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ORIGINAL PAPER

Evaluation of antimitotic and antiangiogenic effect of preoperative subconjunctival application of mitomycin C in primary pterygium: a randomized trial

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Abstract

Purpose To evaluate the influence of preoperative mitomycin C (MMC) on the proliferative behavior of fibroblasts and fibrovascular tissue derived from the primary pterygium using the immunohistochemical method (Ki67 and CD34).

Design Randomized clinical trial.

Subjects, participants and/or controls Sixty-five patients with primary pterygium were randomly selected and divided into one of three groups. The control group had 29 patients that were only submitted to pterygium removal. The group that received the MMC injection a month before surgery had 16 patients, and the group that received the MMC 2 weeks before surgery had 20 patients. Each patient only had one eye operated on.

Methods Sixty-five patients were selected to undergo pterygium excision surgery. We randomly placed the patients into three groups: one without MMC ($n = 29$), one with MMC application 1 month before surgery ($n = 16$) and another with MMC application 2 weeks before surgery ($n = 20$). Subconjunctival injection was applied with 0.1 ml of 0.02% MMC in the pterygium body, and patients were followed for 2 years.

Main outcome measures Proliferative behavior of fibroblasts and fibrovascular tissue using the immunohistochemical method (Ki67 and CD34) comparing the three groups.

Results Of the total 29 patients (44.6%) in the control group (without MMC application), 11 cases had recurrence (37.9%), of which seven (63.6%) were within 3 months of follow-up and four (36.3%) within 6 months of follow-up. The mean proliferation index of the recurrent cases was 4.5%, and of the cases without recurrence, it was 6.1%. There were 16 patients (24.6%) in the MMC application group 1 month before surgery, in which one case (6.25%) recurred at 6 months. In the group with MMC application 2 weeks before surgery, of the total of 20 patients (30.7%), there was one case of recurrence (5%) at 6 months. The proliferation index of the group that had MMC administered and did not have a recurrence was 7.2%, and in the group with recurrence, it was 6.4%. The CD34-labeled cell count was 5.8% among cases with recurrence and 5.6% in cases without recurrence. No side effects of MMC application were reported during the study follow-up period.

Conclusion MMC was efficient to reduce the recurrence index despite the absence of a direct relation with its antimitotic and antiangiogenic effect in the samples that were analyzed.

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Keywords Mitomycin C · Pterygium surgery · Recurrence rate · Immunohistochemistry · Cell proliferation

Introduction

The pterygium is a fibrovascular tissue that starts from the bulbar conjunctiva, overpasses the limbus and reaches the cornea [1]. Men are more commonly affected than women [2]. Symptoms are ocular hyperemia, foreign body sensation and itching. The etiology of the pterygium is not completely clear yet, but it is probably a degenerative process of fibrous connective tissue in response to different stimuli. The risk factors are trauma, hereditary factors and, mainly, exposure to ultraviolet radiation. The latter seems to cause damage to limb stem cells. As a result, there is a migration of fibrovascular tissue toward the cornea. People living in areas near the equator are at increased risk of developing pterygium due to increased exposure to ultraviolet rays [2].

It has recently been suggested that there is a mutation in the p53 gene on chromosome 17 related to the development of pterygium, recurrence after surgery, changes in the expression of basic fibroblast growth factor (bFGF), transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Such growth factors were localized to epithelial cells, vessel endothelial cells, basement membrane of vessels, fibroblasts and inflammatory cells of the pterygium. Thus, a pterygium can be considered the result of an uncontrolled inflammation and cellular proliferation, and not only considered a degenerative disease [3, 4].

The pterygium is characterized by a centripetal and altered growth of limbic epithelial cells, followed by squamous cell metaplasia and goblet cell hyperplasia. Activated fibroblasts and neovascularization have also been related to the pathophysiology of pterygium. The formation and progression of pterygium require the formation of new vessels, and the use of topical bevacizumab to prevent its progression has already been described [3]. The use of lubricant and anti-inflammatory drops improves their symptoms but does not promote regression.

Over the years, many medical treatments have been used, such as bile, urine, acids, radiotherapy, thiotepa, 5-fluorouracil and, more recently, cyclosporine, bevacizumab and mitomycin C (MMC). In the past, horsehair was used to remove pterygium [5]. Surgery is the main treatment indicated when the patient feels discomfort despite the clinical treatment, when there is restriction of the ocular motility, growth in the visual axis and esthetic complaints.

