Benzalkonium Chloride–Induced Denervation of Orbicularis Oculi Muscle in Rabbits

Brian A. Francis, 1 James D. Weiland, 1 Nicholas A. Sachs, 2 and Eli L. Chang 3

PURPOSE. To examine the potential for benzalkonium chloride (BAK) to cause denervation of the orbicularis oculi muscle (OOM) in a rabbit model.

METHODS. Pigmented rabbits were separated into five groups consisting of five rabbits each. Group 1 was injected with 1 mL of BAK 0.25% in the OOM of the upper eyelid. Group 2 was injected with 1 mL of BAK 0.5%. Group 3 included untreated controls. Groups 4 and 5 underwent surgical severing of the facial nerve (to cause complete paralysis of the OOM). Strength-duration curves for electrical stimulation of muscle twitches were measured for each group and chronaxie values were calculated to determine innervation status. Groups 1 and 4 were stimulated at 1 week postintervention while groups 2 and 5 were stimulated at 4 weeks postintervention. The rabbits were then sacrificed and the eyelids sent for histological analysis.

RESULTS. In group 1, all five rabbits demonstrated denervation of the OOM in the injected area. In group 2, one rabbit developed an abscess at the injection site and was sacrificed at 1 week. Of the remaining four rabbits, two showed complete denervation and two showed denervation with evidence of partial reinnervation. The histology demonstrated marked atrophy of the OOM for BAK-treated rabbits when compared with controls. In group 3, all five rabbits showed normal OOM function. In groups 4 and 5, all rabbits showed denervation of the OOM and histological evidence of muscle atrophy similar to groups 1 and 2.

CONCLUSIONS. BAK causes denervation when injected into the OOM in rabbits. The clinical relevance of this finding may be the onset of lagophthalmos and eyelid retraction in patients with chronic BAK exposure. (Invest Ophthalmol Vis Sci. 2013; 54:1868–1872) DOI:10.1167/iovs.12-10792

Surfactants are a group of compounds that possess a number of unique characteristics, including the capacity to reduce the surface tension of water, coalesce to form micelles, and to layer on interfaces. Surfactants can disrupt biological systems by damaging nerve membranes and thereby altering nerve function.

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The cationic surfactant and phase transfer agent benzalkonium chloride (BAK) is a quaternary ammonium compound that is often used in conjunction with disodium EDTA as a preservative in topical ophthalmic preparations. It is a chemical detergent preservative that is stable at a wide range of temperatures and has potent antimicrobial properties. BAK acts upon microorganisms by altering cell membrane permeability and lysing cytoplasmic contents. It has been shown to increase the corneal penetration of some topical medications by causing a separation of the epithelium.1,2 BAK is the most common antimicrobial preservative currently used in topical ophthalmic solutions. Reports have shown that BAK can accumulate in ocular tissues and can contribute to ocular surface inflammation and ocular surface disease.1–5

The orbicularis oculi muscle (OOM) is innervated by the seventh cranial (facial) nerve. The OOM closes the eyelids during voluntary and reflex blinking. Because eyelid closure is the primary protective mechanism of the ocular surface, functional deficiencies in eyelid closure can result in corneal damage, infection, perforation, and loss of the eye. Our clinical observations suggested that several patients who had been exposed chronically to BAK in glaucoma medications were noted to exhibit signs of OOM dysfunction, including incomplete reflex blinking and lagophthalmos on both voluntary and reflex blinking. It is known that BAK has a neurotoxic effect in smooth muscle and has thus been used to create models of myenteric denervation.10–17 This neurotoxic effect has not been studied in skeletal muscle such as the OOM.

Extracellular injection of electrical currents can be used to activate excitable tissues, such as nerve and muscle fibers. The amount of electrical charge necessary to elicit a response depends, to a large extent, on the membrane properties of the tissue being activated.18 A strength-duration curve can be generated by injecting square current pulses and measuring the threshold current necessary to generate a response at a number of different pulse widths. This curve can be parameterized by its rheobase (the current necessary to elicit a response with an infinite pulse width) and chronaxie (the pulse width necessary to elicit a response with a pulse magnitude of twice the rheobase).19 While the rheobase value can be affected by factors such as the distance between the stimulating electrode and tissue being activated, the chronaxie value is a direct measure of the membrane time constant, and therefore indicative of the tissue being activated.20 We used electrical stimulation of eyelid movement to determine the innervation status of OOM muscles in rabbits who had been exposed to BAK and compared them with healthy controls. To our knowledge, our study is the first to examine the potential ability of BAK to cause denervation and paralysis of the OOM in an animal model.

