

Expression of Cyclooxygenase-2 and Ki-67 in Primary and Recurrent Pterygias

Primer ve Nüks Pterjiyumda Siklooksijenaz-2 ve Ki-67 Ekspresyonu

Onur KONUK, MD,^a
Zeynep AKTAŞ, MD,^a
Özlem ERDEM, MD,^b
E. Berrin YÜKSEL KONUK, MD,^c
Mehmet ÜNAL, MD^a

Departments of
^aOphthalmology,
^bPathology
^cMedical Biology and Genetics
Gazi University Faculty of Medicine,
Ankara

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Yazışma Adresi/Correspondence:
Zeynep AKTAŞ, MD
Gazi University Faculty of Medicine,
Department of Ophthalmology, Ankara,
TÜRKİYE/TURKEY
drzeynep2000@yahoo.com

ABSTRACT Objective: To evaluate the expression of cyclooxygenase-2 (COX-2) and Ki-67 in primary and recurrent pterygia. **Material and Methods:** Pterygium excision together with limbal/conjunctival autografting was performed in primary and recurrent pterygium cases who did not have clinically evident inflammatory findings. The excised tissues were stored at -80 °C for the analysis. Ki-67 expression was determined with only immunohistochemically while COX-2 expression was determined with both immunohistochemistry and real-time polymerase chain reaction, which is a more sensitive method. Normal human conjunctival samples from age-matched donors were used as controls. **Results:** Twelve primary and nine recurrent pterygium tissues were included in the study group. The expression of COX-2/ β actin mRNA was not significantly different in primary pterygia, recurrent pterygia and normal conjunctival tissues (0.0082±0.0038, 0.0094 ± 0.0023, and 0.0075 ± 0.0035 respectively (p=0.32). The expression of Ki-67 nuclear antigen was significantly higher both in primary and recurrent cases when compared to control group (5.2±5.0%, 7.1±2.% and 1.29±0.9% respectively (p=0.018 and p= 0.001, respectively). There was no significant correlation between immunohistochemical expression of Ki-67 and COX-2 in primary and recurrent pterygium tissues (p=0.281, r=0.339; p=0.649, r=-0.177, respectively). **Conclusion:** COX-2 expression does not seem to increase in clinically uninflamed primary and recurrent pterygia tissues. Increased Ki-67 expression in primary and recurrent pterygia supports the proliferative nature of the disease which might have implications in regard to treatment options.

Key Words: Pterygium; Cyclooxygenase 2; Ki-67 antigen

ÖZET Amaç: Primer ve nüks pterjiyumda siklooksijenaz-2 (COX-2) ve Ki-67 ekspresyonunun değerlendirilmesi. **Gereç ve Yöntemler:** İnflamatuvar bulguları olmayan, primer ve nüks pterjiyum olgularında, limbal konjonktival otogreft yöntemi ile pterjiyum cerrahisi uygulandı. Tüm dokular analiz için -80 °C'de saklandı. Bu dokularda Ki-67 ekspresyonu sadece immünohistokimyasal olarak incelenirken; COX-2 ekspresyonu hem immünohistokimyasal olarak, hem de daha duyarlı bir analiz yöntemi olan gerçek zamanlı polimeraz zincir reaksiyonu yöntemi kullanılarak değerlendirildi. Pterjiyum dokularındaki Ki-67 ve COX-2 ekspresyon düzeyleri, sağlıklı konjonktival dokulardaki ekspresyon düzeyleri ile karşılaştırıldı. Tüm gruplarda Ki-67 ve COX-2 düzeyleri arasındaki korelasyonlar değerlendirildi. **Bulgular:** On iki primer ve dokuz rekürren pterjiyum olgusu çalışmaya dahil edildi. Ortalama COX-2/ β aktin mRNA ekspresyonu primer, nüks pterjiyum ve kontrol konjonktiva dokularında sırasıyla 0.0082±0.0038, 0.0094±0.0023 ve 0.0075 ± 0.0035 idi (p=0.32). Ortalama Ki-67 nükleer antijen immünopozitifliği primer ve nüks pterjiyum dokularında kontrol grubuna göre anlamlı derecede yüksek olup, sırasıyla %5.2 ± 5.0, %7.1±2.6 ve %1.29 ± 0.9 olarak saptandı (sırasıyla, p= 0.018, p=0.001). Primer ve nüks pterjiyum gruplarında Ki-67 ve COX-2 immünopozitifliği arasındaki korelasyon ise istatistiksel olarak anlamlı değildi (sırasıyla, p=0.281, r=0.339; p=0.649, r=-0.177). **Sonuç:** Primer ve nüks pterjiyumda COX-2 ekspresyonu, hastalığın noninflamatuvar döneminde artış göstermemektedir. Primer ve nüks pterjiyumda Ki-67 ekspresyonunun artmış olması, bu hastalığın proliferatif karakterde olduğunu destekleyen bir bulgudur. COX-2 ekspresyonunun hastalığın proliferatif süreci üzerine etkisi yoktur. İnflamatuvar bulgular taşıyan olgularda COX-2 ekspresyonu araştıran daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Pterjiyum; Siklooksijenaz 2; Ki-67 antijeni

