observed in brown color with the chromogen called diaminobenzidine. Tissues that were counterstained with Mayer hematoxylin (ab128990, Abcam) were taken into toluene after dehydration (passing through the residual alcohol series) and covered with Entellan (Merck, Germany) with a coverslip. For immunohistochemical examination, MMP-2, TGF-β, and α-SMA expressions were evaluated[24-26].

Statistical Analysis The data was statistically analyzed by using the software SPSS 26.0. For descriptive statistics of histopathologic examination data, ratio and frequency values were used. Chi-squared (χ^2) test was performed to analyze

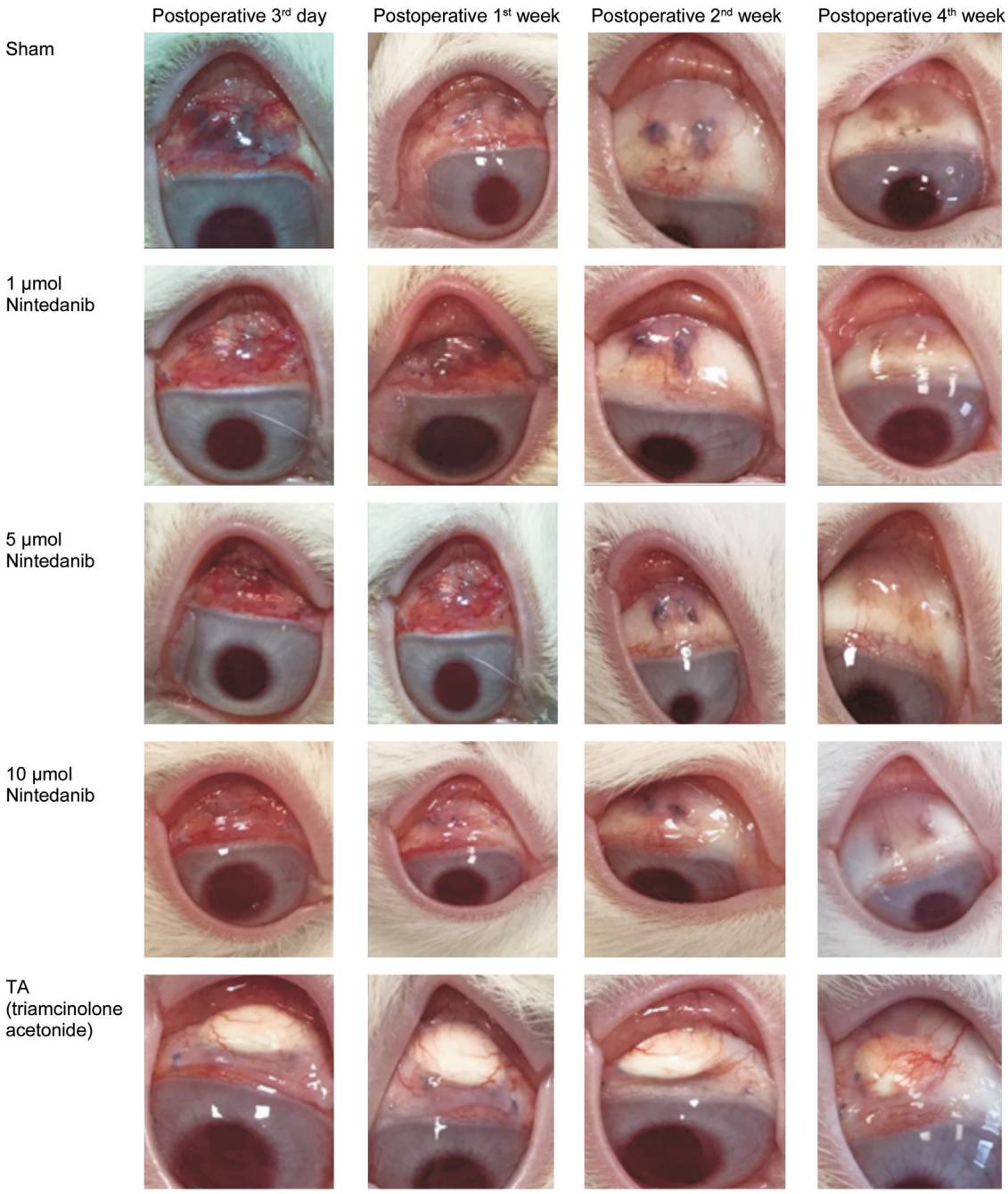

Figure 1 Macroscopic views of the wound sites postoperatively.

qualitative independent data. When χ^2 test conditions were not met, Fischer test was used. The χ^2 test was used to compare the data of conjunctival vascularity, conjunctival and scleral inflammation, as well as of adhesion, SRM and perimuscular fibrosis. For descriptive statistics of immunohistochemical examination data, the lowest and highest values of mean, standard deviation and median, frequency and ratio values were used. Distribution of variables was measured with Kolmogorov-Simirnov test. For analysis of quantitative independent data, Kruskal-Wallis and Mann-Whitney *U* tests were used. Values of $P<0.05$ indicated statistical significance.

RESULTS

All rabbits appeared to be healthy and ate normally. For early postoperative days, conjunctival hyperemia was observed in many surgical sites (Figure 1). There was no evidence of systemic toxicity in any animal.

