ORIGINAL ARTICLE DOI: 10.5935/0034-7280.20140034

Influence of intraoperative episcleral application of topic mitomycin C on proliferation and differentiation of rabbit corneal and conjunctival epithelial cells

Influência da aplicação intraoperatória de mitomicina C tópica episcleral na proliferação e diferenciação de células epiteliais córneo-conjuntivais de coelhos

Marisa Braga Potério[1], Newton Kara José[1], Eliane Maria Ingrid Amstalden[1], Keila Miriam Monteiro de Carvalho[1], Ana Maria Marcondes[1]

ABSTRACT

***Objective**: To evaluate the influence of a three-minute application of 0.02% mitomycin C (MMC) on proliferation and differentiation of rabbit corneal epithelial cells. **Methods**: Topical MMC or 0.9% saline solution was applied to the episclera of the temporal limbal area of both eyes, maintaining intact epithelial tissue in one eye, and after partial corneal deepithelialization in the contralateral eye. The animals were arranged in groups A (20 eyes), B (16 eyes) and C (14 eyes) and sacrificed respectively on the 4th, 15th and 45th postoperative days. In order to stimulate cell proliferation, group C was submitted to central corneal deepithelialization three days before sacrifice. Cell differentiation markers (AE1, AE3 and AE5) were used for differentiation analysis and 5-bromo-2-deoxyuridine (BrdU) for detection of corneal epithelial cells proliferation. **Results**: There was no statistical difference in differentiation cells when drugs or surgical techniques were analyzed. Cell proliferation when using MMC or SF was statis-tically significant at central and temporal areas when applied after partial corneal deepithelialization in all groups. **Conclusion**: These results suggest that the three-minute episcleral application of 0.02% MMC does not affect epithelial cell differentiation; however, when MMC application occurs after corneal deepithelialization, it may affect cell proliferation.*

***Keywords**: Cornea; Epithelial cells/drug effects; Mitomycin/administration & dosage; Rabbits*

RESUMO

Objetivo: Avaliar a influência da mitomicina C a 0,02% (MMC), aplicada em dose única por 3 minutos, na proliferação e diferenciação das células epiteliais da córnea de coelhos. **Métodos**: A MMC tópica foi aplicada na episclera da área límbica temporal, mediante tecido epitelial corneano intacto (um olho) e após desepitelização epitelial parcial da córnea (outro olho). Durante o procedimento cirúrgico, MMC ou solução fisiológica 0,9% (SF) foi aplicada e a solução escolhida para cada animal foi determinada por sorteio. Os animais foram divididos em grupo A (20 olhos), grupo B (16 olhos) e grupo C (14 olhos). Foram sacrificados respectivamente em 4º, 15º e 45º dia de pós-operatório. Para estimular a proliferação celular, os animais do grupo C foram submetidos à desepitelização central da córnea 3 dias antes do dia do sacrifício. Marcadores de diferenciação celular (AE1, AE3 e AE5) e de proliferação (5-Bromo-2-Deoxiuridina, BrdU) foram utilizados. Nas lâminas coradas com BrdU, as áreas nasal, temporal e central foram delimitadas. O número de células coradas pela BrdU foram contadas nos 3 diferentes campos e a média aritmética de cada área foi analisada estatisticamente. **Resultados**: Houve diferença estatística entre MMC e SF nas áreas central e temporal da córnea previamente desepitelizada em todos os grupos. Não houve diferença estatística durante a análise de diferenciação celular. **Conclusão**: A MMC na dose de 0,02%, aplicada por 3 minutos sobre a episclera, interfere na proliferação celular da área exposta à droga e previamente desepitelizada. Não interfere na diferenciação celular e sua ação possui efeito prolongado.

Descritores: Córnea; Células epiteliais/efeito de drogas; Mitomicina/administração & dosagem; Coelhos

[1] State University of Campinas, Campinas/SP, Brazil.

Work conducted at the Department of Experimental Medicine, Faculty of Medical Sciences, State University of Campinas, Campinas/SP, Brazil; Department of Otorhinolaryngology/Ophthalmology, Faculty of Medical Sciences, State University of Campinas, Campinas/SP, Brazil.

The authors declare no conflict of interest.

Received for publication 17/3/2013 - Accepted for publication 23/11/2013

Introduction

Cell growth and proliferation are closely related to cell differentiation. Undifferentiated cells have a high division capacity[1]. Precise regulation of the cell cycle during the embryonic period is crucial to determine cell size and shape and to provide the necessary conditions for cell growth and differentiation[2]. Antimitotic agents can block the cell cycle during cell differentiation[3].

