Glaucoma

Effect of Lovastatin on Wound-Healing Modulation After Glaucoma Filtration Surgery in a Rabbit Model

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Submitted: December 22, 2015
Accepted: March 19, 2016
Citation: Park JH, Yoo C, Kim YY. Effect of lovastatin on wound-healing modulation after glaucoma filtration surgery in a rabbit model. Invest Ophthalmol Vis Sci. 2016;57:1871-1877. DOI:10.1167/iovs.15-19003

PURPOSE. To investigate the efficacy of lovastatin as an antifibrotic agent after glaucoma filtration surgery (GFS) in a rabbit model.

METHODS. Thirty New Zealand white rabbits underwent GFS on the right eye. The rabbits were randomly assigned to one of three groups: (1) the mitomycin-C (MMC) group, which received 0.2 mg/mL MMC-soaked Weck-Cel under the conjunctival flap; (2) the control group, which received postoperative subconjunctival injections with 0.1 mL balanced salt solution (BSS); and (3) the lovastatin group, which received postoperative subconjunctival injection with 0.1 mL lovastatin (10 μM). Intraocular pressure (IOP), bleb survival, and bleb morphology were examined until blebs showed evidence of failure. Three rabbits in each group were killed on postoperative day (POD) 5, and analyzed for histology and immunohistochemistry.

RESULTS. Lovastatin significantly improved bleb survival compared with that in the control group (P = 0.002); however, no significant difference in bleb survival was observed between the MMC and lovastatin groups (P = 0.097). The lovastatin group showed significantly larger and higher blebs than did the control group. Further, the IOPs of the lovastatin and MMC groups were significantly lower than that of the control group (8.0 ± 1.4 mm Hg, 7.9 ± 3.2 mm Hg, and 11.1 ± 2.9 mm Hg, respectively; P = 0.016) on POD 5. Histologic analyses revealed decreased inflammatory response and reduced fibrosis in the lovastatin group than in the control group.

CONCLUSIONS. Postoperative injection of lovastatin improved bleb survival in the rabbit model of GFS. Therefore, lovastatin may have potential as a novel wound-modulating agent after GFS.

Keywords: lovastatin, filtration surgery, rabbit, glaucoma, fibrosis

Glaucoma is a leading cause of irreversible blindness and is expected to afflict 111.8 million people worldwide by 2040.1–3 It is an optic neuropathy that is associated with visual field loss. Because optic nerve damage is progressive and irreversible, accurate diagnosis and prompt treatment are important. Until date, lowering the IOP is the only proven effective in achieving a lower target IOP compared with medical or laser treatments. Glaucoma filtration surgery creates a new drainage channel from the anterior chamber to the subconjunctival area, filtering out aqueous humor, resulting in formation of a subconjunctival bleb and a decrease in IOP. However, despite its promising potential to control IOP, this surgery can fail due to excessive scar tissue formation at the surgical site.6,7 To reduce or prevent scarring, antimetabolites, such as mitomycin-C (MMC) or 5-fluorouracil (5-FU), are commonly used as adjunct agents during GFS and have been shown to improve the surgical outcome. However, because these antimetabolites act in a nonselective manner, there is risk of vision-threatening complications, such as hypotony, leakage, or bleb-related infections.8,9 Therefore, there is need for an alternative agent that selectively targets the antifibrotic process after GFS.

Statin medications lower serum cholesterol by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and are commonly prescribed as a first-line treatment in cardiovascular diseases.10 On the other hand, statins have antifibrotic, anti-inflammatory, and immunomodulatory effects, which are called "pleiotropic effects."12,13 This additional effect on fibrosis has already been proven in pulmonary fibrosis, renal disease, and tissue scarring.14–16

Previous studies have also reported the associated use of statins and various ocular diseases. Statins have been found to prevent the development of AMD and to reduce the incidence of intraocular inflammatory diseases.17–21 Furthermore, statins are known to have a beneficial effect on glaucoma. Several epidemiologic studies suggest that long-term use of statins may reduce the risk of open-angle glaucoma, and slow the progression of glaucoma.22,23 Moreover, statins have been reported to increase aqueous outflow by inducing changes in cell shape and actin cytoskeletal organization in the trabecular meshwork (TM), and by reducing juxtacanalicular extracellular matrix.24,25 A recent study by Villarreal et al.25 demonstrated that incubating TM cells with lovastatin suppressed mRNA and protein expressions of SPARC (secreted protein acidic and rich in cysteine), which is a critical mediator of aqueous outflow, and suggested the possibility of statins enhancing the aqueous outflow facility. In addition, Meyer-Ter-Vehn and colleagues26 showed that lovastatin inhibits TGF-β-induced myofibroblast transdifferentiation in human Tenon fibroblasts by interfering
with Rho-signaling, and suggested that lovastatin may reduce scarring after GFS.

