Mitomycin C Reduces Haze Formation in Rabbits After Excimer Laser Photorefractive Keratectomy

Huizhuo Xu, MD; Shuangzhen Liu, MD; Xiaobo Xia, MD; Peigang Huang, MD; Pingbao Wang, MD; Xiaoyin Wu, MD

ABSTRACT

PURPOSE: To investigate the effects of mitomycin C on haze after photorefractive keratectomy (PRK).

METHODS: Twenty of 24 rabbits underwent bilateral 193-nm excimer laser PRK to correct -10.00 D of myopia; the remaining four rabbits were not operated (no PRK group). The right eyes of the 20 rabbits were treated with 0.02% mitomycin C during surgery (PRK+MMC group) and the left eyes did not receive 0.02% mitomycin C (PRK alone group). Clinical and histopathologic examinations were performed.

RESULTS: The most severe haze in the PRK alone group after PRK reached grade 3; the PRK+MMC group did not exceed grade 1 haze. Statistically significant differences were found between the PRK+MMC and PRK alone groups from week 2 to week 26 after treatment (P<.01). Epithelial thickening appeared for 26 weeks in both PRK groups; no statistically significant differences were found between the two PRK groups (P>.05). A marked reduction of keratocytes in the anterior stroma of the PRK+MMC group was observed. At week 1, 2, and 4 after PRK, keratocytes of the PRK+MMC group were only 3.1±2.6, 6.8±4.7, and 12.4±5.7 keratocytes x 10^4/μm^2, respectively, while those of the PRK alone group were 41.2±80.4, 32.7±7.8, and 40.2±3.3 keratocytes x 10^4/μm^2, respectively. There were statistically significant differences between the two groups (P<.001).

CONCLUSION: A single intraoperative application of topical mitomycin C during PRK in rabbits reduced corneal haze by inhibiting the proliferation of keratocytes. [J Refract Surg 2001;17: 342-349]

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Photoactive keratectomy (PRK) with the 193-nm excimer laser is a promising innovation in refractive surgery. However, the routine clinical application of PRK is hindered by two obstacles—corneal haze and possible regression of the refractive result. In wound healing after PRK, keratocytes in the corneal stroma are activated to proliferate. The collagen tissue produced by these keratocytes is much less organized than normal stromal tissue, shows matrix-free areas and fibers with an irregular stereo-spatial relationship, and is thought to be the cause of corneal haze formation.1 Following PRK, corticosteroids are the most common prophylactic treatment against excessive haze formation and regression. Considering the possible severe side-effects of long-term corticosteroid treatment, it is desirable to find an alternative to prevent haze and regression after PRK. Mitomycin C, a highly potent alkylating agent with antineoplastic and antibiotic activities2, was found to suppress keratocyte proliferation by inhibiting DNA synthesis secondary to alkylation.3-5 This effect is already successfully used and clinically well studied in filtering glaucoma surgery6,7, in which it prevents excessive scar formation of the filtration bleb. Mitomycin C is also effective in preventing recurrence of pterygium8,9 and treating corneal intraepithelial neoplasia.10 However, corneal complications with these procedures have been described by several authors.10-12

The purpose of this study was to determine whether mitomycin C might be an inhibitor for haze formation. A sponge soaked in mitomycin C solution was left on the laser-treated rabbit cornea for 5 minutes. We found that mitomycin C reduced haze formation after PRK in rabbits.

MATERIALS AND METHODS

The study used 24 New Zealand white rabbits weighing between 2 and 2.5 kg; 20 were anesthetized before surgery with intramuscular
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injections of ketamine hydrochloride (30 mg/kg of body weight). All eyes were examined to evaluate corneal clarity before surgery. The remaining four rabbits were not operated (no PRK group).

The VISX 20/20 excimer laser system (VISX Inc., Santa Clara, CA) was used. Fluence was set at 160 mJ/cm², and the ablation rate was 5 Hz. After laser removal of the epithelium, deep keratectomy of 10.00 diopters (D) (120 μm at the deepest point, 6 mm in diameter) was performed on the right eyes of 20 rabbits. Immediately after ablation, a sponge (6-mm diameter) soaked in mitomycin C solution (0.02%) was placed on the laser-treated area of the rabbit cornea for 5 minutes. After removal of the sponge, the eye was irrigated with 20 ml sterile 0.9% saline solution. The left eyes (PRK only group) were operated in the same way but were not treated with mitomycin C. Eye drops (0.3% Norfloxacin) and tetracycline 0.5% ointment were instilled directly after the procedure and three times every day for 1 week.