Mitomycin C (MMC) is an antibiotic isolated from *Streptomyces caespitosus*; it is an antineoplastic agent that selectively inhibits DNA, RNA, and protein synthesis in fast-growing cells. Similar to alkylating agents, it forms covalent bonds with guanine residues in the DNA. It mimics ionising radiation and can produce cumulative effects, and its effect can persist for a long period after treatment is discontinued. Because MMC is a potent inhibitor of fibroblast proliferation it is indicated for prevention of postoperative relapse of pterygium, and it also increases the rate of success and maintenance of antiglaucoma tube-shunt surgery. Furthermore, it is used successfully in the treatment of other eye diseases[4]. Topical MMC can produce drug-related complications[5-8], stressing the need for safe application and adequate dosage[9-11].

Previous studies have shown that the bare sclera technique for pterygium resection is associated with a higher rate of complications, as the sclera remains exposed after topical application of MMC[12-14].

In an attempt to reduce the rate of complications associated with pterygium surgery with topical MMC, a surgical technique has been developed in which the pterygium head in the cornea is removed after application of MMC, so that the corneal tissue remains protected during application of MMC. The good outcomes obtained with the technique suggest that it also influences the rates of drug-related postoperative complications[15].

The aim of this study was to assess the influence of intraoperative episcleral application of topical mitomycin C on the proliferation and differentiation of rabbit corneal and conjunctival epithelial cells using different surgical techniques.

Methods

Double blind study on 24 female New Zealand white rabbits weighing 800-1900 g (mean, 1135.60 g) without ocular abnormalities on slit lamp biomicroscopy. The study was approved by the Animal Ethics Committee (No. 475-2) and animals were treated according to the standards of the Association of Research in Vision and Ophthalmology (ARVO) and the Brazilian College of Animal Experimentation (COBEA). The animals were divided into 3 groups according to the time set for sacrifice. Animals were sacrificed 4 days after surgery in group A, 15 days in group B, and 45 days in group C. After sedation with intramuscular injection of ketamine hydrochloride (50 mg/kg body weight) and xylazine (5 mg/kg), the eyes of rabbits were irrigated with a solution of 0.9% sodium chloride (normal saline, NS) and instilled with one drop of 0.5% tetracaine (1 drop/eye), followed by the surgical procedures described below. Two solutions, NS for the control group and MMC 0.2 mg/ml for the treatment group, had been previously prepared and placed in bottles numbered 1 and 2 by another researcher who did not take part in the surgical procedures. The solution to be applied and the surgical technique employed in each eye were determined randomly.

Study with partial de-epithelialisation of the cornea

The conjunctiva and temporal cornea were marked with an optical zone marker (8.0 mm diameter) previously stained with gentian violet. The optical zone marker was centred in the temporal limbus (Figure 1). The marked conjunctiva was dissected and the episclera was exposed. The epithelial cells of the marked cornea were dehydrated with a cotton swab soaked in 95% ethanol for 30 seconds. After irrigation with NS, the corneal epithelium was removed with a scalpel blade #15 and total de-epithelialisation was confirmed by applying 1 drop of 2% sodium fluorescein. A 5.0 × 3.0 mm cellulose sponge (Weck-Cel Model C00054, Edward & Company, Treton, NJ) soaked in solution 1 or 2 was applied to the episclera for 3 minutes. The episclera was irrigated with 100 ml NS and the conjunctival edges were sutured with 8-0 absorbable sutures. (Figure 1).

Study with an intact cornea

The contralateral eye of each animal was treated as follows: after exposing the episcleral area, a cellulose sponge soaked in the same solution used in the first eye was applied for 3 minutes. The episclera was then irrigated with 100 ml NS and the marked corneal epithelium was dehydrated and removed. The conjunctival edges were sutured with 8-0 absorbable sutures to protect the episcleral area that received the solution. (Figure 2)

All eyes received dexamethasone and chloramphenicol eye drops, 1 drop in each eye every 8 hours for 7 days. External complications such as discharge, de-epithelialisation of the cornea, and conjunctival and palpebral changes were assessed macroscopically and by biomicroscopy.

