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## **TITLE PAGE**

### **Conjunctival short-term evolution after pterygium excision**

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## **ABSTRACT**

**Purpose:** To analyze conjunctival cytological features 1 month after pterygium excision using limbo-conjunctival autograft (LCA) with and without intraoperative mitomycin C and to assess tissue short-term evolution in both situations.

**Methods:** Fifty-nine primary nasal pterygia from 59 patients were excised with LCA. Twenty-nine were treated with intraoperative mitomycin C 0.02% (MMC+) and thirty without it (MMC-). Impression cytology was performed in nasal and temporal conjunctiva before and one month after the excision. Goblet cells density (GCD) and nucleus-to-cytoplasm (N/C) non-goblet epithelial cell ratio were quantified.

### **Results:**

*Surgical strategy comparisons (Intergroup comparisons):* All the preoperative data were, in mean, within the normal range, except for a slight goblet cell hyperplasia in the area of the lesion in MMC+ but no significant differences were found between the groups ( $p=0.079$  for GCD and  $p=0.245$  for N/C; ANOVA). Clinically relevant differences after surgery were only showed in nasal GCD that was significantly lower in MMC+ than in MMC- ( $p= 0.000$ ; ANOVA) being the mean value in MMC+ slightly below normal values while in MMC- remained normal.

*Tissue evolution (Intragroup comparisons):*No clinical relevant changes were found in MMC-. Data from MMC+ displayed no changes one month after surgery, except for nasal GCD that showed a significant reduction ( $p=0.000$ ; paired t-test). Nevertheless, this GCD decrease was more modest than that previously described using mitomycin C without autograft, since in the present

study nasal GCD was not lower but similar to postoperative temporal data of the same eye ( $p=0.164$ ; paired t test).

**Conclusions:** LCA is a good technique for conjunctiva early recovery. When mitomycin C was added, the GCD reduction was lower than described using other surgical techniques. Mitomycin C, in optimal concentration and exposure, associated with LCA could be a good clinical option to minimize pterygium recurrence.

**Keywords:** *pterygium, impression cytology, mitomycin C, limbo-conjunctival autograft, goblet cells.*

## INTRODUCTION

Pterygium is a common ocular surface disease characterized by the encroachment of a fleshy fibrovascular formation from the bulbar conjunctiva across the limbus, invading the cornea. Theories of pterygium pathogenesis have implicated ultraviolet light exposure as a major causative factor<sup>1,2</sup>. Excessive exposure to UVB could damage limbal stem cells, leading to aberrant wound healing responses<sup>3</sup>. This wing-shaped lesion is often associated with inflammation, increased corneal astigmatism, obstructed vision, and unfavorable cosmetic effect<sup>4</sup>.

Different surgical techniques have been used to remove pterygium and prevent recurrence. Evidence indicates that bare sclera excision results in a significantly higher pterygium recurrence rate than excision accompanied by using certain adjuvants<sup>5,6</sup>, such as mitomycin C, and conjunctival (CA) or limbo-conjunctival autograft (LCA). Comparisons recently revealed that to use a combination of CA or LCA with mitomycin C could further reduce pterygium recurrence rate compared with a single adjuvant<sup>6</sup>.

Mitomycin C is an antineoplastic-antibiotic agent produced by *Streptomyces caespitosus* that inhibits the synthesis of DNA, proteins and cellular RNA and is a potent inhibitor of fibroblasts proliferation. Drug use has improved not only recurrence rate after pterygium surgery but also the surgical results in other ocular disease, such as glaucoma, or corneal and conjunctival intraepithelial neoplasia<sup>7,8,9</sup>.

Optimal mitomycin C concentration and exposure time have been analyzed to prevent its deleterious effects<sup>10,11,12,13</sup> and single intraoperative low dose of the

drug is safer than postoperative topical daily application and it is effective reducing pterygium recurrence rates<sup>14,15</sup>.

Delay in conjunctival epithelium healing could be early detected by impression cytology or *in-vivo* confocal microscopy when the chemoadjuvant was applied<sup>16,17</sup>. Important changes, mainly in goblet cell density, were found few weeks after surgery that lasted for long periods, even when mitomycin C was applied in optimal conditions. Nevertheless, tissue evolution after pterygium excision not only depends on mitomycin C use but other adjuvants seem to be determining factors in the process. In fact, autografting (with CA) seems to promote tissue healing compared to bare sclera or bare sclera with mitomycin C<sup>16</sup>. An optimal combination of adjuvants may be the key to reduce recurrences with early tissue recovery or, at least, with minimal alterations in short-term, suggesting a faster conjunctival recovery. However, to our knowledge, conjunctival epithelial phenotypes after LCA or combining LCA with mitomycin C have not been previously reported in short or long-term.

