Injection of Autologous Blood for Bleb Leaks in New Zealand White Rabbits

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Purpose. Bleb leaks after trabeculectomy with antimetabolites can be recalcitrant to therapy. Periblес autologous blood injections are a moderately successful new treatment modality for such leaks. However, it is unclear what mechanism the injections work to achieve leak resolution.

Methods. A randomized, prospective study in the rabbit model was undertaken to evaluate further the clinical and histologic effects of periblе autologous blood injection after leak induction in mitomycin-C exposed blebs, compared to controls that received only periblе balanced salt solution injections.

Results. In the blood-treated eyes, all bleb leaks healed. Control eyes either demonstrated persistent bleb leaks with shallow anterior chambers or failed blebs that were Seidel negative. Histologic results were remarkable for increased periblе cellularity and collagen deposition in the blood-treated eyes, compared to controls.


The administration of antimetabolites has improved dramatically the success rate of primary trabeculectomies and trabeculectomies in eyes with a poor surgical prognosis (i.e., pseudophakia–aphakia, uveitis, neovascular glaucoma, prior failed filter procedure). Unfortunately, the thin avascular blebs associated with antimetabolite use may develop refractory bleb leaks. These leaks can be unresponsive to common conservative management techniques, such as contact lens application, patching, aqueous suppressants, cyanoacrylate glue, trichloroacetic acid, or cryotherapy. Serious sequelae, such as flat anterior chamber, synechiae, cataract, choroidal effusions, hemorrhages, and even endophthalmitis, may develop if persistent bleb leaks are not successfully treated. Recently, we reported a new technique of treating such leaks in humans. Autologous blood was injected into the periblе subconjunctival space in six eyes with discrete bleb leaks with moderate success. We hypothesized that periblе plasma proteins may diffuse to the bleb leak, where subsequent cross-linking of factors seals the leak. In addition, the blood may provide a source of trophic factors and other chemotactic factors, which, in turn, induce migration and proliferation of adjacent fibroblasts, with resultant fibrosis and healing. However, the real mechanism of action as to why periblе blood injections result in bleb leak resolution remained unclear. Therefore, this study in the rabbit model was designed to evaluate further the clinical and histologic effects of periblе autologous blood injection in eyes with bleb leaks after filtering surgery with mitomycin-C (MMC).

MATERIALS AND METHODS. A prospective, randomized study was performed using New Zealand white albino rabbits weighing between 2 and 4 kg. Approval for the protocol was obtained from the University of Florida 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.

A standard posterior lip sclerectomy was performed on 16 right eyes of 16 different rabbits by the same surgeon. General anesthesia was induced with an intramuscular injection of ketamine 50 mg/kg and xylazine 10 mg/kg. A partial thickness 8-0 silk corneal traction suture was placed, and the eye was pulled downward. A superior limbal-based conjunctival flap was raised. Intraoperative MMC (0.4 mg/ml) on a 4 × 1 mm section of cellulose sponge was applied between the sclera and conjunctiva for 5 minutes. The area was then irrigated with 30 ml of balanced salt solution (BSS) before the eye was entered. This concentration of MMC was chosen based on our previous experience, which demonstrated long-lasting blebs and intraocular pressure (IOP) reduction in the rabbit model with this dose of MMC. A linear incision 2 mm long was made, and the anterior chamber was entered. A 1.5 mm cross-section scleral punch was used to remove a standard posterior block of tissue. A peripheral iridectomy was made. The conjunctival incision was closed with a running 8-0 polyglactin suture on a BV 130-4 needle (Ethicon, Somerville, NJ). A 30-gauge paracentesis was made, and BSS was injected into the anterior chamber to confirm the presence of a patent sclerostomy and to inflate the bleb. The conjunctival incision and bleb were inspected to rule out any leaks. Maxitrol ointment was applied at the end of the surgery.

Postoperative examinations were performed under ketamine and rompin anesthesia. The IOP was measured using a Tonopen tonometer (Mentor, Nor-
well, MA), the status of the bleb was assessed on a scale of 0 (none) to 3 (high), the anterior chamber depth was estimated, and fluorescein was used to determine the presence of bleb leaks.

On postoperative day 7, all eyes were examined as described above to insure that the blebs were functioning and that no leaks were present. After the examinations, all blebs were punctured with a 75-Beaver blade to yield a standard 2 mm incision. All incisions were inspected to assure they were Seidel positive. Maxitrol ointment was applied to all eyes after the incision in the bleb was made.

