Mitomycin-C Concentration in Cornea and Aqueous Humor and Apoptosis in the Stroma After Topical Mitomycin-C Application

Effects of Mitomycin-C Application Time and Concentration

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Purpose: To evaluate the effects of the applied mitomycin-C (MMC) concentration and application time on the aqueous MMC concentration and apoptosis in the corneal stroma.

Methods: New Zealand white rabbits underwent mechanical epithelium debridement of the central 7.5 mm of the cornea. A sponge soaked in MMC solution was placed on the denuded corneal stroma. The effect of the exposure times ranging from 15 to 120 seconds and the different MMC concentrations ranging from 0.005% to 0.04% on the aqueous MMC concentration and the apoptosis in the stromal cells were evaluated.

Results: The aqueous concentration of MMC increased linearly with increasing exposure time and MMC concentration. The correlation between the aqueous MMC concentration and the applied concentration ($r = 0.809$, $P < 0.001$) was higher than the correlation between the aqueous MMC concentration and the application time ($r = 0.693$, $P < 0.001$). Terminal deoxyribonucleotidyltransferase-mediated dUTP-digoxigenin nick end labeling (TUNEL)–positive cells were detected in the superficial stroma of the central denuded cornea. The numbers of TUNEL-positive cells increased linearly with increasing concentrations, and the increase was statistically significant ($P = 0.026$). However, the numbers of TUNEL-positive cells increased only slightly with an increasing application time, and the increase was not statistically significant ($P = 0.928$).

Conclusions: Reducing the applied concentration or decreasing the exposure time might be a good modality for reducing the potential MMC toxicity. The applied MMC concentration had greater effects on the aqueous MMC concentration and apoptosis in the stromal cells than the exposure time.

Key Words: apoptosis, aqueous concentration, high-performance liquid chromatography, mitomycin C, terminal deoxyribonucleotidyltransferase-mediated dUTP-digoxigenin nick end labeling stain

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Mitomycin-C (MMC) is a potential modulator of wound healing after photorefractive keratectomy (PRK) in experimental models.[1–3] The clinically successful results of using topical MMC for treating eyes with subepithelial fibrosis after PRK or radial keratotomy and for preventing corneal haze after PRK in high myopia have recently been reported.[4,5] A prophylactic MMC treatment is gaining popularity as a method of reducing the clinically significant haze after surface laser ablation.

The mechanism for the action of MMC in the cornea is controversial. The inhibition of keratocyte proliferation after PRK was originally believed to be the main mechanism of MMC because MMC was reported to have an antiproliferative effect on cultured human keratocytes.[6] However, because MMC was recently reported to cause the apoptosis of cultured human keratocytes,[7,8] the apoptosis of keratocytes is now considered the real mechanism responsible for the effects of MMC.[9]

There are no clinical reports that show a decrease in the endothelial cell density in normal human corneas after a topical treatment of MMC combined with surface laser ablation. However, a case of permanent corneal edema resulting from endothelial cell loss after a topical MMC treatment was recently reported in a patient with basement membrane dystrophy.[10] A decrease in endothelial cell density and early corneal edema after the topical application of MMC was reported in a rabbit model,[11,12] and early apoptotic endothelial cells were observed.[11]

This study investigated the aqueous and corneal pharmacokinetics of MMC after a topical application, in an

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TABLE 1. MMC Concentrations in the Corneal Tissue and Aqueous Humor After Topical Administration (mean ± standard deviation) (n = 4, each time interval)

Interval (hour)	Corneal Concentration (μg/g)	Aqueous Concentration (μg/mL)
0.5	3.728 ± 2.547	0.199 ± 0.067
1	0.756 ± 0.437	0.380 ± 0.038
2	0.049 ± 0.012	0.024 ± 0.010
3	0.038 ± 0.001	0.017 ± 0.010

TABLE 2. Aqueous Concentrations of MMC at Different Exposure Times (mean ± standard deviation) (n = 10, each exposure time)

Exposure Time (second)	Aqueous Concentration (μg/mL)
15	0.062 ± 0.049
30	0.093 ± 0.042
60	0.135 ± 0.084
120	0.220 ± 0.075

attempt to estimate the amount of MMC that reached the endothelial cell layer. In addition, we evaluated the effects of the applied MMC concentration and the application time on the aqueous concentration of MMC and the apoptosis in corneal stromal cells.