Histopathological Findings Five treatment groups showed conjunctival inflammatory cell infiltration in the histological examination of H&E stained sections. Sham group had significantly higher conjunctival inflammation compared to 1, 5, and 10 μmol nintedanib and TA groups (P=0.015, 0.015, 0.002, and 0.002 respectively). Scleral inflammation had statistically significant difference in sham group compared to control, 1, 5, and 10 μmol nintedanib and TA groups (P=0.015, 0.015, 0.015, 0.015, and 0.015 respectively). Conjunctival vascularity was significantly increased in sham group

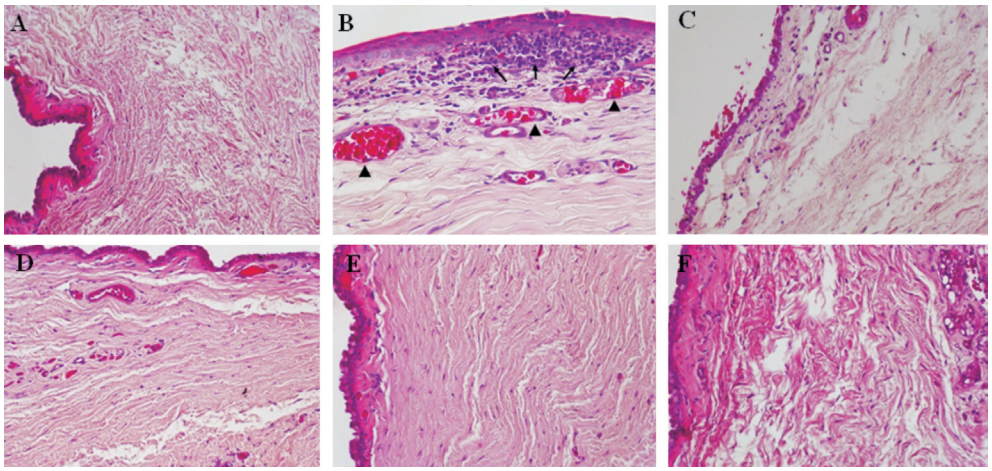

Figure 2 Photomicrographs demonstrating an example of hispathological sections of subconjunctival tissue A: Control group, conjunctival epithelial continuity and healthy morphology in subconjunctiva (H&E, 200× magnification); B: Sham group, black arrow indicates severe leucocytic infiltration; arrowhead indicates increased vascularization (H&E, 400× magnification); C: 1 μmol nintedanib group, some degree of vascularization and inflammation (H&E, 200× magnification); D: 5 μmol nintedanib group, lesser degree of vascularization, edema and inflammation. Conjunctival epithelial continuity is intact (H&E, 200× magnification); E: 10 μmol nintedanib group, minimal vascularization and inflammation, nearly normal morphology is observed (H&E, 200× magnification); F: Triamcinolone acetonide group, some degree of vascularization and inflammation is seen (H&E, 200× magnification). H&E: Haematoxylin eosin.