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Pterygium is a frequent external ocular disease with unknown etiology demonstrating the features of both inflammatory and degenerative pathogenesis.^{1,2} The patients with pterygium usually present with symptoms of inflammatory ocular disease such as redness, irritation, itching and mucoid discharge that usually improve with topical corticosteroids or nonsteroidal anti-inflammatory drugs (NSAIDs).³ Pterygium has tumor-like features as well. It shows abnormal proliferation over cornea and usually invades the Bowman's layer, it has high recurrence rates after excision and may necessitate anti-metabolite agents or irradiation for treatment of recurrences.^{2,4,5} Additionally, histopathological evaluations of pterygium reveal cellular dysplasia, local invasion, abnormal p53 expression and apoptosis.^{2,6-8}

Arachidonic acid metabolism is a well-known pathway in inflammatory process where cyclooxygenase (COX) is a rate limiting enzyme for the expression of eicosinoids including prostaglandins.^{9,10} Two isoforms of COX have been identified; COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced by various factors such as growth factors, tumor promoters and cytokines. Thus, COX-2 is thought to be responsible for prostaglandin production during inflammation.^{9,10} Current literature suggests that COX-2 expression may be involved in cell growth, apoptosis and tumorigenesis in different cell types.^{9,10} Similarly, the degree of cellular proliferation can also be measured by Ki-67 nuclear antigen expression.^{11,12} The aim of this study was to evaluate the expression of COX-2 and Ki-67 in primary and recurrent pterygia.

MATERIAL AND METHODS

This study was performed in September 2006 in Gazi University School of Medicine, Department of Ophthalmology. Pterygium excision and limbal conjunctival autografting was performed in 21 eyes of 21 cases. The excised tissues were prepared for histopathological, real-time polymerized chain reaction (PCR) and immunohistochemical analyses. The tissues were snap frozen in dry ice and stored at -80 °C for quantitative real-time PCR analysis,

and formalin fixed and paraffin-embedded for histopathological and immunohistochemical evaluations. The conjunctival tissues of the control group (10 eyes of 10 patients) were obtained from temporal quadrants of the eyes of age-matched patients without any history of ocular surface disorder during primary retinal detachment surgery .

The data accumulation was in conformity with the Institutional Ethics Committee and the study was in adherence to the tenets of the declaration of Helsinki. The aim of study was described and informed consents were received from the patients.