The eyes were examined daily using the slit lamp until re-epithelialization was complete, usually within 1 week. All eyes were examined at week 1, 2, 4, 12, and 26 after treatment. Corneal haze was defined according to the haze grade of Fantes and colleagues: grade 0.5 indicates trace haze, seen with careful oblique illumination with slit-lamp biomicroscopy; grade 1, more prominent haze that does not interfere with visibility of fine iris details; grade 2, mild obscuration of iris details; grade 3, moderate obscuration of the iris and lens; and grade 4, completely opaque stroma in the area of ablation.

Eyes were enucleated under general anesthesia at week 1, 2, 4, 12, and 26 after PRK. The animals were killed by intravenous injection of 10 ml air. The corneas were excised with a 9-mm trephine and prepared for light and electron microscopy. For light microscopy, tissues were fixed in 4% formalin and processed by routine methods for paraffin sections and hematoxylin and eosin stain. For electron microscopy, the tissues were fixed in 2.5% glutaraldehyde and processed for embedding. Ultrathin sections were contrast-stained with uranyl acetate and lead citrate, then examined with a Hitachi H-600 electron microscope.

Statistical comparisons of corneal haze between the mitomycin C group (PRK+MMC) and the PRK alone group were performed using a rank sum test. The eyepiece micrometer was used to check the thickness of the corneal epithelium and to count the keratocytes in the anterior stroma of the ablation area. Five tissue slices of each specimen were examined and an average was calculated. The Newman-Keuls test was used for statistical comparisons between the PRK+MMC and the PRK alone/no PRK groups.

RESULTS

Clinical Course

All eyes re-epithelialized completely within 5 to 7 days after PRK, although some fluorescein staining was observed during the entire experiment in both groups. A faint subepithelial haze appeared in the area of ablation within the first week. The most severe haze in the PRK alone group reached grade 3; haze in the PRK+MMC group did not exceed grade 1. The haze in the PRK alone group peaked at the fourth week and decreased thereafter. Haze in the PRK+MMC group peaked at the second week (Table 1). There was no statistically significant difference in corneal haze between groups at the first week after treatment (P>.05), but significant differences were found between groups from week 2 to 26 after PRK (P<.01).

Histopathological Findings

Mild to moderate epithelial irregularity and thickening were found at the ablation area in both groups under light microscopy. Epithelial thickening had appeared for 26 weeks after PRK in both groups. As shown in Table 2, the thickness of the normal corneal epithelium was 22.08±1.09 μm. The corneal epithelium of the PRK+MMC group proliferated to a mean of 45.21±5.11 μm at the first week, reached 50.17±5.15 μm at the fourth week, and thereafter decreased to 29.97±3.16 μm at week 26 after PRK. Similar changes were observed in the PRK alone group. The mean thickness of the

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Table 1

<table>
<thead>
<tr>
<th>Haze Grade</th>
<th>PRK + Mitomycin C (weeks)</th>
<th>PRK Alone (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1 1 1</td>
<td>1 1 1</td>
</tr>
<tr>
<td>0.5</td>
<td>19 12 10 7 2 14 3 2 1</td>
<td>1 6 9 4 5 2 3 2 2</td>
</tr>
<tr>
<td>1</td>
<td>3 5 2 3</td>
<td></td>
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<tr>
<td>2</td>
<td>1</td>
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</table>

The haze in the PRK alone group showed a peak at week 4 after PRK; the mitomycin C group peaked at the second week. There was no statistically significant difference between groups at the first week after PRK (P>.05), but significant differences were found between groups from week 2 to week 26 after PRK (P<.01).
Table 2

Corneal Epithelial Thickness (μm, ±SE*) of Rabbit Eyes With and Without Mitomycin C After PRK