All eyes were reexamined on postoperative day 8 and then were randomized in a balanced fashion to receive either subconjunctival peribleb autologous blood or BSS injections. Injections consisted of 0.2 to 0.3 ml of blood or BSS injected in three sites around the bleb using a 30-gauge needle.

Injections were made on either side and directly behind the bleb. No injections were made into the bleb in either group. Autologous blood was obtained from the rabbit's ear vein immediately before injection. All injections of blood were performed within 30 seconds of obtaining the blood, and no anti-clotting medication was used. Figure 1 demonstrates an eye immediately after peribleb blood injections. Maxitrol ointment was applied after the injections.

All eyes were reexamined on postoperative day 11 (3 days after peribleb injection). At this time, four eyes in each group were randomly selected for histologic study. The rabbits were killed by an intracardiac injection of pentobarbital, and the right eye of each, was removed for histologic study. The remaining rabbits were reexamined on postoperative day 14, 21, and 28. After the examination on postoperative day 28, the remaining rabbits were killed, and all eyes in each group were randomly selected for histologic study. The rabbits were killed by an intracardiac injection of pentobarbital, and the right eye of each, was removed for histologic study.

Two sample t-tests and Wilcoxon rank sum tests were used to compare clinical variables of IOP and bleb status (bleb height and presence or absence of bleb leak) before and after injection for treated and control animals. Failure was described at postoperative days 11 and 28 as persistent bleb leak regardless of IOP or bleb status or as IOP within 3 mm Hg of IOP before surgery. Fisher's exact tests were used to test for differences between control and treated eyes. P < 0.05 was considered statistically significant.

The enucleated eyes were fixed in a 10% formalin solution (Buffered Formalde-Fresh; Fisher Scientific, Pittsburgh, PA). After 48 hours in fixative, the bleb was dissected from the surrounding tissues and processed to paraffin. Once embedded in the paraffin blocks, sections were cut at 6 μm intervals. Sections were then placed on slides and stained with Harris' hematoxylin–eosin–phloxine.11

The slides were examined using an Olympus (Tokyo, Japan) BH Binocular microscope, and photomicrographs were obtained. Cell counts were obtained at six sites on two slides from each eye. The slides were examined by an examiner unaware of whether the eye had received peribleb blood or BSS. The six sites counted were as follows: right side of the bleb area, just under the conjunctiva; middle of the collagen–fibroblast response, right side of the bleb; directly under the bleb, not including underlying sclera; middle of the collagen–fibroblast response, left side of the bleb; left side of the bleb area, just under the conjunctiva; subconjunctival space as far as possible from the bleb area. The grid used was a 0.2 × 0.2 mm grid, and all cell nuclei within the grid were counted. The exact areas were located under scanning power, and the magnification was increased to 200X for the count. Multivariate analysis of variance was used to test for treatment, location, and slide number effects and interactions. Transformations were considered for variance stabilization, with the natural logarithm transformation selected for day 11 counts and the square root transformation selected for day 28 counts. When significant differences caused by location were detected, pairwise contrasts were used to identify which locations differed.

RESULTS. Clinical Results. All posterior lip sclerectomies were functioning on postoperative day 7, with a mean IOP of 7.8 mm Hg (range, 6 to 10 mm Hg). This was a decrease from the presurgical average IOP of 16.1 mm Hg (range, 15 to 18 mm Hg). The average bleb height was 2.3 (range, 1 to 3). All anterior chambers were normal depth, and there were no bleb leaks.

Blebs were reexamined after the puncture with the 75-Beaver blade, and all were found to be Seidel positive. Examination on postoperative day 8 (24 hours after the bleb incisions were made) revealed that all blebs were still leaking. The average IOP was 5.3 mm Hg (range, 0 to 7 mm Hg). Two anterior chambers were deemed full depth, and the remaining were shallow compared to the fellow eye. The average bleb height was estimated at 1.3 (range, 0 to 3). At the completion of this examination, the eyes were randomized and received peribleb blood or BSS subconjunctival injections.

On postoperative day 11, all eyes were reexamined. The average IOP of the group (eight eyes) receiving the peribleb blood was 6.7 mm Hg (range, 4 to 10); the average bleb height was graded as 1.8 (range, 1 to 3), no anterior chamber was thought to be shallow, and all bleb leaks had healed. In contrast, eyes that received the peribleb BSS fell into two groups—three eyes had persistent bleb leaks, IOP <
3 mm Hg, average bleb height of 1.6 (range, 1 to 2), and shallow or flat anterior chambers; five eyes had low to flat blebs, average bleb height of 0.4 (range, 0 to 1), with no leaks, average IOP of 11.9 mm Hg (range, 11 to 13), and all anterior chambers were full depth.