ISOLATION OF M RNA AND SYNTHESIS OF C DNA

Total RNA was extracted from 20 mg frozen tissue sample by using High Pure RNA Tissue Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. RNA integrity was electrophoretically verified by ethidium bromide staining and by OD260/OD280 nm absorption ratio of >1.95. One µg of total RNA was used for cDNA synthesis using first strand cDNA synthesis kit for reverse transcription PCR (RT-PCR) (AMV) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

QUANTITATIVE ANALYSIS OF COX-2 M RNA EXPRESSION

Real-time quantitative PCR was performed to assess transcripts of COX-2 relative to the housekeeping gene b-actin by using LightCycler instrument (Roche Diagnostics, Mannheim, Germany) and results were analyzed by LightCycler software 3.0. Primers and probes were designed using Primer Premier 5 software (Premier Biosoft International, USA). All primers and probes were designed to cross intron/exon boundaries to avoid amplification of genomic DNA. All PCR products were sequenced to ensure product validity. The upstream and downstream primer sequences for COX-2 gene were 5' GCTCAAACATGATGTTTGCATTG 3' and 5' GCTGGCCCTCGCTTATGA 3', respectively, and the TaqMan probe selected between the primers was fluorescence labeled at the 5' end with 6-carboxyfluorescein (FAM) as the reporter dye and at the 3' end with 6-carboxytetramethylrhodamine (TAMRA) as the quencher; 5'-FAM- TGCCCAG-

CACTTCACGCATCAGTT –TAMRA-3' (TibMol-Biol, Berlin, Germany) (GenBank no. M90100). The b-actin mRNA was quantified to adjust the amount of mRNA in each sample with b-actin probe and primer set. The upstream and downstream primer sequences were 5' TCACCCACACTGTGCCCAT and 5' TCCTTAATGTCACGCACGATTT 3', respectively, and the TaqMan probe was 5'-FAM-ATCCTGCGTCTGGACCTGGCT-TAMRA-3' (TibMolBiol, Berlin, Germany) (GenBank no. NM_001101). Each 14 µl reaction volume contained 1× FastStart DNA Master Hybridization Probes Mix (Roche Diagnostics, Mannheim, Germany), 4 mM of MgCl₂, 0.5 mM of each primer, 0.2 µM of TaqMan probe and 2 µl of cDNA. The cycling parameters were 10 min at 95°C for activating faststart Taq polymerase, 50 cycles of 10 seconds at 95°C and 20 seconds at 60°C for amplification and quantification. In every PCR reaction, the level of the housekeeping gene b-actin was also quantified with the same PCR conditions described above. We used b-actin as the endogenous internal housekeeping gene that revealed less variability and better reproducibility. Real-time expression values were calculated using the relative standard curve method. Standard curves were generated for each mRNA using 10-fold serial dilutions for both the target of interest and the endogenous control (b-actin) by measuring the cycle number at which exponential amplification occurred in a dilution series of samples. Values were normalized to the relative amounts of b-actin mRNA, which were obtained from a similar standard curve. In real-time PCR reactions, the same initial amounts of target molecules were used, and the cross point (Cp) values (20.8± 0.02) of b-actin mRNA were constant in all samples. Relative expressions were calculated according to mathematical model based on the PCR efficiencies and the crossing points.¹³

HISTOPATHOLOGICAL ANALYSIS

Tissues were fixed in 10% formalin. After routine tissue processing, all blocks were cut at 4 µm thickness and stained with hematoxylin and eosin. All tissues were examined under the light microscope.

IMMUNOHISTOCHEMICAL ANALYSIS

Formalin-fixed, paraffin-embedded tissues were used for immunohistochemistry. Four-micrometer-thick sections from tissue blocks were stained with Ki-67 (Ab-2, Clone MB67, NeoMarkers) and COX-2 (Clone SP21, NeoMarkers) by using the standard streptavidin-biotin indirect method. Primary antibodies were incubated for two hours at room temperature after blocking endogenous peroxides and proteins. AEC (amino-9-ethylcarbazole) was used as a chromogen. Tonsil tissues (for Ki-67) and colon carcinoma tissues (for COX-2) were used as positive controls. Negative controls were processed exactly the same way, but were incubated with PBS only (Phosphate buffered saline) instead of the primary antibody. Nuclear staining for Ki-67 was considered positive and was evaluated throughout whole tissue area on the slide. Cytoplasmic staining for COX-2 was evaluated throughout whole tissue area on the slide and percentage of positive cells was recorded. The pathologist was blinded to the study groups.