The aim of this study is to compare conjunctival cytological features 1 month after pterygium excision using limbo-conjunctival autograft (LCA) with and without intraoperative mitomycin C and to assess tissue short-term evolution in both situations. Analysis of epithelial changes triggered by each specific adjuvant combination could improve the current risk/benefit knowledge of these strategies, and evidence-based decisions in pterygium clinical management.

## **METHODS**

Fifty-nine eyes of 59 patients with primary nasal pterygium, 37 males and 22 females, average age  $43 \pm 11.87$ , range 22-76 years, were enrolled in the

study. Patients with a history of ocular surgery, ocular trauma, contact lens wear, corneal scarring, pseudopterygia or anterior segment diseases, except for pterygium, were excluded from the study. Eyes were randomized into two groups according to the surgical technique used: LCA with mitomycin C (MMC+) and LCA without mitomycin C (MMC-).

Before surgery, informed consent was obtained from each patient, and the study was carried out in accordance with the tenets of the Declaration of Helsinki and with the approval of the Ethics Committee of Consorci Sanitari de Terrassa Hospital de Terrassa (Barcelona, Spain).

After topical and subconjunctival anesthesia, pterygium was removed, starting with a conjunctival incision on the body of the lesion using Westcott scissors (Miltex®). Tenon's capsule tissue under the body of the pterygium was also removed. For the MMC+ group, when the excision was completed, regular-tip microsponges soaked with 0.025% mitomycin C solution (Mitomycin-C™ Inibsa-Hospital) were placed on the exposed sclera surface for 3 minutes and then removed. The exposed sclera was irrigated with 40 ml of balanced salt solution, and an LCA was obtained from the upper conjunctiva. The graft dissection was extended in all the lesions approximately 0.5 mm into the clear cornea to include the Vogt palisades and limbal stem cells and moved to cover the defective area. Then, it was secured with nylon sutures placing the limbal end of the autograft directly over the limbal area. All surgical procedures were performed by a single surgeon (P.P.). Damaged tissue was sent for pathological analysis. Postoperatively, patients were treated with topical tobramycin 3 mg/ml and dexamethasone 1mg/ml (Tobradex®; Alcon Cusi, S.A.Spain) tapered off in one month. Nylon sutures were removed at week 1. The clinical aspect of the

operated area was evaluated using fluorescein staining and slit-lamp examination one, 3, 6 and 12 months after pterygium excision and Prabhasawat criterion<sup>18</sup> was applied to assess recurrence. Clinical adverse effects were also recorded.

Before and one month after the surgery, impression cytology was performed by placing a triangular-shaped mixed cellulose ester filter paper (Millipore Biopore Membrane, code GSTFO1300; Millipore Corporation, BillericaMA) on the interpalpebral nasal and temporal conjunctiva. Filter paper were gently pressed on the ocular surface for 3-5 seconds and then removed. The samples were air-dried for 1 min, fixed with 96% ethanol by immersion for at least 10 min and stained with periodic acid-Schiff–Gill's modified Papanicolaou stain<sup>16</sup>. The specimens were examined by light microscopy and digitally recorded. Nucleus-to-cytoplasm ratio (N/C) of 5-6 non-goblet epithelial cells were quantitatively assessed from 5 areas of each specimen using the Image J processing program (Rasband, National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>). The same process was performed to calculate goblet cell density (GCD, number of goblet cell/mm<sup>2</sup>). The averaged data were included in the statistical analysis as cytological variables. Healthy epithelial assessment was performed using Nelson and Wright criterion for N/C ratio<sup>19</sup> and Rivas and coworkers data for GCD in healthy conjunctival epithelium<sup>20</sup>. All the cytological measurements were made by the same practitioner (S.L.) who was unaware of the patient's treatment.

### **Statistical analysis**

After an exploratory data analysis, the variables were tested for normality by the Kolmogorov-Smirnov test. ANOVA was used to assess differences in the

cytological features between groups. Paired t test was applied to compare epithelial characteristics before and one month after surgery in temporal and nasal conjunctiva of each eye in MMC- , thus analyzing the recovery of the tissue. The same comparison was repeated in MMC+. This test was also applied to quantify alterations in GCD postoperative topographical distribution, comparing nasal with temporal data of the same eye. A p value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS V19 (SPSS Inc, Chicago, Illinois).