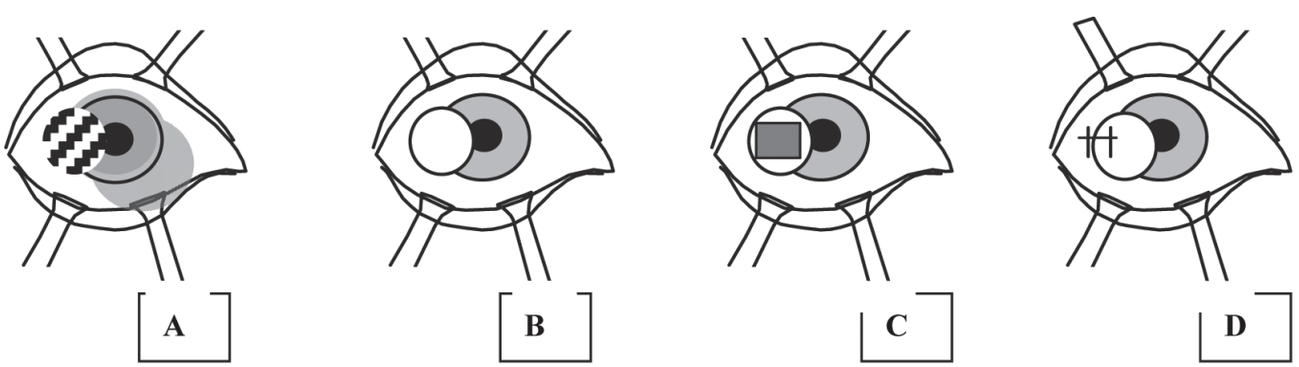

Figure 1. A) 8.0-mm optical zone marker positioned in the temporal limbus of the right eye; **B)** The marked area, with the de-epithelialised cornea and the exposed episcleral area; **C)** The Weck-Cel sponge soaked in solution is applied to the episclera; **D)** The conjunctiva is sutured with 8-0 absorbable sutures.

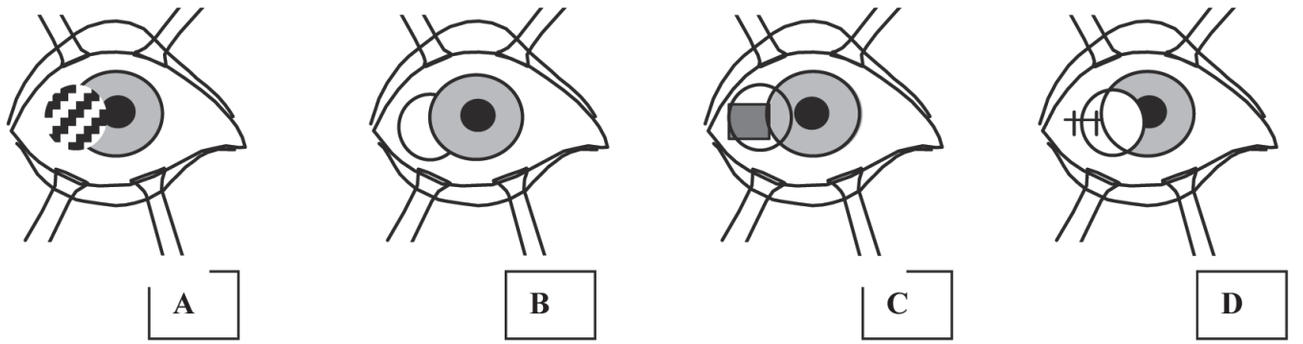

Figure 2. A) 8.0-mm optical zone marker positioned in the temporal limbus of the right eye; **B)** The marked area, with the intact cornea and the exposed episcleral area; **C)** The Weck-Cel sponge soaked in solution is applied to the episclera; **D)** The marked cornea is dehydrated and the conjunctiva is then sutured with 8-0 absorbable sutures.

Procedure performed on animals in group C

To assess the long-term effects of MMC, rabbits in group C were subjected to a new de-epithelialisation of the central cornea 42 days after the initial procedure, in the area previously marked with an 8-mm trephine. The marked cornea was dehydrated and de-epithelialised with a blade #15 in a procedure similar to the temporal cornea.

Applying the BrdU antigen (Calbiochem, Laboratório Sigma)

An intravenous injection of 5-bromo-2-deoxiuridine (BrdU) 100mg/kg diluted in 20.0 mg/ml sterile phosphate (PBS) with pH=7.4 was applied to the rabbit's marginal ear vein 24 hours prior to sacrifice. The cells that were in the S phase of cell division within 24 hours of the BrdU intravenous injection were stained. The number of positive cells in the epithelium was counted using the BrdU reagent and anti-BrdU antibodies.