<table>
<thead>
<tr>
<th>Time After PRK</th>
<th>No PRK Group</th>
<th>Mitomycin C Group (PRK+MMC)</th>
<th>PRK Alone Group</th>
<th>Group-to-Group Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PRK+MMC</td>
<td>PRK Alone</td>
<td>No PRK: PRK+MMC</td>
</tr>
<tr>
<td>22.08 ± 1.09</td>
<td>45.21 ± 5.11</td>
<td>46.49 ± 5.56</td>
<td>P&lt;.001</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>1 week</td>
<td>49.17 ± 4.30</td>
<td>50.28 ± 4.58</td>
<td>P&lt;.001</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>2 weeks</td>
<td>50.17 ± 5.15</td>
<td>51.06 ± 4.75</td>
<td>P&lt;.001</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>4 weeks</td>
<td>33.13 ± 4.96</td>
<td>33.15 ± 3.03</td>
<td>P&lt;.001</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>12 weeks</td>
<td>29.97 ± 3.16</td>
<td>30.13 ± 3.12</td>
<td>P&lt;.001</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>26 weeks</td>
<td></td>
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</tbody>
</table>

*SE = standard error
The corneal epithelium of both the MMC+PRK and PRK alone groups increased after PRK. Using the Newman–Keuls test, there were significant differences between the no PRK group and the MMC+PRK and PRK alone groups (P<.001), but no statistically significant differences were found between the MMC+PRK group and the PRK alone group (P>.05).

Table 3

Number of Keratocytes (keratocytes x 10⁴/μm² ± SE*) in Rabbit Eyes With and Without Mitomycin C After PRK

<table>
<thead>
<tr>
<th>Time After PRK</th>
<th>No PRK Group</th>
<th>Mitomycin C Group (PRK+MMC)</th>
<th>PRK Alone Group</th>
<th>Group-to-Group Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PRK+MMC</td>
<td>PRK Alone</td>
<td>No PRK: PRK+MMC</td>
</tr>
<tr>
<td>16.3 ± 3.1</td>
<td>3.1 ± 2.6</td>
<td>41.2 ± 8.0</td>
<td>P&lt;.001</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>1 week</td>
<td>6.8 ± 4.7</td>
<td>42.3 ± 7.8</td>
<td>P&lt;.001</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>2 weeks</td>
<td>12.4 ± 5.7</td>
<td>40.0 ± 3.3</td>
<td>P&lt;.01</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>4 weeks</td>
<td>14.8 ± 3.3</td>
<td>17.6 ± 3.8</td>
<td>P&gt;-.05</td>
<td>P&gt;-.05</td>
</tr>
<tr>
<td>12 weeks</td>
<td>16.6 ± 2.7</td>
<td>16.5 ± 3.1</td>
<td>P&gt;-.05</td>
<td>P&gt;-.05</td>
</tr>
<tr>
<td>26 weeks</td>
<td></td>
<td></td>
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</tbody>
</table>

*SE = standard error
Compared with the PRK alone group, keratocytes in the anterior stroma of the ablation area decreased significantly in all mitomycin-treated corneas at week 1, 2, and 4 after treatment (P<.001). There was no statistically significant difference in keratocyte density between the PRK alone corneas and mitomycin C + PRK corneas at week 12 and 26 after PRK (P>.05). Compared with the no PRK group, keratocytes increased in the anterior stroma of the PRK alone group at week 1, 2, and 4 after PRK (P<.01). At week 12 and 26, the keratocytes in the anterior stroma of the PRK alone group decreased to a normal level.

epithelium in the PRK alone group at week 1 was 46.49±5.56 μm; week 2, 50.28±4.58 μm; week 4, 51.06±4.75 μm; week 12, 33.15±3.03 μm; and week 26, 30.13±3.12 μm. No statistically significant differences were found between the two groups (P>.05).

Keratocyte density in the anterior stroma of the normal cornea was 16.3±3.1 keratocytes x 10⁴/μm². At the first week after PRK, keratocyte density in the anterior stroma of the PRK+MMC group was only 3.1±2.6 keratocytes x 10⁴/μm² (Table 3). Over time, the keratocytes proliferated and the number of keratocytes in the anterior stroma of the PRK+MMC group increased. At weeks 1, 2, and 4 after PRK, the number of keratocytes was still significantly lower than that of the no PRK group (P<.01). There was no statistically significant difference in keratocyte density between the PRK alone and MMC+PRK groups at week 12 or 26 (P>.05). In the PRK alone group, keratocytes occurred in the anterior stroma at the first week. The number of activated keratocytes in the anterior stroma reached a maximum at week 2 (42.3±7.8 keratocytes x 10⁴/μm²) and then decreased. At week 12 after PRK, the keratocytes in the anterior stroma of the PRK alone group recovered to a normal level (17.6±3.8 keratocytes x 10⁴/μm²) (Fig 1). Both the posterior stroma and the endothelia were normal in all specimens.