## **RESULTS**

Twenty-nine eyes were included in MMC+ and 30 in MMC-. All were classified as grade 1 of Prabhasawat one month after the surgery, when impression cytology was performed. None of the eyes presented mitomycin C clinical adverse reactions during the 12 months follow-up period. Nevertheless, 4 eyes (13%) in MMC- and 1 eye (3%) in MMC+ disclosed recurrence at 12 months. The summary statistic of the cytological variables pre and postoperatively (1 month after surgery) are presented in table 1 (temporal and nasal data of MMC+) and table 2 (temporal and nasal data of MMC-).

### **Preoperative comparison between MMC+ and MMC-**

All the preoperative data were, in mean, within the normal range, except for a slight goblet cell hyperplasia in the area of the lesion in MMC+. Nevertheless, statistical comparison between the two groups displayed no significant differences in all the initial cytological features ( $p=0.079$  for GCD and  $p=0.245$  for N/C; ANOVA).



### **Postoperative comparison between MMC+ and MMC-**

One month after surgery, N/C were significantly higher in MMC+ than in MMC- in both temporal and nasal zones ( $p=0.000$  and  $p=0.007$ , respectively; ANOVA) but the mean exhibited normal values for both groups in all the studied locations. Nasal GCD was significantly lower in MMC+ than in MMC- ( $p=0.000$ ; ANOVA). This difference was clinically relevant since the mean value in MMC+ was slightly below normal values while in MMC- remained normal.

### **Conjunctival short-term evolution. Postoperative vs preoperative cytological features**

Paired t test was applied comparing cytological characteristics before and 1 month after pterygium excision in MMC- and the analysis was repeated for MMC+.

Among the studied variables and locations in MMC-, only temporal N/C showed a slightly reduction ( $p<0.001$ ; paired t-test) but the mean remained within normal values, as we have commented before. In MMC+, temporal conjunctiva displayed no significant modifications and nasal conjunctiva showed similar N/C ratio but a GCD significant reduction ( $p=0.000$ ; paired t-test) of 272.69 cells/mm<sup>2</sup>, in average, (95% confidence interval of the mean difference between 151 and 393 cells/mm<sup>2</sup>), thus leading to an abnormal mean, as mentioned above.

### **Conjunctival short-term evolution. Postoperative Comparison between temporal and nasal conjunctiva**

In order to quantify altered topographical distribution of GCD and subsequently to contrast our results with other previous described in the literature, comparisons between nasal and temporal conjunctiva in MMC- , one month after surgery, were carried out and the same analysis was repeated for MMC+. A greater GCD in nasal than temporal area was found in MMC- ( $p=0.000$ ; paired t test) with a mean difference of 125 cells/mm<sup>2</sup> (95% confidence interval between 87 and 161 cells/mm<sup>2</sup>). This difference is a distinctive feature of healthy conjunctiva<sup>20</sup>. However, postoperative nasal GCD data in MMC+ were similar to temporal data, ( $p=0.164$ ; paired t test). Although this result was indicative of an abnormal topographical distribution, the data revealed a clearly milder GCD reduction in the injured area than that previously described using mitomycin C without autograft, as is discussed in the next section.

### **DISCUSSION**

The findings of this study demonstrate that conjunctival epithelium has already recovered its normal characteristics one month after nasal pterygium excision with LCA. When combined LCA with mitomycin C, a significant reduction of nasal GCD was observed while the rest of cytological features remained normal in the two studied zones one month after surgery. Postoperative nasal and temporal GCD were similar in MMC+. Hence, changes in topographical distribution produced by mitomycin C could be considered as relatively modest when LCA was used.

Preoperative MMC+ mean values showed a slight goblet cell hyperplasia in the area of the lesion, which is consistent with previous reports about epithelial phenotype of pterygium<sup>16,21</sup> but was not disclosed in preoperative MMC- mean values of the same zone. By contrast, the more definitive results of ANOVA displayed no statistical differences in initial conditions between the two groups. Actually, goblet cell density has always shown a high variability in both healthy epithelial conjunctiva<sup>20,22</sup> and pterygium lesion<sup>16,21,23,24</sup> and seems to be closely linked with tear osmolarity and ocular surface inflammation<sup>25,26</sup> both conditions related with pterygium<sup>4,27</sup>. This complex interaction would be the cause of the high variability observed in the studies and could explain the more apparent than real differences in GCD between the groups before the surgery.