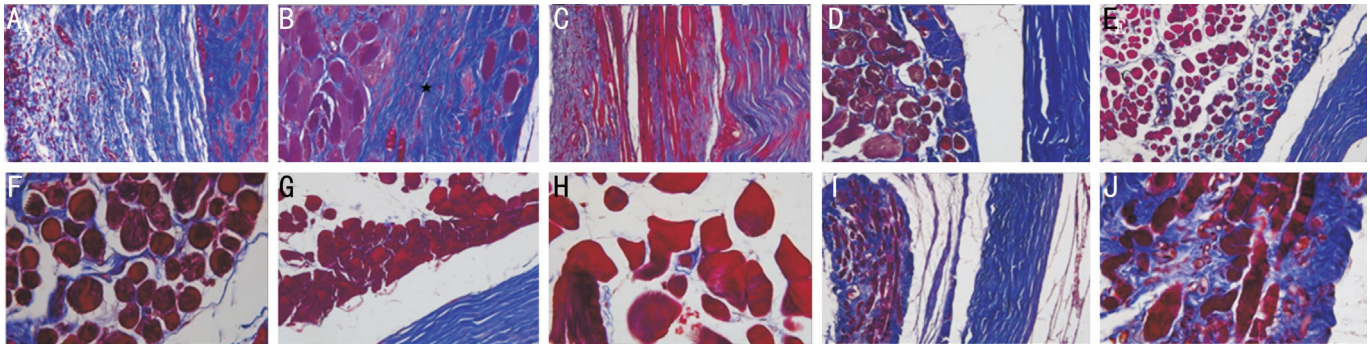

Figure 3 Photomicrographs demonstrating an example of histopathological sections of SRM and perimuscular tissue A, B: Sham group, severe fibrosis in SRM along with local musculoscleral and musculoconjunctival adhesions were detected. The black star indicates severe collagen accumulation. C, D: 1 μmol nintedanib group, fibrotic areas were concentrated in the perimuscular area, musculoconjunctival adhesions were detected in some areas. E, F: 5 μmol nintedanib group, fibrosis in SRM was considerably reduced and there was a very small amount of collagen accumulation in the perimuscular region adjacent to the muscle-sclera. G, H: 10 μmol nintedanib group, fibrosis and adhesion decreased substantionally and showed a morphology close to control. I, J: Triamcinolone acetonide group, SRM fibrosis and adhesion decreased (A, C, E, E, G, I 100×, B, D, F, H, J 400× magnification). SRM: Superior rectus muscle.

compared to control and 10 μmol nintedanib groups (P=0.002 and 0.015 respectively). 10 μmol nintedanib group showed statistically significant difference in reducing conjunctival vascularity compared to sham group (P=0.015; Table 2 and Figure 2).

Five to ten μmol nintedanib and TA groups showed significantly less intense perimuscular collagen staining compared to sham group (P=0.015, 0.002, and 0.002 respectively).

Only 10 μmol nintedanib group had statistically significant difference in reducing rectus muscle fibrosis compared to sham group (P=0.015). Adhesion between SRM and sclera was significantly increased in sham group compared to control group (P=0.002). Neither 1, 5, and 10 μmol nintedanib groups nor TA group differed statistically from sham group with regard to adhesion (P=0.182, 0.061, 0.061, and 0.182 respectively; Table 3 and Figure 3).

Immunohistochemical Findings H-scores of TGF-β, α-SMA and MMP-2 expressions in subconjunctival area and SRM were calculated. The mean values of expressions in the six groups are listed in Tables 4 and 5.