Although there have been studies reporting the beneficial effects of statins on aqueous outflow and wound healing in vitro, their antifibrotic effect after GFS is unknown. We hypothesized that the administration of lovastatin after GFS would reduce the wound-scarring process by inhibiting TGF-β-induced myofibroblast transdifferentiation. In this study, we investigated the effect of lovastatin on the wound-healing response after GFS in a rabbit model.

METHODS

Animals

Thirty female New Zealand white rabbits (2.0–2.5 kg) were included in the study. The rabbits were acclimatized for 1 week before experimentation. Approval for this study was obtained from the Korea University Institutional Animal Care and Use Committee. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The rabbits were randomized to one of three treatment groups: (1) control group (n = 10), (2) lovastatin group (n = 10), and (3) MMC group (n = 10). The experiments were performed in two stages. In the first stage, 21 rabbits underwent GFS on only the right eye, and bleb appearance, survival, and IOP were examined. In the second stage, a subsequent experiment was repeated on the remaining nine rabbits, three rabbits from each group. These rabbits were killed on postoperative day (POD) 5 to compare the histologic features in the three groups.

Surgical Procedure

The animals were anesthetized with an intramuscular injection of 0.2 mL/kg xylazine (Rompun; Bayer Animal Health, Monheim, Nordrhein-Westfalen, Germany) and 0.3 mL/kg zoletapam hydrochloride (Zoletil; Virbac, Carros, France). Topical anesthesia with 0.5% proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX, USA) was applied. A single surgeon (JHP) performed GFS on the right eye of all the rabbits.27 The eyelid was retracted with an eyelid speculum and the eye was rotated inferiorly by a corneal traction suture made in the superior cornea. Next, a fornix-based conjunctival dissection was performed. For rabbits in the MMC group, a 4 × 4-mm Weck-Cel (Alcon Surgical, Fort Worth, TX, USA) soaked in 0.2 mg/mL mitomycin-C was placed between the conjunctiva and sclera at this time point. The Weck-Cel was removed after 3 minutes and the area was washed with 30 mL normal saline. Subsequently, a 25-gauge needle was used to make a scleral tunnel 1 mm behind the limbus for insertion of a 22-gauge cannula (JELCO I.V. Catheters; Smiths Medical Company, Ashford, UK) into the anterior chamber. The cannula needle was removed, and the cannula was positioned such that the distal end of the cannula was beyond the pupillary margin to avoid the tip from being occluded by iris tissue. The cannula was trimmed 1 mm from the insertion site and secured to the scleral bed with 10-0 nylon suture (Ethicon, Somerville, NJ, USA). The conjunctiva was closed in a watertight fashion by a running suture of 8-0 Vicryl (Ethicon) at the limbus.

Rabbits in the control group received 0.1 mL subconjunctival balanced salt solution (BSS) injection into the bleb immediately after surgery and daily for the next 9 days. A 30-gauge needle was used to inject solutions at the nasal margin of the bleb, and each injection was administered after the clinical findings were evaluated.

Rabbits in the lovastatin group received 0.1 mL lovastatin solution (10 μM, #438187; Calbiochem, San Diego, CA, USA) in the same manner as that for the control group. This injection regimen was used to maintain the inhibitory effect at the

### Table 1. Bleb Survival Time of the Three Groups

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean Survival Time, d</th>
<th>Range, d</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>7</td>
<td>6.86 ± 1.46</td>
<td>6–10</td>
<td>0.002</td>
</tr>
<tr>
<td>Lovastatin group†‡</td>
<td>7</td>
<td>10.29 ± 0.76</td>
<td>9–11</td>
<td>0.097</td>
</tr>
<tr>
<td>MMC group§</td>
<td>7</td>
<td>12.00 ± 2.65</td>
<td>7–14</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* Kruskal-Wallis test.
† Mann-Whitney U test with Bonferroni correction for multiple comparisons, P = 0.002 (lovastatin versus control).
‡ Mann-Whitney U test with Bonferroni correction for multiple comparisons, P = 0.097 (lovastatin versus MMC).
§ Mann-Whitney U test with Bonferroni correction for multiple comparisons, P = 0.002 (MMC versus control).