MATERIALS AND METHODS

Materials

New Zealand white rabbits were used in this study. The use of the rabbits conformed to the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research. The MMC powder (Kyowa, Tokyo, Japan) was dissolved in saline to produce the different concentrations, and all of the MMC solutions were prepared immediately before each experiment.

Experiment 1: Aqueous and Corneal Pharmacokinetics of MMC

The rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (40 mg/kg of body weight), and the corneal epithelium of the central area, which was 7.5 mm in diameter, was abraded manually with a golf-club knife. A 6-mm-diameter sponge soaked in a 0.02% MMC solution was placed on the denuded stromal surface for 2 minutes. After removing the sponge, we irrigated the eyes with 30 mL of saline. Approximately 0.1 mL of the aqueous fluid was aspirated from the anterior chamber by using a 1-mL syringe, and the central cornea was taken immediately using a 7-mm vacuum trephine (Katena Products, Denville, NJ) at 0.5, 1, 2, and 3 hours after administration of the drug. Four eyes were assigned to each time. The wet weight of the corneal button was measured with an electronic weighing machine, and the samples were stored at –80°C until analysis.

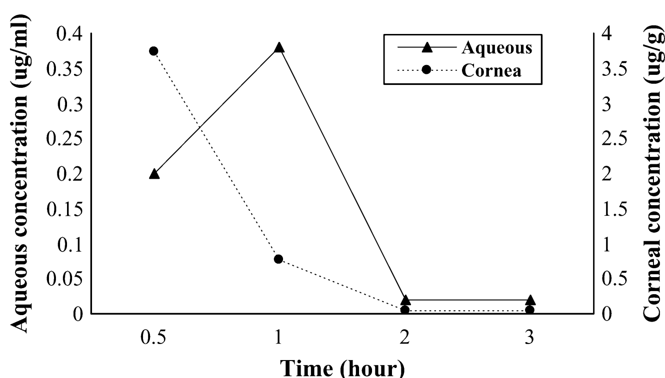

FIGURE 1. MMC concentrations in the aqueous humor and corneal tissue at each time interval after topical administration.

Experiment 2: Effects of MMC Application Time and Applied Concentration

The effects of different application times on the aqueous concentration of MMC were evaluated by placing a sponge soaked in 0.02% MMC solution on the central bare stromal surface for 15, 30, 60, and 120 seconds. Ten eyes were assigned to each application time. The rest of the procedure was the same as that for Experiment 1. Aqueous fluid was aspirated at the time of the maximum aqueous concentration that had been determined from Experiment 1. The impact of the different applied concentrations on the aqueous MMC concentrations was examined by soaking the sponges in 1 of 4 MMC solutions (0.005%, 0.01%, 0.02%, and 0.04%) and placing them on the bare surface of the cornea for 2 minutes. The aqueous fluid was also aspirated at the time of the peak aqueous concentration, and 10 eyes were assigned to each concentration.

The effects of the MMC application time and the applied concentration on apoptosis in the stromal cells were also evaluated by terminal deoxyribonucleotidyltransferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay for the same 4 different application times and 4 different concentrations. In the preliminary study, TUNEL staining was performed at 4 and 24 hours after the application of the MMC on the central bare stromal surface. There were more apoptotic cells in the stroma 4 hours after treatment than there were 24 hours after treatment. Mohan et al[13] also reported that TUNEL-positive cells were the most numerous at 4 hours after PRK. Therefore, corneal specimens were obtained at 4 hours for the TUNEL assay.