Subconjuctival expressions As a consequence of the immunostaining, it was found that TGF-β expression, the marker of cell proliferation and differentiation, in subconjunctival area was highest in sham group compared to control, 1, 5, and 10 μmol nintedanib and TA groups (P=0.004, 0.016, 0.030, 0.004, and 0.004 respectively). TA group had

Table 2 Distribution of conjunctival inflammation, scleral inflammation, conjunctival vascularity according to groups

Parameters	Control		Sham		1 μmol nintedanib		5 μmol nintedanib		10 μmol nintedanib		Triamcinolone acetonide		^cP
	n (%)	Mean±SD	*n* (%)	Mean±SD	*n* (%)	Mean±SD	*n* (%)	Mean±SD	*n* (%)	Mean±SD	*n* (%)	Mean±SD	
Conjunctival inflammation[a]	-	0.16±0.4	-	3.83±0.40	-	1.83±0.75	-	1.66±0.81	-	1.16±0.40	-	1.50±0.54	<0.05
0	5 (83.3)		0		0		0		0		0		
1	1 (16.7)		0		2 (33.3)		3 (50.0)		5 (83.3)		3 (50.0)		
2	0		0		3 (50.0)		2 (33.3)		1 (16.7)		3 (50.0)		
3	0		1 (16.7)		1 (16.7)		1 (16.7)		0		0		
4	0		5 (83.3)		0		0		0		0		
Scleral inflammation[b]	-	-	-	0.83±0.40	-	-	-	-	-	-	-	-	<0.05
0	6 (100)		1 (16.7)		6 (100)		6 (100)		6 (100)		6 (100)		
1	0		5 (83.3)		0		0		0		0		
Conjunctival vascularity[a]	-	2	-	3.33±0.51	-	2.66±0.51	-	2.50±0.54	-	2.16±0.40	-	2.66±0.51	<0.05
2	6 (100)		0		2 (33.3)		3 (50.0)		5 (83.3)		2 (33.3)		
3	0		4 (66.7)		4 (66.7)		3 (50.0)		1 (16.7)		4 (66.7)		
4	0		2 (33.3)		0		0		0		0		

[c]Chi-square test (Fischer test). SD: Standard deviation; [a]Numerical variable; [b]Categorical variable.

Table 3 Distribution of perimuscular fibrosis, rectus muscle fibrosis, adhesion according to groups

Parameters	Control		Sham		1 μmol nintedanib		5 μmol nintedanib		10 μmol nintedanib		Triamcinolone acetonide		^cP
	n (%)	Mean±SD	*n* (%)	Mean±SD	*n* (%)	Mean±SD	*n* (%)	Mean±SD	*n* (%)	Mean±SD	*n* (%)	Mean±SD	
Perimuscular fibrozis[a]	-	-	-	3.33±0.51	-	1.66±0.81	-	1±0.63	-	1±0.63	-	1.33±0.81	<0.05
0	6 (100)		0		0		1 (16.7)		1 (16.7)		1 (16.7)		
1	0		0		3 (50.0)		4 (66.7)		4 (66.7)		2 (33.3)		
2	0		0		2 (33.3)		1 (16.7)		1 (16.7)		3 (50.0)		
3	0		4 (66.7)		1 (16.7)		0		0		0		
4	0		2 (33.3)		0		0		0		0		
Rectus muscle fibrosis[b]	-	-	-	-	-	0.83±0.40	-	0.50±0.54	-	0.16±0.40	-	0.50±0.54	<0.05[c]
0	6 (100)		0		1 (16.7)		3 (50.0)		5 (83.3)		3 (50.0)		
1	0		6 (100)		5 (83,3)		3 (50.0)		1 (16.7)		3 (50.0)		
Adezyon[a]	-	-	-	2.16±0.75	-	0.66±0.81	-	0.33±0.51	-	0,33±0.51	-	0.83±0.98	<0.05
0	6 (100)		0		3 (50.0)		4 (66.7)		4 (66.7)		3 (50.0)		
1	0		1 (16.7)		2 (33.3)		2 (33.3)		2 (33.3)		1 (16.7)		
2	0		3 (50.0)		1 (16.7)		0		0		2 (33.3)		
3	0		2 (33.3)		0		0		0		0		