Non secretory cells of conjunctival epithelium retained their normal phenotype one month after surgery in both surgical strategies and did not show squamous metaplasia, usually associated with keratoconjunctivitis sicca and different cicatrizing diseases and ocular irritation disorders<sup>28,29</sup>. In fact, this is an expected result since none of the surgical techniques, which were used before, seems to trigger this kind of tissue alteration<sup>16,23,30</sup>. However, squamous metaplasia was reported in conjunctival epithelium when mitomycin C drops were used in an intensive topical treatment for primary acquired melanosis with atypia<sup>31</sup>. In the other hand, slight squamous metaplasia has been recently described in 31.3% of the samples (16 eyes) from the pterygium side, 6 months after excision with limbal autograft without mitomycin C<sup>32</sup>. Authors suggested that it might be related to recurrence.

Eyes with LCA without mitomycin C showed the same conjunctival phenotype 1 month after than before the surgery confirming this surgical strategy as a good

solution for a rapid epithelial recovery. Autograft promotion of conjunctival healing may be slower using CA than LCA since 6 months were necessary to achieve similar cytological results than in fellow control eyes<sup>16</sup>. Further comparative studies, applying the same design, are necessary to clarify this question, investigating the limbal postoperative condition with a confocal microscopy analysis.

Despite certain risk of limbal damage at the donor site of the graft<sup>33</sup>, LCA is one of the best choices for pterygium management<sup>5,6</sup>. It is safe, with acceptable recurrence rate, and could be more effective than CA in prevent regrown of recurrent pterygia<sup>34</sup>. Nevertheless, as we have commented in the introduction section, using a combination of conjunctival or limbal autograft with mitomycin C further reduces the recurrence rate compared with conjunctival or limbal autograft or mitomycin C alone<sup>6</sup>.

Eyes with LCA and mitomycin C showed a reduction in nasal GCD that led to abnormal results in this zone. GCD decrease has been also described in previous cytological assessments after bare sclera excision with intraoperative mitomycin C<sup>16,23,30</sup>. The drug seems to delay GCD recovery regardless of the surgical technique chosen. However, this choice could determine cell loss magnitude and duration. Indeed, GCD decrease produced by mitomycin C combined with bare sclera excision seems to be more severe and prolonged than that triggered by the combination of the drug with LCA. Solomon and co-workers<sup>30</sup> reported a 4-fold decrease at the excision area (nasal) when compared with the contralateral nonoperated side (temporal) at a mean of 77.2 months after the surgery (range, 72-84 months after surgery). Far from it, in our study GCD maintained similar values in nasal than temporal side 1 month after

pterygium excision. These differences are plausible since autograft (using CA) has been described as a clear promoter of conjunctival healing in pterygium excision<sup>16</sup>. This quality may be due to the presence of graft basement membrane that serves as a substrate for the regenerating epithelium<sup>16,18</sup>. LCA and mitomycin C combination with a careful patient selection could be a good choice for pterygium management in terms of risk/benefit ratio. The present study provides evidence in this sense. This adjuvant combination not only seems to minimize drug toxicity but reduce the percentage of recurrence. Nevertheless, further assessments with large samples are necessary to ratify this therapeutic issue.

In conclusion, pterygium excision with LCA seems to be an adequate technique to achieve a rapid conjunctival recovery and could minimize goblet cell alteration when mitomycin C was added to further reduce recurrences. The more modest drug epithelial toxicity one month after application clearly evidences the protective value of LCA. Mitomycin C, in optimal concentration and exposure, associated with LCA could be a good clinical option to minimize pterygium recurrence.