METHODS

A total of 25 pigmented rabbits were used in this study. All procedures were approved by the University of Southern California Institutional Review Board.
Animal Care and Use Committee. Rabbits were separated into groups of five. The rabbits in groups 1 and 2 each received an injection of 1 mL of a BAK solution into the OOM of the right-upper eyelid. Group 1 received an injection of 0.25% BAK, while group 2 received an injection of 0.5% BAK. A control group (group 3) consisted of five rabbits that were not treated with injection or surgical severing of the facial nerve. Finally, groups 4 and 5 were selected from our previous study in which rabbits had undergone surgical severing of the right facial nerve in order to induce complete paralysis of the OOM.21,22

After a specified time lapse from the initial intervention (BAK injection or facial nerve transection), rabbits were anesthetized by intramuscular injection of ketamine and xylazine and electrodes were surgically inserted into the right-upper eyelid. These electrodes were used to deliver biphasic, current-controlled stimulation pulses in order to generate strength-duration curves for twitch initiation according to the protocol described in our previous publication.21 Briefly, biphasic square wave current pulses were delivered using a multifunction DAQ (PCI-6025E; National Instruments Corp., Austin, TX) and analog stimulus isolator (Model 2200; A-M Systems, Inc., Carlsborg, WA) at a range of fixed pulse widths, each time increasing the pulse amplitude from zero until the first twitch of the OOM muscle was visible. Rheobase values were estimated as the stimulus amplitude with a 100-ms pulse and chronaxie values were calculated from the strength-duration curves according to the Lapicque law for stimulation by performing a least squares fit.25 The chronaxie depends on the innervation status of tissue being stimulated and is much greater for denervated skeletal muscle than for innervated muscle (in which the innervating motor axons are actually stimulated).24 Calculated chronaxies were compared with previously published values for innervated and denervated OOM.

Groups 1 and 4 were each stimulated 1 week after the initial intervention, while groups 2, 3, and 5 were each stimulated 4 weeks postintervention. At the completion of the stimulation protocol, rabbits were euthanized with an intracardiac injection of pentobarbital (120 mg/kg) and tissue samples were taken from the upper and lower eyelids on both sides for histological analysis.

The histological analysis consisted of Masson trichrome staining and examination under high magnification (>40). The muscle fibers were analyzed in a quantitative fashion following the methods of Sachs et al.21,22 Briefly, the muscle fiber bundles per high power field (>40) were counted and their cross-sectional area calculated. In order to account for the differences in the numbers of muscle fibers counted per HPF in the different groups, the cross-sectional areas were all normalized to the mean of the control group, which was used as the reference (value = 1.00). Thus, a number less than 1.00 indicates lower cross-sectional area (or atrophy), and a number greater than 1.00 indicates greater cross-sectional area (or hypertrophy).

### RESULTS

Stimulation results for all rabbits are listed in Table 1. The mean chronaxie value for group 1 (injection of 0.25% BAK 1 week prior to stimulation) was 31.2 ms (range: 13.9–53.4 ms), compared with values of 0.4 ms (range: 0.2–0.5 ms) in normal rabbits with untreated orbicularis muscle (group 3), and 51.0 ms (range: 33.4–61.6 ms) for rabbits having undergone facial nerve transection the same duration prior to stimulation (group 4). The chronaxies for the BAK-treated group, stimulated at 1 week postintervention, were significantly greater than those of the untreated rabbits (t-test, *P* = 0.019), indicating the presence of denervation in the BAK-treated group. The BAK treated group was not statistically significantly different from the values for rabbits having experienced 1 week of facial nerve transaction (*P* = 0.07).

### Table 1. Chronaxie Values for OOM Stimulation in Rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>Rabbit 1, ms</th>
<th>Rabbit 2, ms</th>
<th>Rabbit 3, ms</th>
<th>Rabbit 4, ms</th>
<th>Rabbit 5, ms</th>
<th>Mean ± SD, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>BAK 0.25%, 1 wk</td>
<td>13.9</td>
<td>47.7</td>
<td>15.4</td>
<td>25.7</td>
<td>53.4</td>
<td>31.2 ± 18.3</td>
</tr>
<tr>
<td>Group 2</td>
<td>BAK 0.5%, 4 wk</td>
<td>6.9</td>
<td>Sacrificed</td>
<td>13.9</td>
<td>4.0</td>
<td>14.8</td>
<td>9.9 ± 5.3</td>
</tr>
<tr>
<td>Group 3, no injection, no nerve transection</td>
<td>0.5</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Group 4, nerve transection, 1 wk</td>
<td>61.7</td>
<td>51.1</td>
<td>50.7</td>
<td>33.4</td>
<td>58.0</td>
<td>51.0 ± 10.8</td>
<td></td>
</tr>
<tr>
<td>Group 5, nerve transection, 4 wk</td>
<td>23.7</td>
<td>24.6</td>
<td>66.0</td>
<td>63.9</td>
<td>58.6</td>
<td>47.3 ± 21.4</td>
<td></td>
</tr>
</tbody>
</table>