[c]Chi-square test (Fischer test). SD: Standard deviation; [a]Numerical variable; [b]Categorical variable.

higher H-scores compared to 5-10 μmol nintedanib groups (P=0.046, and 0.043 respectively). The 1 μmol nintedanib group had higher H-scores compared to 5-10 μmol nintedanib groups (P=0.036, and 0.016 respectively). There was no statistically significant difference between 1 μmol nintedanib group and TA group (P=0.517). Additionally, there was no statistically significant difference between 5 and 10 μmol nintedanib groups (P=0.146).

To assess the degree of transdifferentiation to myofibroblasts, we performed immunohistochemical staining for α-SMA. Sham group had significantly higher values compared to 5-10 μmol nintedanib groups (P=0.01 and 0.004 respectively). TA group had significantly higher values compared to 10 μmol nintedanib group (P=0.01). Additionally, 1-5 μmol nintedanib groups had significantly higher values compared to 10 μmol nintedanib group (P=0.008 and 0.090 respectively).

Expression of MMP-2, the marker of fibroblast cell density, was significantly higher in subconjunctival tissue of sham group compared to 5-10 μmol nintedanib and TA groups (P=0.008, 0.004, and 0.045 respectively). The 1 μmol nintedanib group had significantly higher values compared to 5-10 μmol nintedanib groups (P=0.043 and 0.010 respectively). Additionally, 5 μmol nintedanib group had significantly higher values compared to 10 μmol nintedanib group (P=0.044). There was no statistically significant difference between 1 μmol nintedanib and TA groups (P=0.337).

Superior rectus muscle expressions The statistical analysis showed that the H-scores of TGF-β expression in SRM were significantly higher in sham group compared to 1, 5, and 10 μmol nintedanib and TA groups (P=0.016, 0.004, 0.004, and P=0.048 respectively). It was determined that 1, 5, and 10 μmol nintedanib and TA groups revealed similar TGF-β expressions within SRM (P>0.05).

Expression of α-SMA was significantly higher in SRM of sham group compared to 1, 5, and 10 μmol nintedanib and TA groups (P=0.036, 0.010, 0.010, and 0.030 respectively). Three nintedanib and TA groups revealed similar α-SMA expressions within SRM (P>0.05).

Table 4 Subconjunctival H-scores of TGF-β, α-SMA, and MMP-2 expressions

Parameters	Groups	Subconjunctival H scores			[a]*P*
		Minimum-Maximum	Median	Mean±SD	
TGF-β	Control	5-15	10.0	10.0±4.5	<0.001
	Sham	120-190	153.3	150.0±24.4[b]	
	Triamcinolone acetonide	75-150	98.3	87.5±30.2[b,c]	
	1 μmol nintedanib	70-150	108.3	110.0±30.6[b,c]	
	5 μmol nintedanib	50-100	70.0	65.0±20.9[b,c,d,e]	
	10 μmol nintedanib	30-80	52.5	45.0±22.3[b,c,d,e]	
	Total	5-190	82.1	77.5±50.9	
α-SMA	Control	15-55	29.2	25.0±14.6	<0.001
	Sham	70-160	131.7	145.0±35.4[b]	
	Triamcinolone acetonide	50-105	76.7	75.0±22.7[b]	
	1 μmol nintedanib	50-150	98.3	87.5±37.2[b]	
	5 μmol nintedanib	40-85	59.2	52.5±18.8[b,c,d]	
	10 μmol nintedanib	30-55	40.8	40.0±10.6[b,c,d,e,f]	
	Total	15-160	72.6	55.0±42.3	
MMP-2	Control	10-25	18.3	17.5±6.1	<0.001
	Sham	80-180	143.3	147.5±37.9[b]	
	Triamcinolone acetonide	40-150	92.5	92.5±42.7[b,c]	
	1 μmol nintedanib	60-180	113.3	110.0±44.5[b]	
	5 μmol nintedanib	55-95	70.0	65.0±15.2[b,c,e]	
	10 μmol nintedanib	30-70	49.2	45.0±15.6[b,c,d,e,f]	
	Total	10-180	81.1	70.0±50.6	

