Electroretinographic Effects of an Intravitreal Injection of Triamcinolone in Rabbit Retina

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Objective: To investigate the clinical, histologic, and electroretinographic effects of intravitreal injection of triamcinolone acetonide suspension in the rabbit retina.

Methods: Three groups of 6 rabbits each received intravitreal injections. Group 1 received 4 mg of triamcinolone acetonide, group 2 received an equal volume (0.1 mL) of the corticosteroid supernatant, and group 3 received 4 mg of triamcinolone acetonide with the supernatant replaced with balanced salt solution. Uninjected left eyes served as controls. Electroretinograms were obtained at baseline and at 3 to 4 and 6 to 7 days after injection of triamcinolone. Enucleated eyes were examined histologically.

Results: Ocular examination revealed no differences among the 3 groups. When subjected to stimulation with moderate to high flash intensities, eyes that had received intravitreal injections of triamcinolone (groups 1 and 3) had a 10% to 25% increase in dark-adapted a- and b-wave electroretinographic amplitudes. No histologic differences were observed between injected and control eyes.

Conclusions: Intravitreal injection of 4 mg of triamcinolone acetonide does not cause a toxic reaction in the rabbit retina after 7 days. Triamcinolone therapy may augment the rod-driven electroretinographic responses, suggesting a mechanism by which visual function may improve.

Clinical Relevance: Evaluation of the toxic effects of triamcinolone is useful because of increased applications of intravitreal injection.


Promising results have been observed with the use of intravitreal injection of triamcinolone acetonide to improve the visual acuity of patients with diabetic macular edema and with age-related macular degeneration. This approach may be an emerging remedy for these devastating retinal disorders. The use of intravitreal injection of triamcinolone was initially proposed as a method of inhibiting proliferative vitreoretinopathy, with early studies demonstrating no toxic effects in rabbits. However, caution must be exercised when extrapolating these data to human use because a preparation of pure triamcinolone was used in the animal studies rather than commercial corticosteroid preparations. Toxic preservatives in the vehicles of commercially available corticosteroids have been suggested as possible contributors to proliferative vitreoretinopathy. Sterile endophthalmitis has been reported following intravitreal injection of a corticosteroid, and the toxic effects of commercial vehicle preservatives has been proposed as a possible cause.

Because of the increasing use of intravitreal triamcinolone treatment, despite concerns about the toxic effects, we conducted an investigation in rabbits to determine whether there are differences in the clinical, histologic, and electroretinographic (ERG) responses to intravitreal injections of (1) commercially available triamcinolone, (2) the supernatant, and (3) triamcinolone reconstituted with sterile balanced salt solution. A second aim of the study was to examine the ERG response to determine whether an intravitreal injection of triamcinolone modifies rod and cone pathway function in normal rabbit retina. Although the mechanisms by which triamcinolone therapy aids vision are unknown, the improvement of central visual acuity observed in patients who receive intravitreal injections of triamcinolone is presumably caused by restoration of normal retinal thickness (improvement of macular edema), which primarily affects cone pathway function. In rodent models of retinal degeneration, activation of the glucocorticoid receptor improves retinal morphologic structure and function. However, because retinal thickness is decreased in the rodent model...
of retinal degeneration and because the rodent retina is rod dominant without a cone-rich macula, the therapeutic mechanisms of intravitreal triamcinolone in the human retina may be different from those in the rodent model.

### METHODS

#### ANIMALS

The study was conducted in accord with the Association for Research in Vision and Ophthalmology's statement on the use of animals and was approved by the Animal Care and Use Committee of the University of Missouri–Columbia. Eighteen New Zealand white rabbits, weighing 2.0 to 3.0 kg each, were housed in separate cages under a 12-hour light and 12-hour dark cycle. Albino rabbits were chosen because ocular pigmentation may protect against the toxic effects of the drug. One rabbit's right eye was excluded because it exhibited an unexplained decrease in ERG activity (ie, amplitudes were approximately 20% of normal values) after intravitreal injection of triamcinolone.