Immunohistochemical analysis

After animals were sacrificed with an intravenous injection of 3% thiopental (25 mg/kg body weight) the corneas were fixed in situ by perfusing the anterior chamber with 1% paraformaldehyde diluted in phosphate for 5 minutes. The removed material was preserved in 10% buffered formalin. The nasal cornea opposite to the operated area was marked with a 4-0 silk suture as a reference point. All bottles were identified for later inclusion of the material in paraffin. Once prepared, the slides were submitted for processing of the various reagents (AE1/AE3, AE5 and BrdU). Epithelial differentiation was assessed using monoclonal antibodies AE1/AE3 and AE5. Analyses were performed without prior knowledge of the type of surgical procedure and the study group where the material had been obtained from.

Reagents AE1/AE3 and AE5

Absorption of reagents AE1/AE3 and AE5 by the cells after reaction with keratin from the corneal epithelium was demonstrated using the ABC method (streptavidin-biotin-peroxidase complex revealed). Slides were deparaffinised with quick immersions in xylene I preheated to 110°C in an oven for 30 minutes followed by immersions in xylene II and III at room temperature, absolute ethanol I, II, III, 80, and 50% (at room temperature), and running and distilled water. They were then subjected to blocking of endogenous peroxidase with 10 ml of H_2O_2 (30%) diluted in 90 ml of methanol. Three 3-minute exchanges were performed at room temperature. After being washed in running and distilled water, they were subjected to antigen retrieval for 30 minutes in a steamer at 95°C with 10 mM citrate buffer at pH 6.0 and washed in running water for 5 min, distilled water for 5 min, and PBS for 5 minutes. The antibodies AE1/AE3 and AE5 (1:50) diluted in PBS buffer and BSA (bovine serum albumin at a concentration of 1.25 ml) with pH=7.5 (1:50) were instilled in different slides and incubated in a humid chamber and oven at 37°C for 30 minutes.

The slides were removed from incubation with primary antibodies and washed three times, for 5 minutes each, with PBS in a shaker at room temperature. They then received the secondary antibody, Multi-Link diluted in PBS (1:80) and incubated at room temperature for 1 hour in an oven at 37°C. They were washed three times, for 5 minutes each, with PBS in a shaker at room temperature and dried with filter paper. They then received the ABC complex diluted in PBS (1:100) and were incubated for 40 minutes in an oven at 37°C. The slides were then placed in a PBS buffer and stained for 5 minutes with DAB (diaminobenzidine), washed with running water, counterstained with Mayer's haematoxylin for 30 to 60 seconds depending on macroscopic staining, and washed with ammonia water (NH_4OH) and running and distilled water for a few seconds. The slides were dehydrated and mounted for analysis.

BrdU reagent

The corneas were deparaffinised with xylene I for 15 minutes in an oven at 60°C. They then received xylene II and III for 10 minutes each at room temperature. The following solutions were applied: 100% ethanol 3 times for 1 minute each; 95% ethanol for 1 minute; and 70% ethanol 3 times for 1 minute each. The slides were washed with distilled water 3 times for 1 minute each and with PBS 3 times for 5 minutes each. They were then kept in a bath with 2NHCl for 20 minutes at 31°C. They were then bathed for 2 min in a 0.005 g trypsin solution diluted in 100 ml of PBS at 37°C and placed in Molico™ skimmed milk 1 g in 100 ml of previously homogenised PBS. They remained in this solution for 1 hour at room temperature. The BrdU (1:50) antibodies were diluted in BSA (bovine serum albumin) at 1% and applied to the slides, which remained in a humid dark chamber for 1 hour. After washing the slides 3 times with PBS (5 minutes each), the Multi-Link secondary antibodies (1:80) diluted in BSA were applied, remaining for 1 hour in a humid dark chamber. After washing the slides with PBS 3 times for 5 minutes each and endogenous peroxidase blocking with 3% H_2O_2 for 5 minutes, the slides received the ABC complex in PBS (A and B = 1:50) for 30 minutes in a dark and humid chamber. DAB (1 drop in 1 ml of buffer) was applied, and immediately after the reaction the slides

were washed with running distilled water for 5 minutes and stained with haematoxylin. The slides were washed, dehydrated, and mounted for analysis.