The rate of recurrence after pterygium surgery varies according to the surgical technique, surgeon's experience and use of adjuvant therapy. One of the most common causes of pterygium recurrence is surgical trauma that leads to fibrovascular proliferation. The simple excision of the pterygium has a high recurrence rate: 30–70%. From the technique with conjunctival transplantation, the recurrence rate ranges from 2 to 39% [6, 7].

The purpose of the use of MMC as an adjuvant treatment is to inhibit the growth of episcleral fibroblasts and to avoid recurrence of the pterygium after surgery [8]. MMC is an antineoplastic antibiotic that selectively inhibits the synthesis of DNA, RNA and protein in all cells. Because MMC interferes in cell proliferation, it is expected to also play a role in the control of endothelial cell proliferation in the pathophysiology of angiogenesis. The mechanism has not yet been described, but it should involve intercellular signaling between muscle and endothelial cells and the production of a specific VEGF isoform. The blocking of VEGF may result in reduction in fibrovascular tissue formation and recurrence rate [9, 10].

The purpose of this study was to evaluate the antimitotic and antiangiogenic effect of MMC, in addition to the recurrence rate after the preoperative application of 0.02% MMC in the primary pterygium.

Materials and methods

Sixty-five patients, who presented visual alteration due to irregular astigmatism triggered by the pterygium, or the complaint of refractory discomfort after clinical treatment with lubricant eye drops, participated in a randomized clinical trial and underwent pterygium surgery with conjunctival transplantation. All patients underwent a complete preoperative ophthalmologic examination that showed no alterations beyond the pterygium. Patients were divided into three groups by simple randomization: one without MMC (n = 29), one with MMC application 1 month before surgery (n = 16) and one with application 2 weeks before surgery (n = 20). We applied 0.1 ml of 0.02% MMC to the pterygium body, and patients were followed for 2 years. Only one eye of each patient was operated on. Patients between 21 and 84 years of age and with primary pterygium were included. Individuals with external ocular diseases, such as Sjögren's syndrome and ocular rosacea, and those submitted to previous ocular surgery were excluded.

The study was approved by the Ethics Committee of the University of São Paulo, and a free and informed consent form was given to all patients. This study adhered to the tenets of the Declaration of Helsinki.

The 0.02% MMC was applied preoperatively at a dose of 0.1 ml with a 30-G insulin needle in the pterygium body under local anesthesia with 5.0 mg proxymetacaine hydrochloride drops. After the procedure, the eye was irrigated with 50 ml of balanced saline solution. We prescribed 0.3% ofloxacin eye drops for 1 week after injection under aseptic conditions. All surgeries were performed under local anesthesia. In the surgical procedure, a few drops of proxymetacaine hydrochloride 5.0 mg were applied and followed by subconjunctival injection of 2% xylocaine applied to the body of the pterygium. After limbic peritomy, the limbic plane was deepened and the neck of the pterygium incised with Westcott's scissors, thus separating the head from the body of the pterygium. Next, the pterygium body was separated from the underlying episclera, conjunctiva, tenon and muscle expansions to enable its resection. Then, the pterygium head was removed by superficial keratectomy performed with knife scalpel 15. Finally, the exposed sclera area was covered with a conjunctival transplant using nylon 10.0. All surgeries were performed by the same surgeon.

The removed pterygium was fixed in a sterile vial containing 10% buffered formalin. All fragments were sent to the laboratory to be analyzed by a professional that was blinded, unaware of which patients were submitted to the MMC injections. The fragment was analyzed by the immunohistochemical labeling of Ki67 (Clone MIB-1, Dako, M7240, Glostrup, Denmark) and CD34 (Clone QBEnd10, Dako, M7165, Glostrup, Denmark) (Figs. 1, 2).

In the postoperative period, ciprofloxacin 3.5 mg and dexamethasone 1 mg eye drops were given four times a day for 4 weeks. Follow-up was performed at one day, 1 week, 1 month, 3 months, 6 months,

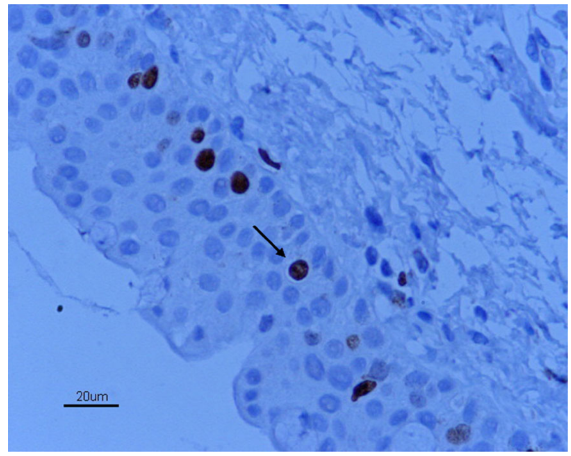

Fig. 1 Immunohistochemical analysis and evaluation of cell proliferation through detection of Ki67 antibody in pterygium sample. Positive epithelial cell for marker Ki67 (arrow)