#### DRUG PREPARATION AND INTRAVITREAL INJECTION

The animals were divided randomly into 3 groups, with 6 animals in each group. Group 1 animals received an intravitreal injection of 4 mg of triamcinolone acetone injectable suspension (0.1 mL of Kenalog-40; Bristol-Myers Squibb Company, Princeton, NJ). Group 2 animals received an intravitreal injection of an equal volume (0.1 mL) of the corticosteroid supernatant. Group 3 animals received an intravitreal injection of 4 mg of triamcinolone acetone with the commercial supernatant removed and reconstituted with 0.1 mL of balanced salt solution. Commercially available triamcinolone (for group 1) was withdrawn from the vial after shaking it to disperse the precipitated suspension. Neither the drug nor vehicle was modified. Triamcinolone supernatant (for group 2) was prepared by first allowing the corticosteroid suspension to settle and then by withdrawing the clear supernatant. No white suspension of corticosteroid was visible in the supernatant solution. Reconstituted triamcinolone (for group 3) was prepared by allowing the corticosteroid suspension to settle before aspirating the supernatant as completely as possible without disturbing the precipitate. A volume of balanced salt solution equal to the amount of supernatant removed was then injected into the vial. The vial was shaken to disperse the suspension before the drug was withdrawn from the vial for injection.

All injections were in the right eye. The uninjected left eye served as a control. Animals were anesthetized with intramuscular ketamine hydrochloride (25 mg/kg) and xylazine hydrochloride (2 mg/kg). The injections were performed using a 27-gauge needle, bevel up, approximately 4 to 5 mm posterior to the corneoscleral limbus in the superotemporal quadrant.

#### OCULAR AND ERG EXAMINATIONS

Ocular examinations were performed immediately before injection (baseline) and at days 1, 3, and 7 following injection. Examinations included slitlamp anterior segment and detailed dilated funduscopy examination of both eyes. A baseline ERG was obtained in each eye of all animals 1 day before intravitreal injection. Following injection, ERGs were obtained twice in all animals, at days 3 and 6 in 3 animals from each group and at days 4 and 7 in the remaining 3 animals from each group. Therefore, ERGs were obtained in all animals at day 3 or 4 and then again at day 6 or 7 following injection.

#### ERG RECORDING

Standard Ganzfeld dark- and light-adapted ERGs were obtained, using a procedure adapted from previous protocols. Recordings and data analysis were made by investigators (B.L. and K.Z.) masked to the experimental groups. Rabbits were dark adapted for at least 1 hour and were anesthetized 20 minutes before ERGs were obtained. The cornea was anesthetized with topical 0.5% proparacaine hydrochloride, and the pupils were dilated with 1% tropicamide. Burian-Allen corneal bipolar electrodes (Hansen Laboratory, Iowa City, Iowa), containing reference and active electrodes integrated into a contact lens, were used, and the ground was placed subcutaneously on the back. Electroretinographic recordings were obtained in both eyes simultaneously, with the head in the upright position.

Signals were amplified at 10,000 gain and bandpass filtered between 0.1 and 1000 Hz (~3 dB points), digitized at 5.12 kHz with a data acquisition device (National Instruments, Austin, Tex), and computer averaged with a custom-built program. Ganzfeld white light illumination was provided by a modified electronic flash (maximum intensity, 2.17 log candelas (cd)/s/m²; Vivator, Santa Monica, Calif). Stimulation intensity was set by neutral density filters (Kodak, Rochester, NY) at 1.0–log unit steps covering a 7.0-log unit range. The light-adapted ERG was recorded on a continuous light-adapting white background of 30 cd/m² after a 10-minute period of light adaptation, with stimulus intensity beginning below the light-adapted b-wave threshold at ~0.83 log cd/s/m².