[a]Kruskal-wallis (Mann-whitney *U* test). [b]Difference with Control group *P*<0.05; [c]Difference with Sham group *P*<0.05; [d]Difference with triamcinolone acetonide group *P*<0.05; [e]Difference with 1 μmol Nintedanib group *P*<0.05; [f]Difference with 5 μmol Nintedanib group *P*<0.05.

Table 5 SRM H-scores of TGF-β, α-SMA, and MMP-2 expressions

Parameters	Groups	SRM H-scores			[a]*P*
		Minimum-Maximum	Median	Mean±SD	
TGF-β	Control	15-50	25.8	20.0±13.9	<0.001
	Sham	115-215	162.5	165.0±33.8[b]	
	Triamcinolone acetonide	50-180	100.8	95.0±48.6[b,c]	
	1 μmol nintedanib	55-155	96.7	90.0±40.3[b,c]	
	5 μmol nintedanib	20-85	59.2	62.5±22.6[b,c]	
	10 μmol nintedanib	25-80	51.7	45.0±23.3[b,c]	
	Total	15-215	82.8	67.5±53.9	
α-SMA	Control	140-260	175.0	165.0±43.1	0.036
	Sham	160-240	219.2	232.5±31.1	
	Triamcinolone acetonide	140-210	179.2	187.5±29.1[e]	
	1 μmol nintedanib	70-230	151.7	150.0±66.4[e]	
	5 μmol nintedanib	50-190	125.8	130.0±55.8[e]	
	10 μmol nintedanib	115-195	148.3	135.0±33.4[e]	
	Total	50-260	166.5	167.5±51.5	
MMP-2	Control	5-15	10.8	10.0±3.8	<0.001
	Sham	110-155	125.8	122.5±15.9[b]	
	Triamcinolone acetonide	60-100	80.8	80.0±16.2[b,c]	
	1 μmol nintedanib	45-105	80.8	80.0±20.3[b,c]	
	5 μmol nintedanib	20-125	58.3	55.0±36.1[b,c,d]	
	10 μmol nintedanib	10-85	40.8	37.5±28.1[b,c,d,e]	
	Total	5-155	66.3	65.0±42.0	

[a]Kruskal-wallis (Mann-whitney *U* test). [b]Difference with Control group *P*<0.05; [c]Difference with Sham group *P*<0.05; [d]Difference with triamcinolone acetonide group *P*<0.05; [e]Difference with 1 μmol Nintedanib group *P*<0.05. SEM: Superior rectus muscle.

The results of the immunostaining for MMP-2 in SRM revealed that positivity was significantly higher in sham group compared to 1, 5, and 10 μmol nintedanib and TA groups (P=0.004, 0.020, 0.004, and 0.004 respectively). When compared to 5-10 μmol nintedanib groups, TA group had statistically increased MMP-2 expression in SRM (P=0.020 and 0.004 respectively). One μmol nintedanib group showed statistically increased expression compared to 10 μmol nintedanib group (P=0.036). There was no statistically significant difference between 1 μmol nintedanib and TA group (P=0.808). Additionally, there was no statistically significant difference between 5 and 10 μmol nintedanib groups (P=0.374).

DISCUSSION

It is possible to see some restrictions in ocular motility after EOM surgery. Various reasons may be responsible for surgical failure. The most challenging one is believed to be the postoperative scar tissue and adhesion formation involving EOM, Tenon's capsule, intermuscular membrane and sometimes orbital fat tissue.