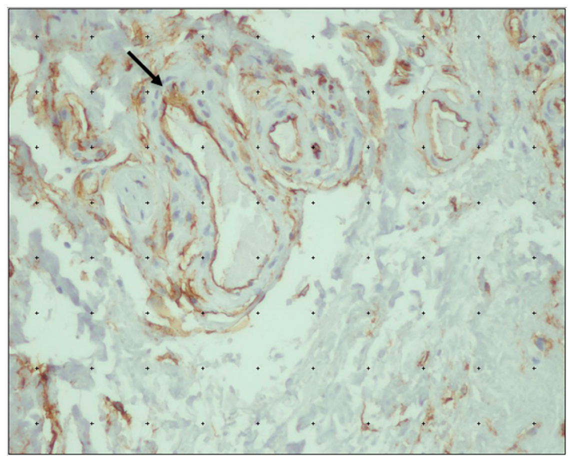

Fig. 2 Evaluation of the positivity for the CD34 marker through immunohistochemical reaction in pterygium sample. Vascular endothelial cells marked in brown (arrow)

1 year and 2 years. At all visits, a complete ophthalmologic examination was performed to check for recurrence and possible complications related to the use of MMC, such as corneal edema, glaucoma, cataract, keratitis and necrosis of the cornea and sclera. Recurrence was defined as any growth of fibrovascular tissue that went beyond the limbus.

Statistical analysis was performed with the Kruskal–Wallis method with posttest Dunn's for the groups labeled with Ki67 and CD34.

Results

Among the 65 patients with primary pterygium that were operated on, 31 (47%) were male and 34 (52%) female. The patients' ages ranged from 21 years to 84 years, with a mean age of 48.3 years. Of the total of 29 patients (44.6%) in the control group (without MMC), 11 had recurrence (37.9%). Of these 11, the recurrence was observed at the 3-month follow-up in seven patients (63.6%) and four cases (36.3%) at the 6-month follow-up. The mean proliferation index of the cases where there was recurrence was 4.5% and of the cases without recurrence 6.1%. Of the 16 patients (24.6%) in the group with MMC application 1 month before surgery, one case of recurrence (6.25%) was observed at the 6-month follow-up. The MMC application group 2 weeks before surgery had 20 patients (30.7%), with a case of relapse (5%) at 6 months. Six patients (16%) submitted to preoperative MMC injection had subconjunctival hemorrhage after the procedure. The recurrence rate was similar in the two groups receiving MMC treatment, and this rate was lower than in the group that did not receive prior MMC subconjunctival injection. In the control group, most recurrences occurred within the first 3 months of follow-up. There was no recurrence in any group after 6 months of follow-up. The mean age of recurrence in the control group was 38 years. The age of recurrence in the group with MMC application 1 month before surgery was 59 years. The age of recurrence in the group with MMC application 2 weeks before surgery was 25 years.

There were no intraoperative complications in either group. Nylon 10.0 sutures were removed with 1-week follow-up from all patients. The proliferation index in the group that received the MMC application and did not have a recurrence was 7.2%, whereas in the recurrent group it was 6.4%. The CD34-labeled cell index was 5.8% among cases with recurrence and 5.6% among cases without recurrence (Table 1). There were no side effects related to the application of MMC during the follow-up period of the study.

Discussion

Recurrence did not have a statistically significant variation between the groups that received preoperative MMC injection at 2 weeks and at 1 month. The cellular and vascular proliferation rates of the analyzed material of the Tenon's capsule and conjunctiva did not demonstrate direct correlation with the recurrence of the cases, which can be explained by the action of the MMC in other regions not analyzed in this study, as, for example, in the fibroblasts of the episclera. There is also the possibility of there being a low rate of cell proliferation because it is a primary pterygium.