High-Performance Liquid Chromatography

The rabbit corneal button was homogenized in 4 mL of acetonitrile with a hand grinder. The mixture was ultra-sonicated for 30 minutes, and the extract was recovered by centrifugation at 3000*g* for 10 minutes. The aqueous solvents were evaporated completely at 40°C under nitrogen gas, and the MMC was resolved using 150 μL of a 0.02 M phosphate buffer. One hundred microliters of the sample was injected into the high-performance liquid chromatograph (HPLC). For the aqueous humor, 3 mL of ethyl acetate was added, and the MMC was extracted from the 2 aqueous phases by vortexing and centrifugation. The upper phase was recovered and dried

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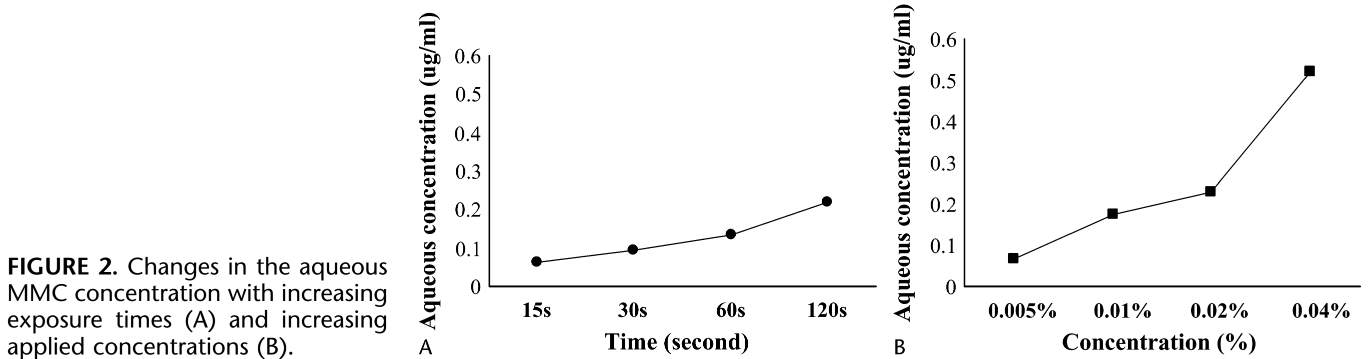

FIGURE 2. Changes in the aqueous MMC concentration with increasing exposure times (A) and increasing applied concentrations (B).

completely at 40°C under nitrogen gas. The MMC was resolved using 150 μL of a 0.02 M phosphate buffer, and 100 μL of the sample was injected into the HPLC. Two gradient mobile systems were used for analysis of MMC: (1) 10% acetonitrile in 20 mM phosphate buffer (pH 7) and (2) 60% acetonitrile in 10 mM phosphate buffer (pH 7). The gradient time program was as follows: 0~1 minutes, 100% mobile in phase 1; 1~6.5 minutes, 40% mobile in phase 1 and 60% mobile in phase 2; and 6.5~9 minutes, 100% mobile in phase 2. The flow rate of the mobile phase was 0.7 mL/min at 30°C, and injection volume at each time was 100 μL. The liquid chromatographic system consisted of a Hewlett-Packard system (1100 model) equipped with an autosampler and quaternary pumping unit and Hewlett-Packard model DAD G1315A programmable UV detector (Agilent Technologies, Palo Alto, CA). Separation was carried out using a 150 × 4.6-mm ID, 5-μm Zorbax eclipse XDB-C18 column (Agilent Technologies). UV wavelength detector was set at 360 nm. The minimal level of detection was 0.001 μg/mL.

Tissue Processing and TUNEL Assay

The rabbits were anesthetized with intramuscular injection of ketamine hydrochloride and were killed by intracardiac injection of 2% lidocaine hydrochloride at 4 hours after administering the drug. The eyes were enucleated, and the posterior one third of the eyes was excised. The lenses were removed through a posterior opening to facilitate frozen sectioning. The eyes were frozen immediately in optimum cutting temperature (OCT) compound (Tissue-Tek; Miles Laboratories, Elkhart, IN) by liquid nitrogen. The frozen tissue blocks were stored at −85°C until they were sectioned.

Central corneal sections (6 μm thick) were cut with a cryostat at −20°C and were placed on silanized microscope slides (Dako, Carpinteria, CA). Some tissue sections were stained with hematoxylin and eosin for histologic observation, and the others were fixed in cold acetone at −20°C for 2 minutes for TUNEL stain. A peroxidase-based TUNEL assay was used according to the manufacturer's instructions (Apoptag, Cat No. S7101; Chemicon International, Temecula, CA). Positive (4-hour mechanical corneal scraping) and negative (unwounded) control slides were included in each assay.