Analysing the pattern of corneal epithelial differentiation

The epithelial cells of the limbal and central corneal regions were analysed. The AE1 reagent stains cells with 40-56 kD keratins, and the AE3 reagent stains cells with 52-67 kD keratins. The keratins stained by AE1/AE3 reagents are mainly found in the cytoplasm of basal and suprabasal epithelial cells near the limbus or at the peripheral cornea, and the AE5 reagent stains 64 kD keratins specific to mature or suprabasal corneal cells[16].

Counting cells

Cell nuclei stained with BrdU were counted using the following methodology. Under light microscopy (Nikon microscope, Labophot) with a 4x magnification and using a marker pen, the two ends of the cornea (temporal and nasal) were marked. The tissue was then divided into 3 equal parts using a millimetre ruler, based on the previous marking. One central and two peripheral areas of the cornea were established. The tissue in the slide was compared with the tissue in paraffin (with the silk thread in the nasal region) and the peripheral areas of the slides (temporal and nasal) were identified. For microscopic analysis three fields in each region were randomly selected using an objective lens with a 40x magnification. Using a millimetre Kpl (Carl Zeiss, Germany) 8x ocular lens with the 40x objective, the number of stained cells in each field (corresponding to 0.25 mm) was counted. Cell counts in three fields for each region (0.75 mm) comprised approximately 90% of the corneal epithelium's total surface area. Intact, round and intensely stained cells were considered positive. Cells that were not intensely stained were considered negative[17].

Statistical analysis was based on the arithmetic mean for the 3 fields in each area (nasal, central and temporal). A factorial model for analysis of variance was used to test the effect of drugs in each group for each region of the cornea and for each surgical technique. All statistical tests used the descriptive measure p with a significance level of 5% ($p<0.05$)[18]. Data were edited using Microsoft Excel for Windows 98.

Results

Macroscopic and biomicroscopic analysis

No hyperaemia or discharge were observed in the eyes of animals preoperatively. In the postoperative period, leukoma was observed in the de-epithelialised area of one eye in an animal from group A (who received MMC with partial de-epithelialisation prior to MMC). A moderate amount of yellow discharge was observed in 3 eyes: 2 from group A (of which one received MMC with prior partial de-epithelialisation and the other received NS with subsequent partial de-epithelialisation) and 1 from group B (which received NS with prior partial de-epithelialisation). The latter presented scleral thinning in the same eye with tissue bulging in the operated area.

AE1/AE3 and AE5 reagents

Cells considered as positive, or those marked by primary AE1/AE3 or AE5 antibodies, were expressing keratins in the basal and suprabasal epithelial layers. Analysis of the pattern of differentiation of basal epithelial cells in the peripheral and central regions of the cornea showed that cells in both regions reacted with the AE1/AE3 antibodies in all groups, with both surgical techniques and regardless of the drug used (Table 1). Figure 3a illustrates the pattern of AE1/AE3 positivity among keratins in the corneal epithelium. The pattern was similar in the different groups, with the different drugs (MMC and NS) and with both surgical techniques (previous partial de-epithelialisation and intact cornea). The epithelial cells in the central cornea reacted with the AE5 antibody in all groups (Table 2 and Figure 3b). For this primary antibody, the pattern of cell differentiation was similar for animals receiving MMC and NS, regardless of the surgical technique or group.

Reagente BrdU

Cells considered as positive, or those marked by BrdU (Figure 3c), were present in the basal and suprabasal layers of the epithelium in the three groups. Table 3 shows that in this experimental model, for the technique of prior partial de-epithelialisation, there were statistically-significant differences between NS and MMC in the central and temporal regions and in both regions combined ($p<0.05$), i.e., MMC significantly affected cell counts in these regions.

Statistical analysis showed that for the intact cornea technique, there were no statistically-significant differences between NS and MMC. There were statistically-significant differences between groups in the temporal region and in both regions combined ($p<0.05$), i.e., for this technique, the cell counts in these regions were statistically different between groups A and B. There were no statistically-significant differences for group C. The interaction of variables "drug" and "group" showed no statistical differences. (Table 4)

Discussion

Ophthalmic drugs used topically on the cornea can influence epithelial cell kinetics[19,20]. We chose to study the corneas of rabbits due to their similarity to the human cornea. They have

Figure 3. a) Optical microscopy of a specimen containing corneal epithelium. Example of basal cells and reticular lamina cells marked with AE1/AE3 (400x). b) Optical microscopy of a specimen containing corneal epithelium. Example of basal cells and reticular lamina cells marked with AE5 (400x). c) Optical microscopy of a specimen containing corneal epithelium. Example of basal cells and reticular lamina cells marked with BrdU (400x).