Flashes were controlled by an electronic timer (UNIBLITZ; Vincent Associates, Rochester). In the dark-adapted ERG recording, the intrastimulus interval at low intensities was 15 seconds; at high intensities, the intrastimulus interval was longer than 150 seconds. Luminance was calibrated with an IL-1700 integrating radiometer-photometer (International Light Inc, Newburyport, Mass).

#### ERG ANALYSIS

We studied ERG signals off-line with a custom-compiled analysis program. Dark-adapted and light-adapted a- and b-wave peak amplitudes were measured from the prerecord baseline or, in the case of the b-wave when an a-wave was present, from the a-wave trough. A ratio of the amplitudes in the injected and control eyes was then derived for analysis. The use of a ratio overcomes the variance between the 2 eyes, as well as that introduced by the injection procedure, anesthesia, body temperature, and circadian rhythm.

To demonstrate whether statistically significant differences existed between injected and control eyes, multivariate analysis of variance was performed using SPSS for Windows (version 10; SPSS Inc, Chicago, Ill). The primary outcome variable was the ratio of the injected and control eyes of each rabbit for the dark-adapted a- and b-wave and light-adapted b-wave amplitudes at baseline, at 3 to 4 days after injection, and at 6 to 7 days after injection. Predictor variables were ERG amplitudes and treatment groups. When a significant difference (<.05) was found between groups, 1-way analysis of variance was performed at each intensity level, and post hoc tests were used. The significance between the experimental groups was determined using Bonferroni correction.
Animals were humanely killed with intracardiac pentobarbital overdose. Ten eyes were enucleated for histologic evaluation from group 1 (3 eyes), group 2 (2 eyes), group 3 (2 eyes), and the controls (3 eyes). Enucleated eyes were fixed with Davidson fixative for 48 hours and then rinsed with tap water and transferred to a 10% neutral-buffered formalin solution for shipment to a commercial histology laboratory (Research Pathology Services, New Britain, Pa), where the eyes were processed for paraffin embedding, sectioned through the 12-o’clock position of the limbus and the optic nerve (dorsal-ventral), and stained with hematoxylin-eosin.

RESULTS

CLINICAL OBSERVATIONS

Slitlamp examination at baseline and following injection at days 1, 3, and 7 did not reveal any cell, flare, or hypopyon in the anterior chamber of injected eyes or control eyes. No cataract formation was seen. Fundus examination showed normal retinal appearance, with no detachment or opacity of the retina seen. In eyes injected with commercially available triamcinolone (group 1) and reconstituted triamcinolone (group 3), clumped white triamcinolone precipitate was seen ophthalmoscopically in each injected eye. Triamcinolone became more dispersed by day 6 or 7.

DARK-ADAPTED ERG

At baseline, the a- and b-wave thresholds and amplitudes were almost identical in both eyes in all groups over a wide range of intensities, as shown in the plots of ERG amplitude compared with flash intensity curves (Figure 1). These waveforms and amplitudes are comparable to those observed in previous studies.22,23 At postinjection days 3 to 4 and 6 to 7, the a- and b-waves, thresholds, and amplitudes were comparable in all rabbits (Figure 1 and Figure 2). However, in both groups injected with triamcinolone, a 10% to 25% increase in the amplitudes of the a- and b-waves in injected eyes, relative to their respective control eyes, was observed from moderate intensities near the b-wave plateau (−1.83 to 0.17 log cd·s/m²) to the high intensities (>0.17 log cd·s/m²) (Figure 1). Below the plateau intensity, the response amplitudes in all injected eyes injected with triamcinolone became more dispersed by day 6 or 7.
eyes were similar to those in the corresponding control eyes. In group 2 (supernatant injected), an increase in a- and b-wave amplitudes was not observed.

To better compare differences in ERG amplitudes among groups across the intensity range, we plotted the mean ratio of the injected and the control eyes for each group and performed statistical analysis (Figure 3). Baseline dark-adapted b-wave amplitude ratios were almost 1.0 in all 3 groups. At days 3 to 4 and 6 to 7, the ratios of groups 1 and 3 (triamcinolone injected) were above 1.0, while the ratios of group 2 (supernatant injected) were consistently around 1.0. The dark-adapted b-wave amplitude ratio of group 1 was significantly higher than that of group 2 at −0.83 log cd·s/m² at days 3 to 4. The ratio in group 2 (supernatant injected) remained almost 1.0 at both postinjection time points (Figure 4). However, there was not a statistically significant difference (P > .05) at any time point among the 3 groups.