TABLE 3. Aqueous Concentrations of MMC at Different Applied Concentrations (mean ± standard deviation) (n = 10, each applied concentration)

Applied Concentration (%)	Aqueous Concentration (μg/mL)
0.005	0.068 ± 0.028
0.01	0.177 ± 0.041
0.02	0.228 ± 0.074
0.04	0.519 ± 0.229

Cell Counting for Quantitation of TUNEL Assays

Four corneas were evaluated for each concentration and for each application time. The total number of TUNEL-positive cells in 1 microscopic field under high magnification (×400) was counted in each specimen for quantitative comparison.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) software (version 10.1; SPSS, Chicago, IL) was used for the statistical analysis. The ANOVA test was used to compare the mean aqueous MMC concentrations at each exposure time and each concentration. For comparison of the mean number of TUNEL-positive cells, a nonparametric Kruskal–Wallis test was used, because samples were not enough for parametric test. The correlations between the aqueous MMC concentration and the application time and between the aqueous MMC concentration and the applied concentrations were assessed using Pearson correlation analysis. The Spearman rank correlation was performed to evaluate the correlations between the number of TUNEL-positive cells and the MMC application time and between the number of TUNEL-positive cells and the MMC applied concentration. $P < 0.05$ was considered statistically significant.

RESULTS

Experiment 1: Aqueous and Corneal Pharmacokinetics of MMC

The mean concentrations of MMC at different times after topical administration are shown in Table 1 and Figure 1. The peak corneal concentration of the MMC was 3.728 ± 2.547 μg/g at 30 minutes after its administration. The corneal concentration decreased to 0.756 ± 0.437 μg/g at 1 hour after its administration. However, the aqueous concentration

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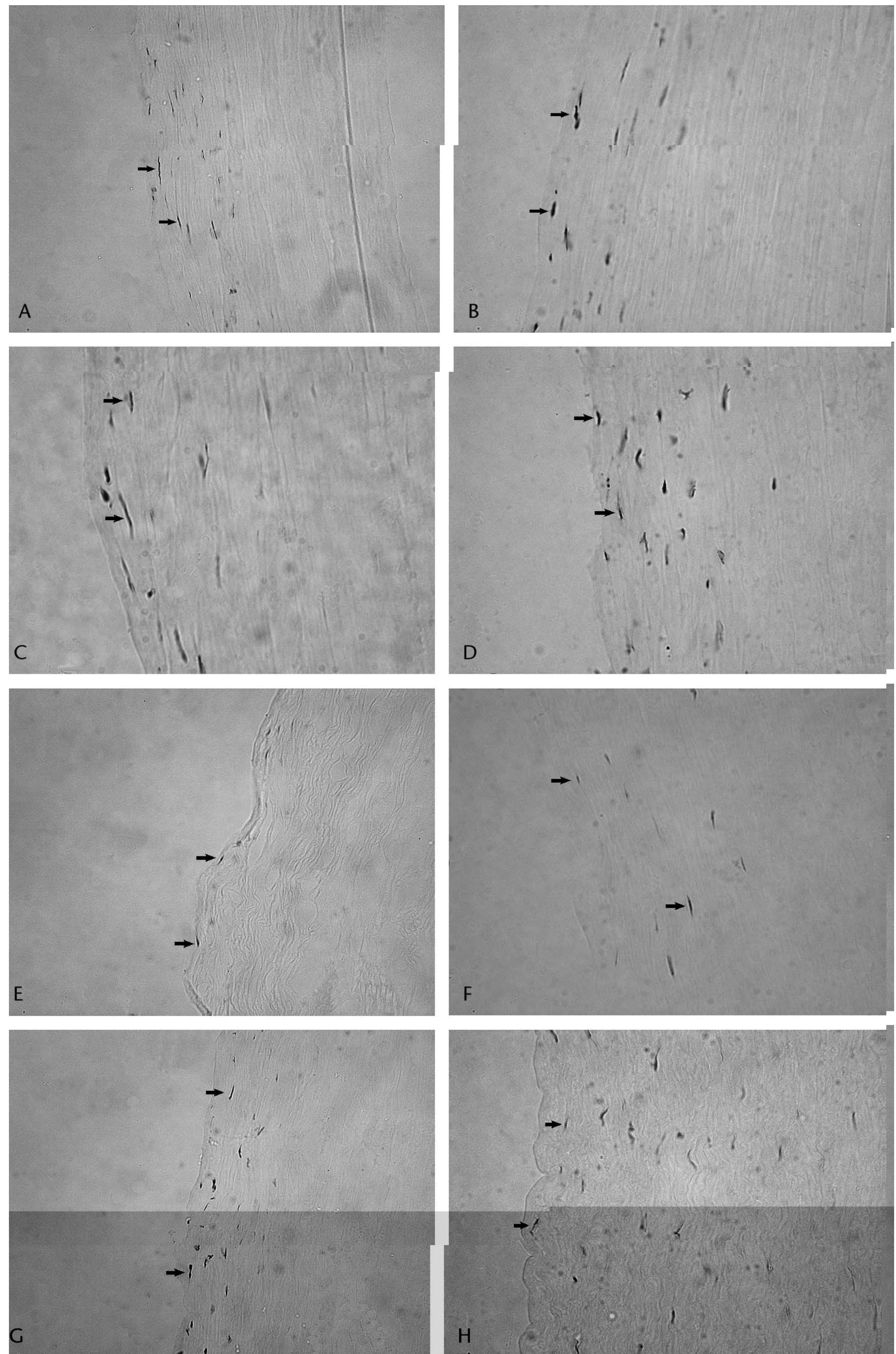