STATISTICAL ANALYSIS

Kruskal-Wallis test were used for comparisons of the age of the patients, disease durations, COX-2 mRNA expression levels, immunopositivity for COX-2 and Ki-67 nuclear antigens in cases with primary pterygia, recurrent pterygia and control subjects. Dunnett's multiple comparison post hoc test was performed for statistically significant data ($p < 0.05$). The correlation between COX-2 and Ki-67 immunopositivity was calculated with Spearman test. SPSS version 11.0 system for personal computer was used and $p < 0.05$ was regarded as statistically significant for all statistical analyses (Table 1).

RESULTS

Twelve primary and nine recurrent pterygium cases (13 males, 8 females) had surgery. There was no statistically significant difference between the groups in terms of the age of the patients; mean ± SD ages of primary, recurrent pterygia and control groups were 38.1±6.7 years (range= 30-49 years), 37.1±4.4 years (range= 28-54 years) and 40.3±6.2 years (range= 33-51 years), respectively ($p = 0.434$).

TABLE 1: Number, mean, standard deviation and medians of the results.

	COX-2/ β actin mRNA				Percentage of COX-2 immunopositivity (%)				Percentage of Ki-67 immunopositivity (%)			
	Mean	\pm SD	Range	Median	Mean	\pm SD	Range	Median	Mean	\pm SD	Range	Median
	Primary pterygium group (n:12)	0.0082	\pm 0.0038	0.0028-0.0142	0.074	1.0	\pm 2.1	0-5 %	1	5.2	\pm 5.0	1-15
Recurrent pterygium group (n: 9)	0.0094	\pm 0.0023	0.0070-0.0131	0.085	1.4	\pm 1.9	0-6%	3	7.1	\pm 2.6	2-10	8
Control group (n: 10)	0.0075	\pm 0.0035	0.0016-0.0118	0.074	0.9	\pm 1.1	0-3%	1	1.29	\pm 0.9	0-2	1
P value	0.320				0.220				0.002			

All of the cases had a history of topical NSAID or corticosteroid treatment to decrease inflammatory signs and symptoms of pterygium during the course of their disease and all were referred to the hospital for surgery due to ongoing complaints such as photophobia, pain, foreign-body sensation, tearing and proliferation of pterygium tissue over the cornea. Only five recurrent pterygium cases showed mild ocular inflammatory signs, and the remaining cases presented with non-inflammatory pterygia. None of the cases had a history of ocular surgery or anti-inflammatory treatment in the previous six months.

The mean \pm SD disease duration was 24.1 \pm 10.5 months (range: 12-48 months), and 24.8 \pm 10.1 months (range: 12-42 months) in the primary and recurrent pterygium cases, respectively (p: 0.74). The mean \pm SD postoperative period in cases with recurrent pterygia was 20.5 \pm 6.8 months (range: 11-34 months).

Histological examination revealed focal lymphocyte infiltration without significant tissue edema in four tissues from patients with primary pterygia. These cases did not have any clinical signs of inflammation prior to the surgery. There were no inflammatory cells in the rest of the tissues from primary and recurrent pterygium cases the control group.

The mean \pm SD expression of COX-2/ β actin mRNA was 0.0082 \pm 0.0038 (range: 0.0028-0.0142), 0.0094 \pm 0.0023 (range: 0.0070-0.0131) and 0.0075 \pm 0.0035 (range= 0.0016-0.0118) in primary pterygium, recurrent pterygium and control cases, respectively (p= 0.32). The mean \pm SD percentage of COX-2 immunopositivity in primary, recurrent

pterygium and control cases was 1.0 \pm 2.1 % (range: 0-5 %), 1.4 \pm 1.9% (range: 0-6%), and 0.9 \pm 1.1% (range: 0-3%), respectively (p= 0.22) (Figure 1). The mean \pm SD immunopositivity for Ki-67 nuclear antigen was 5.2 \pm 5.0% (range: 1-15 %), 7.1 \pm 2.6 (range: 2-10%) and 1.29 \pm 0.9% (0-2 %) in primary pterygium, recurrent pterygium and control cases, respectively (p= 0.002). Although Ki-67 expression did not show any difference between primary and recurrent pterygia (p= 0.27), both groups showed significantly higher Ki-67 immunopositivity than the control group (primary pterygium; p= 0.022, recurrent pterygium; p=0.002; Dunnet's correction) (Figure 2). There was no correlation between Ki-67 and COX-2 expressions in either primary (p= 0.797) or recurrent pterygium tissues (p=0.787).