![Figure 1. Kaplan-Meier survival curve for the three groups. The lovastatin and MMC groups showed significantly prolonged bleb survival after GFS compared with the control group (Log rank test; overall P < 0.001). *Log rank test with Bonferroni correction for multiple comparisons.](ovs/10.1126/iovs.15-18427/figure1-01.jpg)
critical period of active wound modulation and tissue-remodeling process, based on previous studies.\textsuperscript{28,29} Injections of BSS and lovastatin were scheduled up to POD 9, unless the bleb showed evidence of failure. A bleb was deemed to have failed if it was observed to be flat and scarred with a deep anterior chamber on two consecutive examinations. The first date of the two examinations was considered the date of failure. When the bleb failed before POD 9, injections were performed until the second time point of consecutive examination of the failed bleb.

At the end of the surgery, ofloxacin and dexamethasone ointment were topically applied. In all the eyes, topical levofofaxacin solution (Cravit eye solution; Santen, Osaka, Japan) and prednisolone acetate 1% suspension (Pred Forte eye solution; Allergan, Irvine, CA, USA) were instilled four times a day for 7 days following surgery.

### Clinical Evaluations

Before surgery, the IOP was measured with a portable applanation tonometer (Tonopen AVIA; Reichert, Depew, NY, USA) after topical instillation of 0.5% proparacaine hydrochloride. The Tonopen was calibrated according to the manufacturer’s instructions before measurements at each time point. The IOP value for each measurement was the mean of two consecutive measurements that were within 2 mm Hg and had less than 5% error as indicated on the Tonopen, or the median of three measurements if the first two differed by 3 mm Hg or more. After surgery, the eyes were examined daily during the first postoperative week and three times per week until the bleb showed clinical signs of failure.

Bleb size, height, and vascularity were evaluated under a slit lamp and were documented as follows: the width and length were measured using a caliper and the height was determined semiquantitatively on a 4-point scale (0, flat; 1, shallow <1 mm; 2, elevated <2 mm; 3, high <3 mm). Bleb vascularity was graded from 0 to 3 (0, avascular; 1, normal vascularity; 2, hyperemic; 3, very hyperemic). Anterior chamber depth was also evaluated by slit-lamp microscope examination and graded semiquantitatively (0, flat; 1, shallow; 2, deep). Anterior chamber inflammation was also graded from 0 to 3 (0, no inflammation; 1, cells present; 2, fibrin formation; 3, hypopyon present).

### Histology

Operated eyes from three rabbits of each group were enucleated on POD 5, fixed in 4% paraformaldehyde (pH 7.4), dehydrated, and embedded in paraffin. Because none of the blebs showed failure until POD 5 in the first experiment, we selected this same time point to compare the histologic findings when all the blebs were elevated. The upper eyelid was attached to the enucleated globe to preserve the histologic structure of the drainage site. Sequential sections (5 µm) were then cut and histologic staining was performed with hematoxylin-eosin (HE) to observe the inflammatory cells, with Masson’s trichrome to identify the area of fibrosis, and with α-smooth muscle actin (α-SMA) immunohistochemistry to determine the number of myofibroblasts. The numbers of inflammatory cells and myofibroblasts were calculated based on the average cell numbers per high-power field (>400) from four consecutive central bleb cross-sections of each specimen. Fibrosis was examined by taking a photograph of a representative part of the fibrotic area and the proportion of the fibrotic area was calculated with the help of the National Institutes of Health ImageJ image analysis software version 1.52 (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The aforementioned histopathologic analysis was performed by a masked observer.

### Statistical Analysis

Statistical analysis was performed using the SPSS software version 21.0 (SPSS, Inc., Chicago, IL, USA). The Kruskal-Wallis test was used to compare time to failure and IOP among the

![Figure 2](https://example.com/figure2.png) Intraocular pressure changes before and after GFS. Intraocular pressures in the lovastatin and MMC groups were significantly lower than that in the control group (8.0 ± 1.4 mm Hg, 7.9 ± 3.2 mm Hg, and 11.1 ± 2.9 mm Hg, respectively; Mann-Whitney U test; both, \( P = 0.016 \) on POD 5. Postoperative day 0 represents the day of surgery. *Time points at which the postoperative IOP was significantly lower than baseline IOP.