Under electron microscopy the arrangement of lamellae in the anterior stroma of the PRK alone group was wavier than that of the MMC+PRK group. The arrangement of lamellae in the anterior stroma of both groups was normal at week 12 after PRK (Fig 2). In both groups, Descemet's membrane appeared as a thin basophilic layer of electron-dense material posterior to the area of ablation. This line was found more anteriorly with the passage of time after surgery.
DISCUSSION

The cornea forms a part of the tough outer protective coating of the eye and it is also the primary refractive component. Transparency is generally believed to depend on a restriction in the cross-sectional diameter of the collagen fibrils and regularity in the packing of the collagen fibrils, which produce destructive interference of scattered incident light.\textsuperscript{13,14} Corneal haze is believed to be closely related to corneal wound healing after PRK. Two or three weeks after PRK, keratocytes were activated to proliferate and migrated into the existing normal structure in numbers many times that of normal keratocytes.\textsuperscript{1} Vacuoles, abnormal large proteoglycan filaments, amorphous material, and newly synthesized collagen have been found in rabbit corneas after PRK.\textsuperscript{15} The collagen tissue produced by these keratocytes was much less organized than normal stromal tissue. The results showed that corneal transparency decreased. After approximately...
12 weeks of intense activity, with keratocytes decreased and less new extracellular matrix formed, the stromal structure became more normal, and the corneal haze diminished simultaneously. Corticosteroids have been used to inhibit haze formation by clinicians after PRK.\textsuperscript{16,17} The main mechanism may be that corticosteroids can inhibit collagen synthesis. However, long-term corticosteroid treatment will cause corticosteroid glaucoma. The side-effects of corticosteroids prompted clinicians to search for another substance to inhibit haze formation induced by PRK. Mitomycin C is an antibiotic-antineoplastic agent that selectively inhibits the synthesis of DNA, RNA, and protein.\textsuperscript{18} In the latest decade, a single intraoperative dose of mitomycin C has been used in glaucoma-filtering procedures\textsuperscript{19} and pterygium surgery.\textsuperscript{8,9} Mitomycin C was chosen for our study because of its fast-acting and long-lasting suppression of keratocyte activity after only a single application. Although topical application of mitomycin C after operations may cause ocular injection, ocular pain, punctate keratopathy, scleromalacia, corneal perforation, glaucoma, and iritis, most of these occurrences are related to higher concentrations of mitomycin C and mainly to the uncontrolled prolonged use of the drugs by patients.\textsuperscript{20,21} Advantages of a single intraoperative use of mitomycin C are the lack of compliance problems and minimal risk of side-effects.

Mitomycin C can inhibit various cell proliferation. Yamamoto and colleagues found that mitomycin C 0.001%, 0.01%, and 0.1% could suppress proliferation of keratocytes in vivo, and the effect increased as the concentration of mitomycin C increased.\textsuperscript{22} In our study, there were fewer keratocytes in the anterior stroma of the mitomycin C group than in the PRK alone group within 4 weeks after PRK. This indicated that a single intraoperative dose of 0.02% mitomycin C could effectively inhibit keratocyte proliferation. Ando and colleagues found that mitomycin C lower than 0.04% had no effect on corneal epithelial growth.\textsuperscript{11} Manning\textsuperscript{8} and Frucht-Pery\textsuperscript{9}, who used mitomycin C 0.04% and mitomycin C 0.02% in pterygium surgery, found no corneal epithelial delayed healing. In our study, both the PRK+MMC group and the PRK alone group re-epithelialized completely within 5 to 7 days after PRK, and there was no difference in corneal epithelial thickness between the two groups, indicating that mitomycin C 0.02% had no effect on corneal epithelial healing.