The most commonly used anti-inflammatory and antifibrotic agents in EOM surgery are steroids. Although some published studies have revealed that subconjunctival steroid injection has cytocidal effects on adjacent fibroblasts and causes breakdown of the collagen fibers[27], steroids may induce significant side effects, such as cataract formation and increased intraocular pressure. Currently, the most suitable material or pharmacologic agent has not been yet identified. Therefore, there is a need for experimental researches that aim to search for new agents that are easily applicable and obtainable with negligible side effects.

In this study, we investigated the effects of three doses of subconjunctival nintedanib injection on postoperative inflammation, fibrosis and adhesion formation after experimental EOM surgery in a rabbit model. The current data shows that nintedanib can effectively reduce scar formation. In histological sections of 10 μmol nintedanib applied animals, a considerable decrease in severity of fibrosis was found compared to TA. Although nintedanib was found to be statistically insignificant in terms of reducing adhesion in this study, we cannot ignore its successful antifibrotic effect. Because, when histopathological examinations were evaluated, some degree of adhesion developed in all eyes in sham group, whereas adhesion did not develop in 1, 5, 10 μmol nintedanib groups at a rate of 50 percent or more. In addition, it can be said that the applied cautery is not sufficient since the percentage of Grade 3 adhesion extending to the Tenon capsule and subconjunctival area is only 33.3%. From this point of view, the reason why we could not obtain statistically significant results may be the insufficient number of subjects and the insufficient effect of cautery.

Nintedanib (BIBF 1120) is an orally bioavailable intracellular inhibitor of multiple receptor and non-receptor tyrosine kinases, with significant antiangiogenic, antifibrotic and antineoplastic activities[18-19]. Recent studies have reported that nintedanib can be used to target tumor growth, metastasis and angiogenesis in some cancers (lung, ovary, colorectal)[28]. Knowing that PDGFR and FGFR, the targets of nintedanib, are involved in the pathogenesis of intraocular fibrosis[29], these findings may have direct implications for assuming nintedanib as an option for treating postoperative adhesion related to EOM surgery.

To our knowledge, there are no published reports about the histopathologic and immunohistochemical data in the current literature evaluating the effect of nintedanib application in ophthalmic surgery. Data from *in vitro* studies have shown that nintedanib interferes with fibrotic processes such as TGF-β induced fibroblast proliferation, migration and differentiation, and the secretion of ECM[20,30]. In the literature, there is only one *in vitro* study on the use of nintedanib as an antifibrotic agent in human tenon fibroblasts (HTFs), obtained form tissue explants taken during strabismus and glaucoma filtering surgery. In the study of Lin *et al*[31], nintedanib exhibited a potent antifibrotic effect in HTFs, through inhibition of TGF-β induced cell proliferation and migration, myofibroblast differentiation, and 3D collagen gel contraction. Furthermore, nintedanib has shown consistent antifibrotic and anti-inflammatory activity in animal models of lung fibrosis[19].

The use of nintedanib in *in vitro* experiments has been reported in a wide range of concentrations in the literature[20,22,31]. We opted to use the drug at concentrations 1, 5, and 10 μmol that have been widely used and recommended in *in vitro* studies. In the literature, low concentration of nintedanib (1 μmol) was found to be effective in reducing fibrotic gene expression, fibroblast proliferation, migration, myofibroblast differentiation and collagen secretion[21,31]. In our study, contrary to published articles, in respect to all data obtained, 10 μmol nintedanib was found to be more effectively reducing postoperative fibrosis.

No ophthalmologic formula is available for nintedanib. We preferred to perform subconjunctival injection. Subconjunctival administration can provide better results because it allows direct access of the drug to the target site. However, in subconjunctival application, the drug can be rapidly eliminated due to the blood and lymphatic circulation in the conjunctiva. By preparing any colloidal form of the drug such as liposome, nanoparticle, microemulsion and nanoemulsion, the effectiveness of the drug can be achieved by applying the drug with less frequency by providing continuous and controlled release in the target area[16].