FIGURE 3. TUNEL-positive cells (arrows) at each application time (A–D) and at each applied concentration (E–H). The 0.02% solution of MMC was applied on the corneal surfaces for 4 different exposure times: 15 seconds (A), 30 seconds (B), 1 minute (C), and 2 minutes (D). A sponge soaked in 1 of the 4 MMC solutions was placed on the bare stroma for 2 minutes: 0.005% (E), 0.01% (F), 0.02% (G), and 0.04% (H).

of the MMC peaked at 1 hour after topical application rather than at 30 minutes. The mean aqueous concentration of the MMC was 0.199 ± 0.067 μg/mL at 30 minutes and 0.380 ± 0.038 μg/mL at 1 hour. Although the concentrations of the MMC were minimal, it remained in the aqueous humor and the corneal tissue for 3 hours after topical administration.

Experiment 2: Effects of MMC Application Time and Applied Concentrations

The aqueous humor was aspirated 1 hour after the topical application in Experiment 2, based on the results of Experiment 1. The mean aqueous concentrations of MMC at the different application times are shown in Table 2. The aqueous concentrations of MMC increased linearly with increasing

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exposure times (Fig. 2A), and the changes in the concentrations at the different exposure times were statistically significant ($P < 0.001$). Table 3 shows the mean aqueous concentrations of MMC at different applied concentrations. The aqueous concentration increased linearly with increasing applied concentrations (Fig. 2B), which were also statistically significant ($P < 0.001$). The correlation between the aqueous MMC concentration and the applied concentration ($r = 0.809$, $P < 0.001$) was greater than the correlation between the aqueous MMC concentration and the application time ($r = 0.693$, $P < 0.001$), even though both of them were statistically significant.

TUNEL-positive cells were detected in the superficial stroma of the central denuded cornea at 4 hours after administering the MMC (Fig. 3). Table 4 shows the mean number of TUNEL-positive cells at each application time and at each applied concentration. Although the number of the TUNEL-positive cells increased slightly with increasing application time (Fig. 4A), it was not statistically significant ($P = 0.928$). However, the numbers of TUNEL-positive cells increased linearly with increasing applied concentrations (Fig. 4B), which were statistically significant ($P = 0.026$). In the correlation analysis using Spearman rank correlation, the correlation between the numbers of TUNEL-positive cells and the applied MMC concentration was statistically significant ($r = 0.784$, $P < 0.001$). However, the correlation between the numbers of TUNEL-positive cells and the application time was not significant ($r = 0.091$, $P = 0.737$).

DISCUSSION

Since some cases of iatrogenic keratectasia after laser in situ keratomileusis (LASIK) were reported,[14–17] more attention has been paid to the residual corneal thickness, and there is a renewed interest in surface laser ablation procedures, such as PRK, laser subepithelial keratomileusis (LASEK), and Epi-LASIK to increase the remaining corneal thickness. However, haze formation is still a major complication of surface laser ablation, particularly in cases of high myopia. Many studies have been performed to identify the most effective prophylactic treatments.