### References


### Figures

**Figure 1.** (A) Histologic section of untreated, control rabbit orbicularis muscle (group 3). Note the abundance of muscle fiber bundles with intervening connective tissue and blood vessels. (B) Histologic section of rabbit orbicularis muscle 1 week after facial nerve transection. Note the decrease of muscle fiber bundles and the replacement with connective tissue corresponding to denervation. (C) Histologic section of rabbit orbicularis muscle 4 weeks after facial nerve transection. Note the moderate decrease of muscle fiber bundles and the replacement with connective tissue corresponding to severe denervation.
One rabbit in group 2 developed an untreatable infection (eyelid and orbital abscess) and was sacrificed prior to stimulation. For rabbits receiving stimulation, the mean chronaxie value for group 2 (injection of 0.5% BAK 4 weeks prior to stimulation) was 9.9 ms (range: 4.0–14.8 ms), compared with values of 0.4 ms (range: 0.2–0.5 ms) in normal rabbits with untreated orbicularis muscle (group 3) and 47.3 ms (range: 23.7–66.0 ms) for rabbits having undergone facial nerve transection the same duration prior to stimulation (group 5). The chronaxies for the BAK-treated group were significantly greater than those of the untreated rabbits (t-test, \( P = 0.037 \)), but significantly less than the values for rabbits having experienced 4 weeks of facial nerve transaction (\( P = 0.015 \)). This likely indicates a mixture of innervated and denervated muscle in the BAK-treated group.

Histology sections from the untreated controls show robust, densely packed muscle fibers, connective tissue, and vasculature (Fig. 1A). After severing of the facial nerve and sustained paralysis of the OOM, atrophy of the muscle fibers is evident at 1 week (Fig. 1B) and becomes severe by 4 weeks (Fig. 1C). Similarly, the histology of group 1 shows attenuation of muscle fibers 1 week following BAK injection (Fig. 2A). The histology of group 2, however, does not show as severe a degree of atrophy as that seen in group 5 (Fig. 2B). This is likely due to the fact that some of the muscle fibers have become reinnervated and atrophy is thereby being reversed. Quantitative analysis of muscle histology is presented in Table 2, and shows evidence of muscle atrophy in all treatment groups (BAK and facial nerve transection) as compared with control group 3.

Thus, results from both electrophysiological and histological analysis appear to indicate that a single injection of BAK into the OOM of rabbits causes acute denervation with the potential for at least partial nerve regeneration beginning sometime between 1 and 4 weeks postinjection time.

**DISCUSSION**

The technique of using chronaxie measures to diagnose muscle innervation status is well established. In general, the chronaxie values for denervated muscle tend to be at least an order of magnitude greater than those of healthy innervated muscle due primarily to the fact that motor axons, which have a much shorter electrical time constant than muscle fibers, are stimulated first and subsequently activate the muscle in the normal case. The chronaxie values reported here for BAK-treated OOM generally fall within previously published ranges for denervated skeletal muscle and for denervated OOM specifically, while the values for normal OOM are more consistent with previously published values for innervated OOM and motor nerve.

The exception to the above statement includes the lowest two chronaxie values for BAK-treated OOM, measured 4 weeks postinjection. The chronaxie values for these samples lay between the normal ranges for healthy and denervated OOM. Our previous study found that rabbit facial nerve regenerated following transection in a manner that is more robust than typically seen in human facial nerve. It has been reported that chronaxie values decrease during the reinnervation process. In the case of OOM stimulation following nerve transection, we have previously seen strength-duration curves that exhibited characteristics of both innervated and denervated OOM at different pulse widths, representative of mixed denervated and reinnervated muscle fibers, during the reinnervation process. This could potentially explain the atypical chronaxie values, as our curve fitting process may atypical electrical time constant for the nerve. In either case, the lower chronaxie values seem to be explainable by the presence of partial reinnervation of the OOM.

The fact that evidence of reinnervation was present at a much earlier time following BAK injection than we previously

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**Table 2.** Histological Comparison of Muscle Fiber Atrophy

<table>
<thead>
<tr>
<th>Group</th>
<th>BAK 0.25%</th>
<th>1 wk</th>
<th>BAK 0.5%</th>
<th>4 wk</th>
<th>no injection, no nerve transaction, 4 wk</th>
<th>nerve transaction, 1 wk</th>
<th>nerve transaction, 4 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibers Counted, Mean ± SD, ms</td>
<td>Cross-Sectional Area, Normalized, Mean ± SD, ms</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group 1</td>
<td>98 ± 5.1</td>
<td>0.66 ± 0.33</td>
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<tr>
<td>Group 2</td>
<td>88 ± 6.8</td>
<td>0.81 ± 0.55</td>
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<tr>
<td>Group 3</td>
<td>76 ± 6.2</td>
<td>1.00 ± 0.60</td>
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<tr>
<td>Group 4</td>
<td>105 ± 8.1</td>
<td>0.70 ± 0.33</td>
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<tr>
<td>Group 5</td>
<td>86 ± 5.6</td>
<td>0.63 ± 0.38</td>
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</table>