DISCUSSION

The inflammatory cyclooxygenase, COX-2 is usually expressed at extremely low levels under normal basal physiological conditions, but it is highly inducible by several factors such as cytokines and mitogens.^{9,14-17} The expression of COX-2 enzyme and possible treatment modalities including selective COX-2 inhibitors have been evaluated in various inflammatory and proliferative diseases.¹⁸⁻²² However, its relation with inflammatory ocular surface disorders has not been studied in the literature.

The pathogenesis of pterygium is still obscure, but one of the most common proposed hypotheses for pterygium pathogenesis consist of the disruption of the limbal corneal-conjunctival epithelial barrier followed by progressive "conjunctivalization" of the cornea.^{1,2} According to clinical course

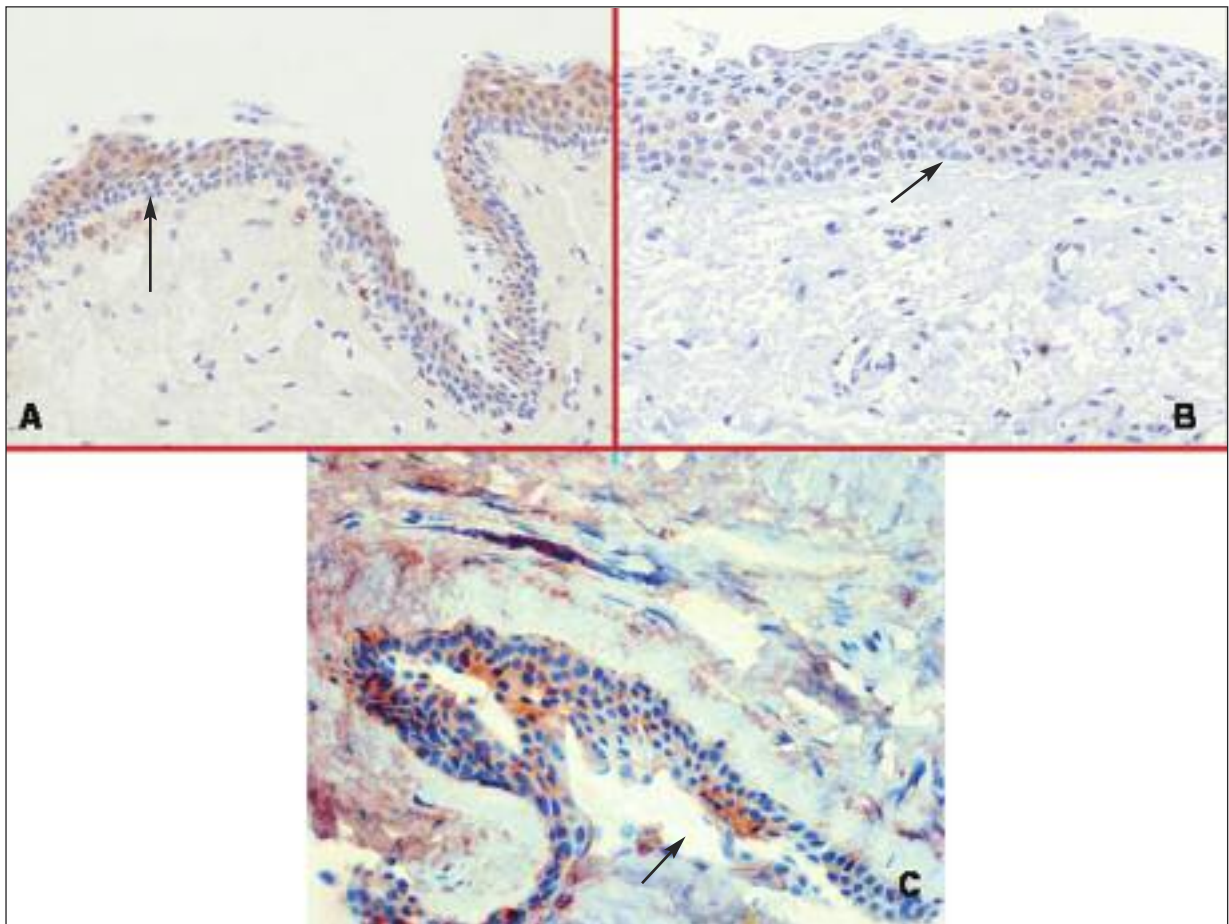


FIGURE 1: Immunohistochemical analysis of COX-2 in (A) normal conjunctiva (B) primary pterygium, and (C) recurrent pterygium. Arrow points the areas with red stain which represent positive cytoplasmic staining with COX-2. AECx200.

of the disease, there are various medical and surgical treatment options. In the presence of inflammatory signs and symptoms, it is usually treated with anti-inflammatory treatment, mainly with topical steroids or NSAIDs. However, recurrence of the symptoms after cessation of the topical medication, and proliferation of pterygium tissue over the cornea during the follow-up period is a common feature. It is demonstrated that topical NSAIDs and corticosteroids are equally effective for the treatment of inflammatory pterygium, and both drugs inhibit the arachidonic acid pathway where COX is the rate limiting enzyme.^{3,23} In this study, we compared the expressions of COX-2 enzyme in primary pterygia, recurrent pterygia and normal conjunctival tissue, and found no statistically significant differences. However, we evaluated the expression of COX-2 only in non-inflamed ptery-

gium tissues since we usually treat these cases with a course of topical anti-inflammatory agents since we assume that excising the pterygium at its inflammatory stage might increase postoperative complications and recurrence rates. COX-2 expression was found to be increased in recurrent pterygium cases in studies by Karahan et al.²⁴ and Adiguzel et al.²⁵ However, our study demonstrated that the expression of COX-2 is not elevated in non-inflamed primary and recurrent pterygium cases. Further, in clinical practice, topical steroids or NSAIDs usually provide only temporary relief in these cases without any significant benefit in terms of halting the disease process. We may conclude that the limited effects of these drugs on non-inflammatory chronic phase of the disease may be related to the low expression levels of COX-2 in those cases.