Corticosteroids are a frequently used drug group because of their anti-inflammatory, antiallergic and immunosuppressive

effects on almost every organ. Steroids inhibit angiogenesis, proliferation and migration and delay the inflammatory phase and wound healing accordingly. TA, steroid with a long half life, has a wide therapeutic range due to its anti-inflammatory and immunosuppressive properties. TA has been shown to inhibit fibroblast proliferation and collagen synthesis. In this way, it alters wound healing[32].

In the study conducted by Oh and Lee[33], sodium hyaluronate and TA were used to evaluate their effectiveness in reducing adhesions after strabismus surgery and their effects were compared. As a result, which lasted for 4wk, wrapping the tissues with sodium hyaluronate preserved the tissue and reduced postoperative adhesions. Additionally, TA was not significantly different in postoperative adhesion when compared with the untreated control group. In the study of de Carvalho *et al*[23], two groups were used which investigates the effect of TA on the inflammatory response in experimental strabismus surgery. This study showed that the granuloma response in eyes treated with TA was dramatically decreased compared to the control group. In our study, contrary to this study, TA was found to be ineffective in reducing postoperative fibrosis compared to sham group. However, TA effectively reduced conjunctival and scleral inflammation.

One-time application of TA after surgery is an important option because it increases treatment compliance in the postoperative period. The formation of a white mass in the subconjunctival area and the disappearance of 6-8wk may cause cosmetic problems (Figure 1).

The results of histopathological evaluation were supported by the immunohistochermical staining processes for TGF-β, α-SMA and MMP-2. Lymphocytes, platelet cells and fibroblasts secrete TGF-β, which is the most important fibrogenic mediator. Main functions of TGF-β are angiogenesis stimulation, collagen production and induction of HTF proliferation and migration. In our study, we found statistically significantly lower levels in the surgical scar areas in three doses of nintedanib than in the sham group. This difference was found to be statistically insignificant in comparision to TA group.

Myofibroblasts are characterized by high expression of α-SMA, which is seen in small amounts in fibroblasts. In this study, we showed an increase in the level of α-SMA in the subconjunctival area and SRM in sham group. α-SMA increase secondary to surgery decreased in all experimental groups in SRM, but 10 μmol nintedanib was the most effective agent in reducing a-sma level in the subconjunctival area, while 1 μmol nintedanib and TA were ineffective. We think that decrease in α-SMA expression can be considered as an important evidence that nintedanib reduces fibrosis in the late period.

MMPs are proenzymes and secreted from osteoblasts, fibroblasts, connective tissue cells, endothelial cells and chondrocytes. They have substantial roles in wound healing embryogenesis, angiogenesis normal tissue remodeling and wound healing[34]. Immunohistochemical staining of MMP-2 was evaluted in our study, and the least expression was found in both 5 and 10 μmol nintedanib groups; moreover, the difference between 10 μmol nintedanib group and TA group was statistically significant.

Limitations of our study are as follows: First, human and rabbit eyes have anatomical differences. There is less subconjunctival tissue in rabbit eyes. Second, follow-up period (4wk) is partly short, because collagen maturation takes place for 12-18mo. Finally, we used the grading scales which were subjective judgement of the observer for evaluating adhesion, inflammation, and fibrosis.

In conclusion, we show that subconjunctival nintedanib application showed unique properties, as both an anti-inflammatory and antifibrotic agent. The antifibrotic activity of nintedanib was found to be immunohistochemically and histopathologically higher than TA. It was observed that the most successful group in terms of reducing postoperative inflammation and fibrosis was the 10 μmol nintedanib group. TA showed its main effect as an anti-inflammatory agent, its antifibrotic effect was weaker. Although we think that nintedanib may have a role in improvement of EOM surgery success, further investigations are needed to determine long term toxic effects and duration, application and dosage of nintedanib treatment.

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