Number of Fibers Counted is per ×40 HPE Cross-sectional areas are all normalized to the mean value of the control group (group 3). Groups 1, 2, 4, and 5 are all statistically different than reference group 3 (\( P < 0.05 \)).
reported for nerve transection (4 weeks compared with 8 weeks) is easily explained by the location of the induced injury. The transection study severed the nerve approximately 2 to 5 cm from the lateral canthus, meaning that following recovery from the initial trauma, several weeks would have been required for the nerve to regrow the distance to the eyelid before reinnervation could begin to occur. In contrast, the damage due to BAK injection would likely have been local to the OOM; therefore, reinnervation could potentially have occurred after recovery from the injury without the need to wait for the nerve to grow a significant length.

The qualitative and quantitative analyses of the histological images were consistent with the quantitative electrophysiological analysis presented above. Together, these indicate that injection of BAK at moderate doses into the OOM caused complete denervation and dysfunction of the muscle in a rabbit model. The amount of denervation was similar to that found in rabbits in which the facial nerve was surgically severed. Since BAK is locally neurotoxic, however, regeneration occurs more quickly than after transection of the facial nerve. Therefore, the partial innervation noted in rabbits that were stimulated at 4 weeks postinjection of BAK was likely due to partial nerve regeneration.

Chronic exposure to BAK has been linked to chronic inflammation in ocular surface structures such as the cornea and conjunctiva, which may adversely affect glaucoma filtration surgery as well as symptoms of ocular surface disease.1,2,7,8

Chemical biomarkers and histological evidence of inflammation in ocular surface tissues have been shown to be greater in BAK containing drug compounds compared with their non-BAK counterparts.5,3,4,6,9

The presence of ocular surface disease in over 100 glaucoma patients was analyzed in a study by Leung et al.25 Symptoms of dry eye were reported in 59% of patients, with 27% being severe. Signs of ocular surface disease were also seen with an abnormal Schirmer test in 61% and lissamine green staining of the conjunctiva and cornea in 22%. Abnormal tear quality was seen in 78% of patients by tear breakup time, with 65% of those severe. Importantly, after adjusting for age and sex, multivariate regression showed a 2-times greater likelihood of abnormal lissamine green test with each additional BAK-containing eye drop used.

Higher daily doses of BAK in glaucoma medications have also been linked to greater severity of ocular surface disease that decreases quality of life measures.20 In this study, the Ocular Surface Disease Index (OSDI) and Glaucoma Quality of Life-15 questionnaires were administered to mild, moderate, severe glaucoma patients and controls. The OSDI scores and the number of patients with OSD increased with increasing severity of glaucoma, and quality of life measures decreased with greater severity. A higher daily dose of BAK was significantly predictive of OSDI score in multivariate modeling.

While our study demonstrates denervation of the OOM in rabbits following acute BAK intramuscular injection, we hypothesize that a chronic exposure of BAK can result in a similar condition. A link between topical medications and the loss of orbital changes has already been established clinically with the use of prostaglandin analogs and the loss of orbital adipose tissue.27 Therefore, we believe that chronic topical BAK administration may be absorbed through the conjunctiva in quantities sufficient to affect the orbicularis oculi muscle. The clinical implications of BAK denervation of the OOM in humans is that chronic use of BAK-containing ophthalmic solutions may result in weakness of eyelid closure with lagophthalmos that can create exposure keratopathy and exacerbate ocular surface disease symptoms.

There are several limitations to our study that should be mentioned. First, the concentrations of BAK used in our rabbit model exceed those used in topical preparations by approximately 10-fold. Second, the injection of BAK into the OOM may not act in the same way as chronic topical exposure. However, since the nerve regeneration is extremely robust in the rabbit model, we felt that it was necessary to use an acute model to demonstrate the effects of BAK exposure. Future studies may look at lower doses and topical administration, but this may not be feasible in a rabbit model. We plan to conduct a study of clinical glaucoma patients using the OSDI and Glaucoma Quality of Life Index along with measures of BAK exposure and the correlation with ocular surface disease signs and clinical lagophthalmos. In addition to the daily dose of BAK medications, we suggest using a BAK drop-year index, with the daily number of BAK medications used multiplied by the number of years of use (analogous to a pack-year history of smoking).

To our knowledge, this is the first demonstration of skeletal muscle denervation associated with BAK exposure. The possibility of a similar effect due to chronic, topical exposure in humans should be explored. The clinical implications are a syndrome of lagophthalmos and corneal exposure that has the potential to cause or exacerbate ocular surface disease and decrease quality of life for patients.

References

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