The difference in IOP between the control group and the blood-treated group was statistically significant ($P < 0.001$) using the two sample t-test. The difference in bleb height was significantly different using the Wilcoxon rank sum test ($P = 0.014$). Using the Fisher exact test for surgical failure (as defined in Materials and Methods), the difference in surgical success between the groups also was statistically significant ($P = 0.007$). Four eyes in each group were removed at this time for histologic evaluation, and four eyes in each group were reexamined on postoperative day 28.
FIGURE 3. (A) Photomicrograph at higher power of the increased cellular response at postoperative day 11, after peribleb injection of autologous blood. Magnification, ×100. (B) Photomicrograph at higher power of the mild cellular response at postoperative day 11, after peribleb balanced salt solution injection. Magnification, ×100. Note the increased number of fibroblast nuclei and the denser eosinophilic material (consistent with collagen deposition) after injection of peribleb blood, compared to peribleb balanced salt solution.

On postoperative day 28, the average IOP in the peribleb blood-treated group was 8.8 mm Hg, the average bleb height was graded as 1.3, and all the anterior chambers were fully formed. No eye had a bleb leak. In eyes that received the peribleb BSS, endophthalmitis developed by postoperative day 15 in one eye that had a persistent leak, and the rabbit was killed. The other three had an average IOP of 15.7 mm Hg on postoperative day 28. No eye had an elevated bleb, and no bleb leaks were identified. All anterior chambers were fully formed. All animals were killed at postoperative day 28, and the right eyes were removed for histlogic evaluation. The difference in IOP observed at postoperative day 28 between the control group and the blood-treated eyes was statistically significant ($P = 0.01$). The difference in bleb grading between groups at this time was not found to be statistically significant ($P = 0.057$), probably because of the small size of the groups; the Wilcoxon rank sum test has insufficient power to detect differences in such small groups. Again, using the Fisher exact test for surgical failure (as defined in Materials and Methods), the

FIGURE 4. (A) Photomicrograph of intrableb contents at postoperative day 11, after peribleb injection of autologous blood. Magnification, ×100. (B) Photomicrograph of intrableb contents at postoperative day 11, after peribleb balanced salt solution injection. Magnification, ×100. Note the increased cellular response and denser eosinophilic strands within the bleb after peribleb injection of blood, compared to peribleb injection of balanced salt solution.
difference in success rate between the peribleb blood-treated and the BSS groups at this time was statistically significant \((P = 0.029)\).

**Histologic Results.** In those eyes removed at postoperative day 11, there were marked differences in histologic appearance between the peribleb blood-treated and the BSS groups. Sections from those eyes that received peribleb blood demonstrated an increased cellular response in the areas of blood injection adjacent to the bleb. Most of these cells had polygonal nuclei, a fact that is consistent with fibroblast proliferation. In addition, there was a far denser homogenous eosinophilic staining response, consistent with greater collagen deposition, in eyes that received peribleb blood than in BSS eyes (see Figs. 2, 3). Histologic evaluation of intrableb contents in eyes that received peribleb blood also revealed a greater number of polygonal nuclei, again consistent with fibroblast proliferation. Sections of blebs from the peribleb blood-treated group showed a more loculated bleb appearance, with greater collagen deposition than similar bleb sections from the BSS group (see Fig. 4).

This increased cellularity of areas adjacent to the bleb in eyes that received peribleb blood was still noted in sections from eyes removed at postoperative day 28. Eyes that received peribleb BSS had failed blebs, and histologic evaluation revealed a moderately cellular, collagenous response filling the entire bleb space.

The number of fibroblasts from two slides from each eye were counted as described in Materials and Methods. There was no statistically significant difference between the two slides from each eye, so the cell counts were averaged for each animal. The average number of fibroblast nuclei at each site for control and peribleb blood-treated eyes is shown in Table 1. Cell counts revealed a statistically significant greater number of cells from those eyes treated with peribleb blood at postoperative days 11 \((P = 0.016)\) and 28 \((P = 0.004)\) when compared to eyes that did not receive blood. There was a statistically significant difference in the number of cells, depending on the location of the count. Site 6, selected as the furthest from the bleb on all slides, contained a significantly lower number of cells in peribleb blood-treated and BSS eyes than all other sites counted.