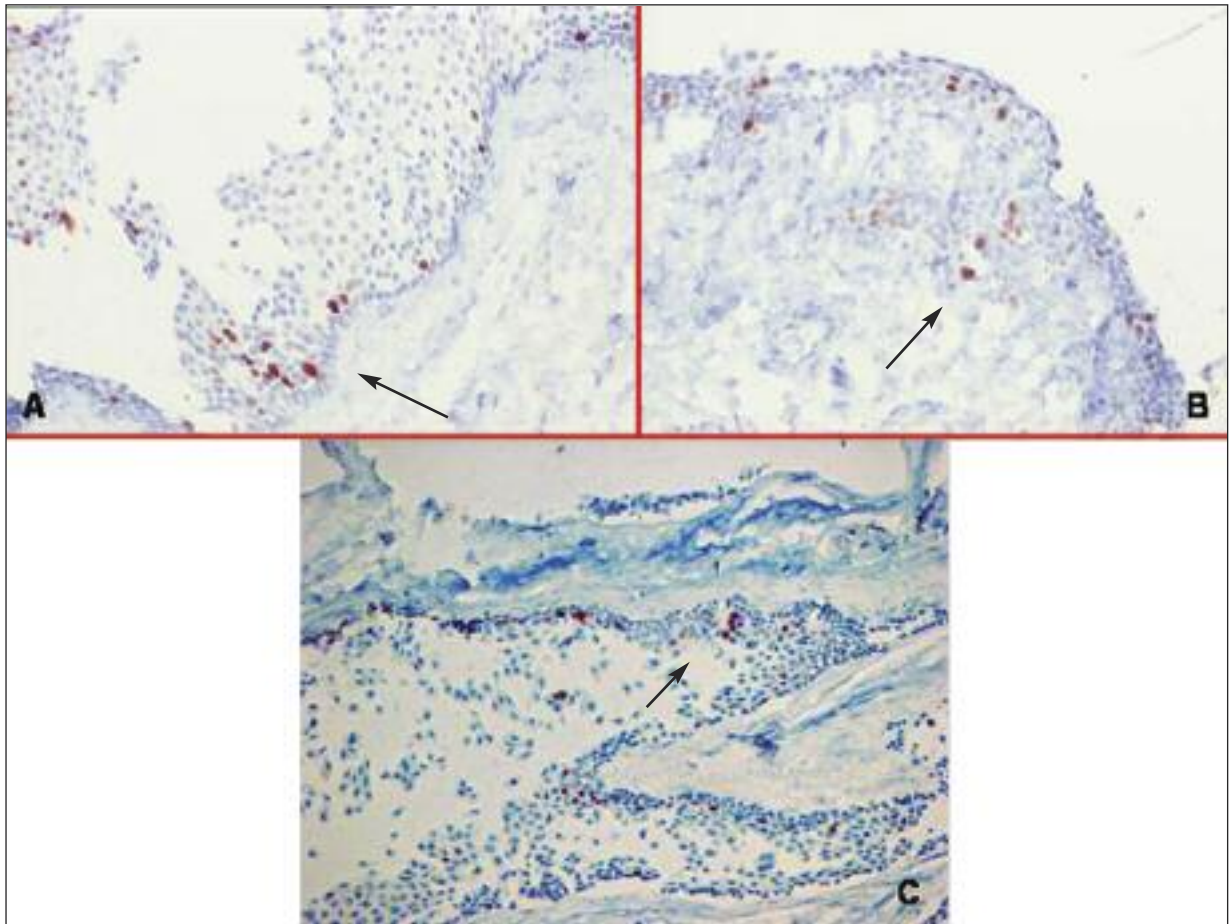


FIGURE 2: Immunohistochemical analysis of Ki-67 in (A) normal conjunctiva (B) primary pterygium, and (C) recurrent pterygium. Arrow points the areas with brown stain which represent positive nuclear staining with Ki-67. AECx200.

Surgical management is the primary treatment for primary and recurrent pterygia. Adjuvant anti-metabolites are used to decrease the recurrence rate of the pterygium, and medical therapies including steroids and NSAIDs are usually used during the early postoperative period to reduce postoperative inflammation. In clinical practice, both primary and recurrent pterygium cases usually had history of steroid or NSAID treatment for management of inflammatory symptoms, however they usually experience recurrence of ocular inflammatory symptoms with progression of the disease and need surgery. Similarly, in our study, all cases had a history of topical corticosteroid and/or NSAID treatment with no benefit in terms of preventing the progression of the pterygium onto the cornea. In our study, primary and recurrent pterygium cases showed statistically significant increase in the

expression of Ki-67 when compared to control group demonstrating the proliferative feature of the disease. Interestingly, Adyanthaya et al.²⁶ recently reported the beneficial effects of topical mitomycin C in halting the progression of acutely recurring pterygium cases.

There was no correlation between the Ki-67 immunopositivity and expression of COX-2 enzyme in our study. This may show the irrelevance of the expression of COX-2 enzyme with the proliferation of the pterygium tissue where inhibition of COX-2 may not change the proliferative course of the disease. Additionally, the clinical and histopathological examination of the primary and recurrent pterygia demonstrated no evidence of inflammation which was in concordance with low levels of COX-2 expression. This may support that progression of the disease can continue with-

out any sign of inflammation and the absence of inflammatory signs in the pterygium tissue does not diminish the proliferative character of the disease.

To the best of our knowledge, this is the first in situ study that reports COX-2 expression in primary and recurrent pterygia, and evaluates its rela-

tionship with Ki-67 expression. We conclude that expression of Ki-67, but not COX-2, is relevant in terms of the pathogenesis of recurrence after complete removal. Further studies are needed to assess the advantage of inhibiting Ki-67 in halting or perhaps reversing the growth of primary as well as recurrent pterygia.

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