Table 1

Results of immunohistochemical analysis of cytokeratin expression by epithelial cells of rabbit corneas in all 3 groups, with AE1/AE3 antibodies, distributed according to the surgical technique (prior partial de-epithelialisation; intact cornea) and the type of solution (MMC; NS).

Group	Solution	Animal number	Technique			
			Partial de-epitelization		Intact cornea	
			Peripheral	Central	Peripheral	Central
A	NS	3	+	+	+	+
		6	+	+	+	+
		8	+	-	+	+
		17	+	-	loss	loss
		19	+	+	+	+
	MMC	4	+	+	+	+
		5	+	+	-	+
		7	+	+	+	+
		16	+	-	+	+
		18	+	-	+	+
B	NS	23	-	-	-	-
		24	-	-	+	+
		25	+	-	-	-
		26	+	+	+	+
	MMC	20	-	-	-	-
		21	+	+	+	+
		22	-	-	-	-
		27	+	-	+	-
C	NS	10	-	+	-	-
		12	-	-	+	-
		14	+	+	+	+
	MMC	9	+	-	+	-
		11	+	+	+	+
		13	-	-	-	-
		15	+	-	+	+

Group A: animals sacrificed on the 4th postoperative (PO) day; Group B: 15th PO day; Group C: 45th PO day and 72 hours after a new procedure for de-epithelialisation of the central cornea; MMC, Mitomycin C; NS, Normal saline; Loss, insufficient material, not assessed; +, Keratin expression; -, No keratin expression.

Table 2

Results of immunohistochemical analysis of cytokeratin expression by epithelial cells of rabbit corneas in all 3 groups, with AE5 antibodies, distributed according to the surgical technique (prior partial de-epithelialisation; intact cornea) and the type of solution (MMC; NS).

Group	Solution	Animal number	Technique			
			Partial de-epitelization		Intact cornea	
			Peripheral	Central	Peripheral	Central
A	NS	3	+	+	+	+
		6	+	+	-	-
		8	+	+	+	+
		17	+	+	loss	loss
		19	+	+	+	+
	MMC	4	+	+	+	+
		5	+	+	+	+
		7	+	+	+	+
		16	+	+	+	+
		18	+	+	+	+
B	NS	23	+	+	+	+
		24	+	+	+	+
		25	+	+	-	+
		26	+	+	-	-
	MMC	20	+	+	+	+
		21	+	+	+	+
		22	-	-	+	+
		27	-	-	-	-
C	NS	10	-	+	-	+
		12	loss	loss	-	+
		14	-	+	-	+
	MMC	9	-	+	-	-
		11	-	+	-	+
		13	-	+	-	+
		15	-	+	-	+

Group A: animals sacrificed on the 4th postoperative (PO) day; Group B: 15th PO day; Group C: 45th PO day and 72 hours after a new procedure for de-epithelialisation of the central cornea; MMC, Mitomycin C; NS, Normal saline; Loss, insufficient material, not assessed; +, Keratin expression; -, No keratin expression.

a quick turnover of epithelial cells and their pattern of keratin expression is very similar to the human cornea[21-23]. MMC, a drug used as adjuvant therapy in many ophthalmic procedures, acts as an inhibitor of cell proliferation and has been linked to postoperative complications such as corneal erosion, conjunctival hyperaemia, blepharospasm, corneal and scleral thinning, and perforations[8,10]. In the present study, no complications such as discharge, hyperaemia, or corneal or scleral thinning were associated with 0.02% MMC applied to the episcleral region in a single dose for 3 minutes. Sampaio et al. reported similar findings after instilling 0.02% and 0.04% MMC in the eyes of female rats[24].

The regulatory mechanism for the number of basal cell penetrating the cornea coming from the stem cells located in the limbus, which is responsible for controlling basal cell proliferation, differentiation and shedding, is still unknown[25]. The literature stresses that MMC is a potent anticancer agent capable of inhibiting cell proliferation, thus contributing to the success of many ophthalmic procedures. For example, by decreasing cell proliferation in tenonian and conjunctival tissue after tube-shunt surgery, reducing of the rates of relapse following pterygium surgery, and reducing the rates of postoperative haze in refractive surgery. Its low cost and simple manipulation justify its use and the need to better understand its potential complications[5,6,10,26.]