Donnenfeld reported the efficacy and safety of preoperative MMC injection of 0.1 ml (0.15 mg/ml) in the pterygium body 1 month prior to surgery to avoid recurrence. The results showed less vascularization and inflammation within the pterygium 1 month after the injection of MMC with a recurrence of 6% after 24.4 months of follow-up. The results of the study showed a dose of 0.10 mg/ml inhibited fibroblast replication, and a dose greater than 0.3 mg/dl caused fibroblast death [11]. The main risk involved in the preoperative injection results from the impossibility of washing the MMC, which can be toxic when restricted to the subconjunctival space. According to studies, the subconjunctival injection of 0.2 ml (0.4 mg/ml) MMC injected at 2 mm posterior to the limbus is capable of causing cellular alterations in the ciliary body epithelium, leading to reduction in aqueous humor production in the month following

Table 1 Evaluation of the number of vascular endothelial cells labeled with CD34

Controls without recurrence × controls with recurrence	P value = 0.35
Controls without recurrence × MMC 15 days without recurrence	P value = 0.83
Controls without recurrence × MMC 1 month without recurrence	P value = 0.27
Controls with recurrence × MMC 15 days without recurrence	P value = 0.54
Controls with recurrence × MMC 1 month without recurrence	P value = 0.97
MMC 15 days without recurrence × MMC 1 month without recurrence	P value = 0.33
One-way ANOVA (all groups)	P value = 0.60
One-way ANOVA (without the group MMC with recurrence)	P value = 0.60

the application. Carrasco et al. [12] reported a case of scleral necrosis in a patient with severe dry eye and a history of lacrimal cauterization, who received a subconjunctival injection of MMC 0.15 mg/dl 1 month prior to pterygium surgery with transplantation of conjunctiva. The local accumulation of MMC associated with dry eye, besides the use of anti-inflammatory eye drops and corticoids in the postoperative period, interfered in the healing process. Thus, although MMC reduces the recurrence of the pterygium, its dose and the method of administration should be appropriately chosen according to patient profile [10–15].

Conjunctival transplantation was first described by Kenyon et al. [8] with a recurrence rate of 5.3%. In our study, the recurrence rate was 6.25% in cases with preoperative MMC application 1 month prior and 5% in cases with preoperative MMC application 2 weeks prior.

From our results, we noticed that mitomycin C 0.02% is a good therapeutic option for preoperative use via subconjunctival injection, reducing the risk of relapse and presenting no complications with its use. We chose to apply MMC directly to the pterygium body to try to focus its action and avoid damage to the corneal epithelium. The use of MMC has already been described before, during or after surgery, also in the form of eye drops. Inhibition of MMC-induced episclera fibroblast proliferation is restricted to its site of application and has a prolonged duration, preventing pterygium recurrence. Longer duration and concentration of MMC are more associated with complications, such as epithelial lesion, corneal ulcer, iritis, necrotizing scleritis, cataract, glaucoma and ciliary body lesion [8]. The subconjunctival injection of MMC guarantees drug efficacy and prevents inappropriate penetration that may occur with topical application [9].

Immunohistochemical methods with the detection of monoclonal antibodies can be used to evaluate cell proliferation. The most frequently used Ki67 antibody targets nuclear antigens which are in proliferating cells throughout the cell cycle, especially in the G2 and mitosis phases. The proliferative potential of a tissue can also be measured via cell proliferation markers. Among the methods used, the most traditional are those that count the mitoses. They have the disadvantage that mitosis is the fastest phase of the cell cycle, in which the proliferating cell population can be underestimated with this type of reading [7, 8]. CD34 is an endothelial cell marker that we used to identify vessels in the pterygium tissue.

Knowing the behavior of the epithelial tissue and fibroblasts that make up the pterygium, its proliferative potential, its interaction with several drugs, its ultrastructure and its behavior compared to fibroblasts of other tissues, we will have valuable information that can guide the establishment of more effective techniques and be less iatrogenic, all or any of which can prevent recurrence of the pterygium after its removal, which is the most commonly attributed to surgical failure [8, 16–18].

A publication by Tan et al. [19] using flow cytometry to analyze the proliferative potential of the healthy conjunctiva epithelium, comparing it with the potential of the epithelium covering the pterygium and its fibrovascular tissue, opened new perspectives for the research of treatment methods directed primarily to the fibrovascular tissue of the pterygium. Their study concluded that the proliferative potential of the primary or recurrent pterygium is due to the behavior of fibroblasts and vascular tissue, but not to the epithelium that covers it. This theory had already been proposed by Girolamo et al. [20, 21] who found no differences between the behavior of epithelia cultivated in the laboratory, extracted from the pterygium, and those extracted from normal conjunctiva under the conditions studied.

The number of patients in our study was probably insufficient, and statistical significant results could be obtained with a greater number of patients. The episclera was not analyzed, and its analysis could help understand the effects of MMC.

New studies with a greater number of patients and analyses of other regions, such as the episclera, may be necessary to correlate the cell proliferation index and the antiangiogenic effect of MMC, in addition to its possible action in the inhibition of other cellular growth factors, such as factors of TGF-β, as well as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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