## **ACKNOWLEDGMENTS**

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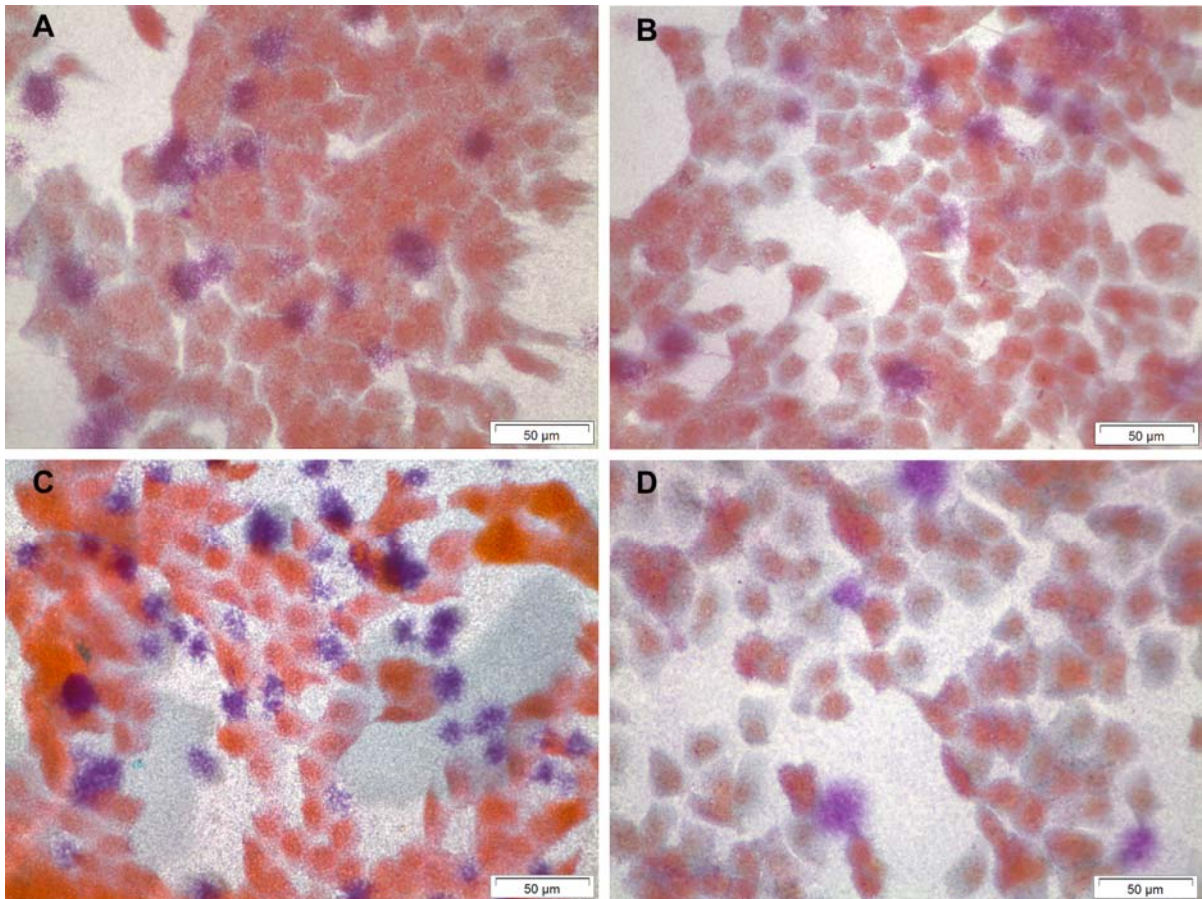


Figure 1

Examples of impression cytology in MMC- (images A and B) and in MMC+ (images C and D), before (left) and after (right) lesion excision. Periodic acid-Schiff-Gill's modified Papanicolaou stain.

**Table 1.** Summary statistics of the cytological variables pre and postoperatively in MMC+ group.

Variable	N (MMC+)				T (MMC+)			
	Preoperatively		Postoperatively		Preoperatively		Postoperatively	
	GCD	N/C	GCD	N/C	GCD	N/C	GCD	N/C
<b>Average</b>	385	0.6	113	0.6	112	0.6	126	0.7
<b>Median</b>	351	0.6	103	0.6	99	0.6	93	0.7
<b>S.D.</b>	259	0.2	113	0.2	85	0.2	89	0.2
<b>Minimun</b>	18	0.2	4	0.4	5	0.3	28	0.1
<b>Maximun</b>	1019	1.0	554	1.0	318	1.0	381	1.0

GCD (goblet cell density), N/C (nucleus-to-cytoplasm ratio). Nasal GCD normal range.<sup>20</sup>= 159-323 cells/mm<sup>2</sup>. Temporal GCD normal range<sup>20</sup>= 65-265 cells/mm<sup>2</sup>. Normal ratio N/C<sup>19</sup> ≥0.5

**Table 2.** Summary statistics of the cytological variables pre and postoperatively in MMC- group.

Variable	N (MMC-)				T (MMC-)			
	Preoperatively		Postoperatively		Preoperatively		Postoperatively	
	GCD	N/C	GCD	N/C	GCD	N/C	GCD	N/C
<b>Average</b>	229	0.5	213	0.5	106	0.6	86	0.5
<b>Median</b>	192	0.5	225	0.5	87	0.6	69	0.5
<b>S.D.</b>	184	0.1	74	0.1	59	0.1	63	0.1
<b>Minimun</b>	40	0.3	53	0.3	43	0.4	10	0.4
<b>Maximun</b>	773	0.7	329	0.8	310	1.1	298	1.0

GCD (goblet cell density), N/C (nucleus-to-cytoplasm ratio). Nasal GCD normal range.<sup>20</sup>= 159-323 cells/mm<sup>2</sup>. Temporal GCD normal range.<sup>20</sup>= 65-265 cells/mm<sup>2</sup>. Normal ratio N/C<sup>19</sup> ≥0.5