Recently, Majmudar and colleagues\textsuperscript{23} reported results of topical mitomycin C for subepithelial fibrosis after refractive corneal surgery. In eight eyes of five patients who had central subepithelial fibrosis after radial keratotomy or PRK, a no. 64 Beaver blade was used to remove the corneal epithelium and mechanically remove as much of the fibrosis as possible. A sterile, 6-mm circular sponge soaked in mitomycin C 0.02% was applied to the corneal surface for 2 minutes. The sponge was then removed and the ocular surface was irrigated with 30 ml of balanced salt solution. The eye was then covered with antibiotic-corticosteroid ointment. Serial examinations were performed to evaluate corneal clarity and best-corrected visual acuity (BCVA). Corneal clarity and BCVA were improved in each eye. However, corneal haze in the eyes after PRK was not mentioned in this study. It is well known that the more severe the haze, the lower the corneal clarity. Our study showed that corneal haze in the PRK+MMC group was less than that in the
Figure 2. Transmission electron micrographs of PRK alone and PRK+MMC group corneas, ×6000. A) At 1 week after PRK, the arrangement of lamellae (L) in the anterior stroma of the PRK alone group cornea was much wavier than normal. B) At 1 week, the lamellae in the anterior stroma of the PRK+MMC group were arranged more smoothly. C) At week 12, the lamellae in the anterior stroma of the PRK alone group were arranged more smoothly than those at the first week. D) At week 12, the lamellae in the anterior stroma of the PRK+MMC group were arranged normally.
PRK alone group and proliferation of keratocytes was inhibited in the PRK+MMC group. As far as corneal clarity is concerned, the results of our study corresponded well with those of Majmudar.  

Cellular morphologic changes consistent with apoptosis are observable in keratocytes immediately after PRK. The keratocytes that die are thought to be replaced over a period of a few days by proliferation and migration of remaining keratocytes from the posterior and peripheral stroma. The activated keratocytes that replenish the anterior stroma, however, produce increased amounts of disorganized collagen and other components associated with stromal healing. As a result, transparency of the cornea decreases. Because keratocyte apoptosis is the earliest stromal change observable after epithelial injury associated with corneal surgical procedures, it seems reasonable to speculate that it is an initiator of the corneal wound healing response. A signal is transmitted to other keratocytes in the posterior and peripheral stroma through some unknown way. Consequently, the keratocytes are activated to proliferate. Therefore, we thought that if apoptosis of keratocytes is the initial corneal wound healing response, activation and proliferation of keratocytes may be the key to the formation of corneal haze. If so, the inhibition of keratocyte proliferation could result in less corneal haze. As shown in our study, corneal haze in the PRK+MMC group was less than in the PRK alone group, which may have resulted from inhibition of keratocyte proliferation after treatment with mitomycin C. 

In the PRK+MMC group, corneal haze showed a peak at the second week after treatment, although it did not exceed grade 1. We think this faint subepithelial haze may be due to other causes, such as transient anterior stromal edema and corneal epithelial hyperplasia.  

The arrangement of lamellae is regular in the normal corneal stroma; keratocytes are interspersed between the lamellae, which form an interlinking network through the cornea. We found the lamellae in the anterior stroma of the PRK alone group arranged in a wavelike pattern and those in the PRK+MMC group arranged more smoothly. In our opinion, this may be related to the activated keratocytes that migrated into the anterior stroma and disturbed the alignment of the lamellae. 

The first application of topical mitomycin C as a modulator of corneal wound healing after PRK was suggested by Talamo and colleagues. They found that mitomycin C and corticosteroids could inhibit subepithelial collagen synthesis. Schipper and colleagues carried out a similar experiment and also found that the number of keratocytes in the anterior stroma of the mitomycin C group was less than that of the balanced salt solution (BSS) group. However, there are several differences between our findings and those of Schipper and colleagues. They found the corneal epithelium of the mitomycin C group was thinner than that of the BSS group. They also found no difference in haze between the two groups. Additional investigation is needed to explain these differences. 

Our study has shown that a single intraoperative treatment of mitomycin C in rabbit eyes can effectively suppress proliferation of keratocytes and corneal haze formation. Mitomycin C may be a useful drug for preventing corneal haze after PRK.

REFERENCES