As MMC probably induces greater damage to intensely-proliferating cells, the drug must act specifically on DNA synthesis and the mitotic spindle of limbal stem cells.[5,27] In 1992, Kawase et al.[28] measured the concentration of MMC in the conjunctiva, sclera and aqueous humour of rabbits after subconjunctival injections at concentrations of 0.002 to 0.2 mg/ml. The authors observed that MMC has a half-life of 0.18 to 0.48 hours. Its

Table 3

Factorial model for statistical analysis considering the following variables: drug (MMC and NS) and group (A, B and C). Study considering cell proliferation across the entire cornea and in each specific region after prior partial de-epithelialisation.

Technique	Region	Variable	F Value	Pr>F*	Significance
	Nasal+central+	Group	0,48	0,5067	n.s.
	temporal	Drug	10,00	0,0023	*
		GroupX Drug	0,36	0,6993	n.s.
Previous	Nasal	Group	2,37	0,1210	n.s.
Partial		Drug	1,56	0,2266	n.s.
De-epitelization		GroupX Drug	2,19	0,1399	n.s.
	Central	Group	2,95	0,0767	n.s.
		Drug	5,81	0,0263	*
		GroupX Drug	1,13	0,3447	n.s.
	Temporal	Group	0,39	0,6803	n.s.
		Drug	4,60	0,0451	*
		GroupX Drug	0,03	0,9737	n.s.

Group A: animals sacrificed on the 4th PO day; Group B: 15th PO day; Group C: 45th PO day and 72 hours after a new procedure for de-epithelialisation of the central cornea; *, significant at a 5% level; n.s., not significant.

Table 4

Factorial model for statistical analysis considering the following variables: drug (MMC and NS) and group (A, B and C). Study considering cell proliferation across the entire cornea and in each specific region using the intact cornea technique (i.e., partial de-epithelialisation after applying the solution).

Technique	Region	Variable	F- value	Pr>F	Significance
	Nasal+central	Group A and B	3,19	0,0480	*
	+temporal	Drug	2,90	0,0955	n.s.
		Group X Drug	0,07	0,9305	n.s.
	Nasal	Group	1,25	0,3078	n.s.
Intact cornea		Drug	0,58	0,4546	n.s.
		Group X Drug	1,03	0,3775	n.s.
	Central	Group	1,50	0,2493	n.s.
		Drug	3,90	0,0629	n.s.
		GroupX Drug	2,94	0,0773	n.s.
	Temporal	Group A and B	4,57	0,0240	*
		Drug	3,41	0,0804	n.s.
		Group X Drug	0,03	0,9711	n.s.

Group A: animals sacrificed on the 4th PO day; Group B: 15th PO day; Group C: 45th PO day and 72 hours after a new procedure for de-epithelialisation of the central cornea; *, significant at a 5% level; n.s., : not significant.

concentration was reduced to 1/15 when the conjunctiva and sclera were irrigated with 200 ml of normal saline, however the drug's half-life remained unchanged. These results show that the drug disappears rapidly from tissues and that irrigation significantly reduces its concentration. In this study, irrigation of the episcleral area treated with MMC with 100 ml of NS was enough to prevent the drug from interfering with cell proliferation in the nasal cornea (Tables 3 and 4).

Studies on the regenerative potential of corneal epithelial cells for rapid tissue repair suggest that the regeneration process can occur by centripetal migration of stem cells from the limbus or circumferentially, as cells adjacent to the de-epithelialised area slide into it.[23,28,29]. Despite the rapid regenerating potential, significant differences were found in cell proliferation from the central and temporal peripheral regions of animals receiving NS or MMC and evaluated on the 4th, 15th and 45th PO days, in eyes

subjected to prior partial de-epithelialisation of the cornea (Table 3). These results suggest that MMC acted in those regions, i.e., the cell inhibition found in the temporal and central regions suggests that MMC influenced cells exposed by the epithelial defect and did not affect cells with an intact epithelium. Using surgical techniques that preserve the corneal epithelium's integrity during MMC administration can help improve wound healing in the postoperative period. The inhibition of cell proliferation observed in later stages (15th and 45th PO day) suggests that MMC has a long-term inhibitory effect on corneal epithelial cells of rabbits (Table 3).