Topical MMC was first used to treat human corneas with subepithelial fibrosis after PRK or radial keratomy.[4] It was suggested that the topical application of MMC might prevent the recurrence of subepithelial fibrosis. Carones et al[5] clinically evaluated the prophylactic effect of MMC on the formation of haze after PRK and reported that the prophylactic use of MMC resulted in lower haze rates, better uncorrected and best-corrected visual acuity, and a more accurate refractive outcome than those achieved without MMC treatment. A confocal microscopic study recently showed that activated keratocytes and extracellular matrix were more evident in untreated eyes than in MMC-treated eyes.[18]

Although MMC was originally used as a systemic chemotherapeutic agent, topical MMC has been used for ophthalmic indications with increasing frequency. Topical MMC has become popular in glaucoma filtering surgery,[19] pterygium surgery,[20,21] and in the treatment of conjunctival and corneal intraepithelial neoplasia.[22,23] However, serious ocular complications have been reported after the use of MMC in pterygium surgery, including corneal edema, corneal perforation, and scleral melting.[24,25]

Although there are no clinical reports of corneal complications after prophylactic MMC use in combination with surface laser ablation, a few experimental studies reported early corneal edema in rabbit eyes after topical MMC application.[11,12] Early apoptotic changes in endothelial cells of rabbit cornea were also shown.[11] Humans do not have the same type of corneas as rabbits. The rabbit cornea does not contain a Bowman membrane, and it is thinner than a human cornea. Furthermore, the corneal endothelium of rabbits is capable of mitosis.[11] Therefore, the effect of MMC on the human cornea might be different. However, with the exception of the mitotic capacity of the endothelium, the rabbit cornea is similar to a markedly ablated human cornea in that there is no Bowman membrane and the corneal thickness is similar.[11] Therefore, clinicians need to be alert to corneal changes that can occur after the topical application of MMC in high myopic patients.

To the best of our knowledge, this is the first study to investigate the aqueous and corneal pharmacokinetics of MMC in rabbit eyes after topical application to the central bare cornea. This topical application is the same method used clinically to prevent corneal haze after surface laser ablation. The maximal corneal concentration of MMC was achieved 30 minutes after its topical administration and the aqueous concentration peaked at 1 hour. Although the concentration was minimal, MMC remained in the aqueous humor and corneal tissue for at least 3 hours after topical administration. There are a few reports on the MMC concentration in ocular tissues after topical application.[26–29] However, the methods for the administration of MMC were different from those used in this study. Sarraf et al[26] applied a cellulose sponge soaked in a 0.04% MMC solution on the conjunctival bleb for 5 minutes to study the aqueous concentration of MMC in rabbit eyes that had undergone filtering surgery. Nevertheless, the peak aqueous concentration was achieved 1 hour after administration, which was identical to the results of this study. However, despite the higher MMC concentration and longer application time, the mean aqueous concentration was lower than it was in this study. The penetration of MMC through the central bare cornea appeared to be more effective than through the conjunctiva and sclera, and this application method might carry a higher risk of endothelial cell damage. Recently, Torres et al[30] showed the presence of MMC in aqueous humor of hen eyes after topical MMC application on the central cornea.

TABLE 4. The Number of TUNEL-Positive Cells at Each Application Time and Each Applied Concentration (mean ± standard deviation) (n = 4, each exposure time and each applied concentration)

Exposure Time (second)	No. TUNEL-Positive Cells	Applied Concentration (%)	No. TUNEL-Positive Cells
15	27.50 ± 13.60	0.005	13.25 ± 6.75
30	26.75 ± 10.21	0.01	19.75 ± 5.25
60	28.75 ± 5.38	0.02	31.75 ± 13.40
120	31.75 ± 13.40	0.04	41.75 ± 17.93