![Figure 3](https://example.com/figure3.png) Appearances of filtration blebs in the three groups 5 days after GFS. The bleb was narrow and flat in the control group (A), whereas blebs in the lovastatin (B) and MMC (C) groups were diffuse and elevated. Black arrows indicate the borders of the blebs.
groups. The Mann-Whitney U test with Bonferroni correction was used to compare IOPs and survival times among the groups. For analysis of the data of repeated measures, repeated measures ANOVA was used to compare the overall differences among groups. A P value less than 0.05 was considered statistically significant unless the Bonferroni correction method for multiple comparisons was applied, in which case a P value less than 0.017 was considered significant. Kaplan-Meier analysis was performed to evaluate the cumulative probability of success.

RESULTS

Bleb Survival

Table 1 shows the bleb survival times. Rabbits receiving BSS had an average time to failure of 6.9 ± 1.5 days. The bleb survival times in the lovastatin and MMC groups were significantly longer than that in the control group (Mann-Whitney U test; both, P = 0.002); however, there was no significant difference in bleb survival time between the lovastatin and MMC groups (P = 0.097). The bleb survival among the groups showed a significant difference for the Kaplan-Meier survival curve (log rank; P < 0.001; Fig. 1). All blebs had failed in the control group on POD 10, whereas 40% of blebs in the lovastatin group and 70% in the MMC group survived at this time point.

Intraocular Pressure

The mean day when the IOP reached or was higher than the baseline IOP was POD 4.4 ± 1.6 (range, 2–7) in the control group and POD 8.7 ± 1.5 (range, 6–10) in the lovastatin group; the difference was significant (Mann-Whitney U test; P = 0.001; Fig. 2). Moreover, IOPs in the lovastatin and MMC groups were significantly lower than that in the control group on POD 5 (8.0 ± 1.4 mm Hg, 7.9 ± 3.2 mm Hg, and 11.1 ± 2.9 mm Hg, respectively; Mann-Whitney U test; both, P = 0.016). Statistical analysis of the repeated IOP data revealed a significant difference among treatment groups at a 95% confidence interval (P = 0.028).

Clinical Findings

The groups differed in the appearance of filtration blebs on POD 5 (Fig. 3). Although the blebs were elevated and diffuse in the lovastatin and MMC groups, they were flat and scarred in the control group. Bleb area and height were also significantly different among the groups until POD 6, with flatter and narrower blebs in the control group than those in the lovastatin and MMC groups (repeated measures ANOVA; bleb width, P = 0.034; bleb height, P = 0.002). The MMC and lovastatin groups showed no significant difference in bleb area and height (repeated measures ANOVA; bleb width, P = 0.307; bleb length, P = 0.646; bleb height, P = 0.407). Moreover, there was a significant difference in the vascularity score; blebs in the MMC group had lower vascularity score than blebs in the
lovastatin and control groups (repeated measures ANOVA; \( P = 0.033 \)). Scores for the anterior chamber depth and inflammation showed no statistically significant differences.

**Histologic Findings**

The microscopic structures of the filtration blebs were analyzed and compared at the same time point (POD 5) in three rabbits from each group (Figs. 4, 5). Analysis of the histologic structure was performed by a pathology specialist who was blinded to the experiment (Table 2; Fig. 6). The mean number of inflammatory cells increased in the order of MMC, lovastatin, and control groups, and the difference showed marginal significance (\( P = 0.051 \)). In addition, although not statistically significant, the mean number of myofibroblasts was reduced in the lovastatin and MMC groups compared with that in the control group. The lovastatin group also showed the least proportion of fibrotic area compared with the other two groups.

**DISCUSSION**

This study demonstrated the effect of lovastatin on wound-healing process after GFS in rabbits. Lovastatin significantly improved bleb survival and reduced IOP compared with the control group. Although it was not superior to MMC, lovastatin showed potential as an adjuvant option for reducing the scarring response after GFS. A recent study by Villarreal and coworkers\(^2\) demonstrated the effect of lovastatin on SPARC expression in human TM cells and explored the underlying molecular mechanism. A prototypical matricellular protein, SPARC has a critical role in aqueous outflow and has been associated with increased tissue fibrosis and aberrant tissue

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**Table 2.** Mean Number of Inflammatory Cells and Myofibroblasts, and Mean Percentage of Fibrotic Area