**DISCUSSION.** Antimetabolites increasingly are used as an adjunct in filtering surgery to increase the success rate and to help achieve lower final IOP in patients.\(^1\text{-}^5\) However, these greater success rates and lower IOP are not achieved without cost. As longer follow-up is obtained on these eyes, we are finding an increased incidence of persistent bleb leaks, with their associated complications.\(^6\text{-}^7\) It is imperative that we develop a more effective method to deal with these leaks.

**TABLE 1. Average Cell Counts**

<table>
<thead>
<tr>
<th>Site</th>
<th>S/P Autologous Blood</th>
<th>S/P BSS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POD 11, 3 days after injection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32*</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>45*</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>24*</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>36*</td>
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<tr>
<td>5</td>
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<td>20</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>31*</td>
<td>16</td>
</tr>
<tr>
<td><strong>POD 28, 20 days after injection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
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<td>20*</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>16*</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>20*</td>
<td>10</td>
</tr>
</tbody>
</table>

All counts are averages of two slides from each animal. There were four animals in all groups except the POD 25, BSS injection group, which had three because of endophthalmitis in one eye.\(^*\) Statistical significance compared with similar site of BSS injected eyes \((P < 0.05)\). S/P = status post; BSS = balanced salt solution; POD = postoperative day.

Autologous blood peribleb and intrableb subconjunctival injections have been used successfully to treat some bleb leaks in humans.\(^8\text{-}^{12}\) However, it is unclear how or why these injections work.

Previously, rabbit and monkey models have been used to evaluate the effects of antimetabolite application on filter surgery survival. Mitomycin-C has been demonstrated to increase substantially the filter surgery success rate in these animals eyes. Wilson et al\(^{13}\) administered MMC \((0.2 \text{ mg/ml})\) subconjunctivally at the time of filter surgery in the rabbit model and demonstrated effective prolongation of filtration surgical success. Similarly, Pasquale et al\(^{14}\) noted increased surgical success after MMC use in monkeys undergoing filter surgery. Histologic examination of these treated eyes has shown patent sclerostomies and hypocellular, well-formed bleb cavities.

Our prospective, randomized study in the rabbit model addressed both the clinical and the histologic effects after bleb leak induction in filtered eyes after MMC, which then were treated with either subconjunctival peribleb autologous blood or BSS. Clinically, all eight eyes receiving subconjunctival peribleb blood injection experienced leak resolution within 3 days, which was associated with bleb maintenance. This was in marked contradiction to the eight failed filters in the BSS group. Two kinds of eyes experienced failure: eyes with persistent bleb leaks, hypotony, and shallow or flat anterior chambers and eyes in which the bleb
flattened secondary to the leak and the sclerotomy subsequently healed at the episcleral level. More interestingly, there was a significant difference in cellular response between the peribleb blood- and BSS-treated groups. Previously, we hypothesized that peribleb blood injections may function to seal leaks by plasma protein diffusion to the area of leak, with subsequent cross-linking of factors and leak seal. However, our histologic analysis demonstrated probable fibroblast proliferation with collagen deposition around and within the bleb of the blood group. Thus, although this does not rule out coexisting plasma protein diffusion, it supports another theory, which is that blood may provide a source of trophic factors that in turn induce migration and proliferation of adjacent fibroblasts, with the promotion of healing around and/or within the bleb. Also of note, increased cellularity was greatest at the sites of blood injection around the bleb, and it diminished significantly in areas farther away from the injections and the bleb.

It is interesting that the blood injection resulted in bleb leak seal in all eight treated rabbits. In our clinical experience, it works approximately 60% of the time in humans. This may be related to the greater healing powers of the rabbit eye, especially when induced by blood injection. We know that mitomycin-exposure can cause irreversible human fibroblast inhibition and even death in cell culture, as well as human vascular endothelium inhibition. The fresh, but limited, fibroblast load brought by peribleb blood injection in humans may be insufficient in some cases to overcome the antiproliferative, antihealing qualities of the initial MMC exposure at the time of filter surgery.

One drawback of our study, as alluded to, involves our inability to deduce whether more than one healing cascade (i.e., fibroblast reproduction and plasma protein diffusion—cross-linking) occurs simultaneously. The next step in our study involves separating blood into plasma and cellular components and then proceeding with injections as discussed here.

Key Words
antiproliferative effects, autologous blood, bleb leak, glaucoma surgery, rabbit

References