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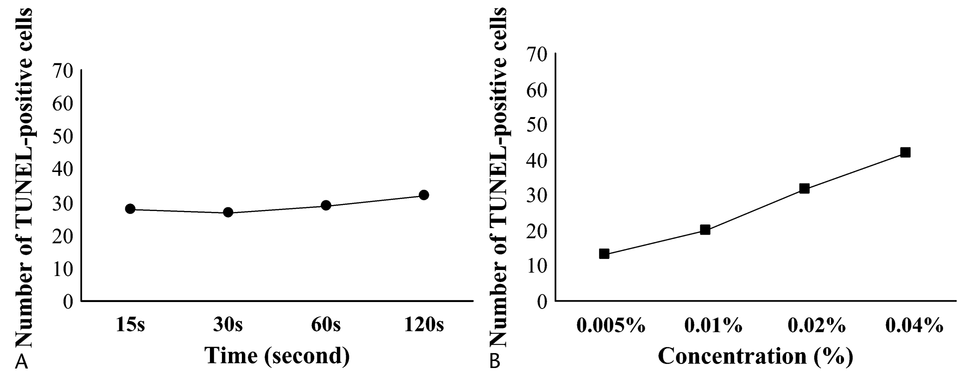

FIGURE 4. Changes in the mean number of TUNEL-positive cells in the stroma with increasing exposure times (A) and increasing applied concentrations (B).

Hen eyes have not been used popularly in the animal models of corneal refractive surgery. Although the authors did not explain the reason they had used hens as an animal model, there seems to be a difference in the aqueous pharmacokinetics of MMC between rabbits and hens, because maximal aqueous concentration of MMC was obtained at 10 minutes in hens but at 60 minutes in rabbits.

The current MMC treatment of preventing corneal haze is performed by placing a 0.02% MMC–soaked sponge over the ablated cornea for 2 minutes.[5] However, there has been a tendency to use a lower MMC concentration for a shorter exposure time to reduce the potential MMC toxicity. Camellin[31] used 0.01% MMC on LASEK-treated corneas and reported success in decreasing the extent of subepithelial haze. Seldomridge et al[32] suggested a 12-second application of 0.02% MMC to prevent corneal haze after PRK. Netto et al[33] showed the prevention of corneal haze in rabbit eyes with the use of 0.002% MMC and exposure times from 12 seconds to 2 minutes. However, 0.002% MMC was not as effective in treating existing corneal haze. In this study, the aqueous MMC concentration decreased linearly with decreasing applied concentrations and application times. Reducing the applied concentrations or exposure times seems to be an effective modality for decreasing the potential endothelial toxicity. However, from the results, the applied MMC concentrations had a higher correlation with the aqueous concentration than did the exposure times.

Keratocyte apoptosis is a controlled form of cell death that normally occurs without significant release of lysosomal enzymes or other intracellular components that could damage the surrounding tissues or cells.[34] This keratocyte apoptosis is hypothesized to be an initiating event in the wound healing process after PRK[35] and most commonly detected with the TUNEL assay.[13] Activated keratocytes replenish the anterior corneal stroma after keratocyte apoptosis, and they are associated with increased collagen deposition and collagen disorganization correlating with corneal haze and regression after PRK.[36,37] Recently, MMC was reported to induce keratocyte apoptosis in both in vitro and in vivo studies.[7,12] Although corneal epithelial injury and corneal operations, such as PRK and LASIK, can also cause keratocyte apoptosis,[38,39] additional MMC application induces more apoptosis in keratocytes.[12] In this study, TUNEL-positive cells were detected in the superficial stroma of the central denuded cornea after the administration of MMC. The number of TUNEL-positive cells increased linearly with increasing applied concentrations of MMC, and the correlation between the number of TUNEL-positive cells and the applied MMC concentration was statistically significant. However, although the number of TUNEL-positive cells increased slightly with increasing application times, the correlation was not significant. Therefore, lower aqueous MMC concentrations and less apoptosis in keratocytes could be obtained by reducing the topically applied MMC concentration, and lower aqueous concentrations and similar apoptosis could be obtained by reducing the exposure times. If the main mechanism of MMC that prevents the corneal haze after PRK is the induction of keratocyte apoptosis, reducing the exposure time without a change in the concentration seems to be an effective modality for reducing the potential endothelial toxicity while maintaining its preventive effectiveness. Further studies are needed to determine the ideal combination of concentration and application time for the prevention of corneal haze and to minimize the toxicity to the corneal tissue.

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