<table>
<thead>
<tr>
<th>Group</th>
<th>Inflammatory Cells Per Field, mm(^2)</th>
<th>Myofibroblasts Per Field, mm(^2)</th>
<th>Fibrotic Area Per Field, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group 3</td>
<td>99.67 ± 18.64</td>
<td>84.50 ± 21.61</td>
<td>27.12 ± 11.52</td>
</tr>
<tr>
<td>Lovastatin group 3</td>
<td>54.58 ± 21.36</td>
<td>52.00 ± 24.70</td>
<td>19.04 ± 7.46</td>
</tr>
<tr>
<td>MMC group 3</td>
<td>37.58 ± 20.53</td>
<td>43.92 ± 12.90</td>
<td>26.84 ± 12.90</td>
</tr>
<tr>
<td>( P )†</td>
<td>0.051</td>
<td>0.177</td>
<td>0.561</td>
</tr>
</tbody>
</table>

\(^*\) Magnification: ×400.
\(^†\) Magnification: ×200.
\(\ddagger\) Kruskal-Wallis test.
remodeling. Lovastatin significantly suppressed SPARC mRNA and protein levels in TM cells without cellular toxicities. In addition, lovastatin has been shown to inhibit TGF-β-induced CTGF transcription, α-SMA expression, and myofibroblast transdifferentiation in human Tenon fibroblasts in vitro. The present study extends this in vitro study to show that lovastatin exerts its antifibrotic effect after GFS in a rabbit model.

In this study, lovastatin significantly reduced the IOP and prolonged bleb survival compared with the control group. Although the MMC group had a lower IOP than the baseline at all time points, which was not observed in the lovastatin group, the time required for postoperative IOP to exceed the baseline IOP was significantly extended when the bleb was treated with lovastatin than when treated with BSS. Bleb appearance was also significantly different among the groups. Blebs in the control group were significantly narrower and flatter than those in the lovastatin and MMC groups. Histologic examination showed that lovastatin and MMC similarly reduced fibroblast transdifferentiation compared with the control. The numbers of inflammatory and myofibroblast cells were reduced in the lovastatin group than when treated with BSS. Although the histologic difference did not reach statistical significance, these findings indicate that injection of lovastatin after GFS may reduce fibrotic reactions and may improve the surgical outcome of filtration surgery. Further, lovastatin did not cause bleb avascularity, which was observed in the MMC group. Even though we injected lovastatin several times, there were no cases suggestive of inflammation caused by multiple injections, such as conjunctival injection or upper eyelid swelling. Although explaining the exact mechanism of the antifibrotic action of lovastatin in the rabbit model of GFS is beyond the scope of this article, we hypothesize that this is a result of reduced myofibroblast transdifferentiation and subsequent reduced extracellular collagen fiber deposition on lovastatin injection. Improved bleb survival, with fewer complications, renders lovastatin more suitable for reducing wound scarring after GFS.

However, it is important to note that this study has its own limitations. First, we used the previously reported experimental concentration of lovastatin. Although the concentration of lovastatin used in this study showed excellent inhibition of myofibroblast transdifferentiation of Tenon fibroblasts without cellular toxicities, future studies are needed to determine the optimal dosage, concentration, and application method of lovastatin on wound modulation after GFS are specific for inhibiting myofibroblasts, or affect other cell types and cytokines involved in the wound-healing process as well. Third, the histologic findings in the study groups showed no statistically significant differences; the small sample size may have contributed to this result. Fourth, the material of cannula used here was different from the one commonly used in previous studies. The difference in the cannula material might have influenced the inflammatory response, and may have affected the bleb survival time. Finally, the inhibitory effect of lovastatin on GFS was not superior to MMC. However, we can expect that combined use of lovastatin and MMC may lower the dose and exposure time of MMC, thereby improving the safety profile of MMC, and may have beneficial effects on the surgical outcome after GFS. Further studies investigating the effect of this combination on wound modulation may enlighten us about the additional role of lovastatin.

In conclusion, lovastatin improved the GFS outcome in a rabbit model by reducing subconjunctival scarring in the bleb tissue. This indicates the potential of lovastatin as a novel wound-modulating agent after GFS.

Acknowledgments

The authors thank KiSeok Jang, Department of Pathology, Hanyang University College of Medicine, for assistance in histological analysis. The authors alone are responsible for the content and the writing of the paper.

Disclosure: J.-H. Park, None; C. Yoo, None; Y.Y. Kim, None
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