In this study we used not only AE1/AE3 antibodies to mark proliferating cells, but also AE5, a monoclonal antibody highly specific to 64 kD keratins which is considered a specific marker of differentiated corneal epithelial cells[22]. Differentiated cells presenting keratins that reacted with AE5 were found in all groups (Tables 1 and 2). Holzchuh[16] noted in 1999 that after instillation of 0.02% MMC or distilled water, cells in the intact epithelium of rabbits showed a pattern of differentiation when marked with AE1 and AE5 antibodies, with limbal cells marked by AE1 and central cells by AE5 in all groups. In our study, animals sacrificed on the 4th and 15th PO days presented basal cells marked with AE5 in the peripheral cornea (Table 2). In 1986, Schermer et al.[23] used AE5 to study epithelial cell kinetics and showed that 55/64 kD keratins are expressed in the suprabasal layer and the limbus. In contrast, basal cells of the central corneal epithelium expressed 64 kD keratins. These findings suggest that the limbal basal cells are more differentiated than stem cells. The keratocytes of the basal layer in cultured corneal tissue can be marked with AE5, suggesting that the expression of 64 kD keratins is not limited to the suprabasal layer. In our study, there were no differences in the pattern of differentiation of animals receiving NS (control) or MMC in all groups, therefore MMC did not affect the pattern of cell differentiation regardless of the surgical technique.

In 1982 Gratzner[30] developed and tested BrdU to study cell kinetics. The author suggested that BrdU could be an efficient, quick and simple alternative to demonstrate the process of cell division in the corneal epithelium. BrdU replaces thymidine and is incorporated into cell DNA during the S phase of cell replication. In the 1990s, Raska et al.[31] and Szerenyi et al.[17] reported that exposure time to BrdU influenced marking rates, with the optimal time interval depending on the duration of the cell cycle. In our study, BrdU was chosen because it is easy to handle, not radioactive, and effective in determining proliferating cells. An intravenous injection was applied 24 hours before sacrificing the animals, thus covering the circadian rhythm of epithelial cell mitosis that peaks at 9 hours, as described by Haaskjold et al.[32] in 1992. Sacrificing animals 24 hours after BrdU administration allowed its incorporation into cell DNA and subsequent counting of corneal cells

During BrdU analysis it was found that MMC affected cell proliferation in the temporal and nasal regions of previously de-epithelialised corneas. In our study, MMC acted locally and did not affect areas with intact epithelium; thus, in procedures where the corneal epithelium is removed, it is advisable to apply MMC before de-epithelialisation[17,33,34].

MMC is currently used in surgical procedures such as photorefractive keratectomy to correct hyperopia following radial keratotomy or to reduce the incidence of postoperative haze in refractive surgery. The fact that the drug acts only at the administration site and after prior de-epithelialisation of the cornea can help inform further studies aiming to assess the efficacy and safety of MMC during photorefractive surgery[35,36].

Our results, as well as those of Mattar et al.[33], Lacayo et al.[37], Mietz et al.[38] Holzchuz et al.[39], and Ando et al.[40], suggest that MMC, due to its antimitotic properties, can interfere with the cell regeneration process. Its use should therefore be controlled, minimising contact with the cornea. Handling and administering the drug properly, choosing the right surgical technique, and determining the minimal concentration to inhibit cell proliferation are crucial for surgical or medical therapeutic success and to reduce the rates drug-related complications.

Conclusion

In this study, Mitomycin C at a concentration of 0.02%, soaked in a cellulose sponge, was applied for 3 minutes to the temporal episcleral region of rabbit eyes, followed by assessments in PO days 4, 15 and 45 days. Our findings suggest that the clinical complications seen in the eyes of animals during the postoperative period can not be attributed to MMC. The presence of differentiated cells at all postoperative time-points suggests that MMC does not interfere with the cell maturation process.

The effects of MMC are restricted to the application site and to cells exposed through de-epithelialisation, inhibiting corneal cell proliferation. Irrigation with 100 ml of NS after intraoperative administration of MMC was enough to prevent the inhibition of cell proliferation in other corneal regions with an intact epithelium for both surgical techniques. The persistence of MMC effects on the corneal epithelium at the 45th PO day after a single administration suggests that the drug has a long-term effect and points to the need for further studies aiming to better assess the drug's potential toxicity.

Acknowledgements

The authors would like to thank the staff of the Centre for Experimental Medicine and Surgery for their assistance during the surgical procedures and animal care; Prof. José Butori Lopes de Faria, Ph.D., head of the Nephrology Laboratory, Medical School of the State University of Campinas, for offering his laboratory for slide preparation; and Prof. José Vassalo, Ph.D., from the Department of Pathology, for his guidance in preparing the slides for analysis of cell differentiation.

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Corresponding author:
Marisa Braga Potério
Rua Duque de Caxias, nº 780, Cj 91 – Centro
Campinas, SP, Brazil
E-mail: poterioma@hotmail.com