The light-adapted a-wave contains responses generated from cone photoreceptors and second-order neurons, including cone bipolar cells and horizontal cells.29 Because the relative contributions of the cone photoreceptors and the bipolar cells are unknown in the rabbit, we did not analyze the light-adapted a-wave amplitude ratios.

HISTOLOGIC FINDINGS

Light microscopic examination showed no difference between the 3 groups and the control eyes in the central retina (immediately superior to the optic nerve) or in the peripheral retina (superior periphery). The photoreceptor layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, and ganglion cell layers appeared to be of normal thickness. The cell nuclei were well oriented and appeared normal. No quantitative measurements were made.

We demonstrated that intravitreal injection of 4 mg of triamcinolone acetonide does not cause a toxic effect in rabbit retina for at least 7 days. In addition, we showed that triamcinolone transiently augments the rod-driven ERG responses, suggesting that triamcinolone treatment may improve visual signal transmission in the retina.

Because the possibility of toxic effects from the preservatives in triamcinolone has been proposed as a cause of sterile endophthalmitis in humans, we wanted to determine whether the occurrence of a transient toxic reaction could be documented by the retinal appearance, function, or histologic findings in these experimental eyes. We did not observe sterile endophthalmitis or any other evidence of clinical toxicity. Results of the histologic examinations were normal. No decrease in ERG response was found in the rabbits that received the vehicle with associated potential toxic preservatives (groups 1 and 2). Plausibly, a decrease in the ERG response in group 1 because of preservatives may have been masked by the antiinflammatory effects of the large bolus of corticosteroids. However, a similar effect would not be expected in group 2, yet an ERG decrease was not observed in either group. Admittedly, although no precipitated corticosteroid was present in group 2, triamcinolone was presumably present in solution. Triamcinolone in solution (without the precipitated corticosteroid) has a short half-life of 2.9 hours and would not be expected to persist long enough to affect the ERG responses during the time frame of this study.30 An evaluation of the effects of the vehicle devoid of corticosteroid has been performed.15 The findings of that study are consistent with those of a previous investigation examining the toxic effects of triamcinolone.15
Curiously, we found an increase in the rod ERG b-wave in triamcinolone-injected eyes. To our knowledge, this is the first report that triamcinolone enhances the ERG response in normal retina. Across all study groups, the ERG waveforms were consistent with those of previous rabbit studies. However, compared with baseline values, the maximum rod b-wave amplitudes were increased in the eyes injected with triamcinolone and in the eyes injected with the supernatant without drug precipitate.

As the only objective retinal function test for animals, the ERG reveals retinal function layer by layer (mainly the photoreceptors and bipolar cells) and column by column (rod and cone pathways). The dark-
adapted b-wave is generated primarily from rod bipolar cells and the a-wave primarily from rod photoreceptors. For light intensity lower than −0.83 log cd·s/m², the b-wave is generated mainly from rod bipolar cells and is an indicator of rod pathway function. For light intensity higher than 0.17 log cd·s/m², at which the a-wave emerges, the cone pathway system becomes activated, and the dark-adapted b-wave contains input from rod and cone pathways.

We found the dark-adapted b-wave amplitude to be higher at all intensity levels in eyes injected with triamcinolone (groups 1 and 3) than in the supernatant-injected eyes (group 2). However, only the ratio between groups 1 and 2 at −0.83 log cd·s/m², which elicits the maximum rod-driven responses, showed a statistical difference. At the lower intensities, the b-wave threshold and b-wave ratios were not significantly higher. These results suggest that triamcinolone increased the rod bipolar cell responses but did not affect the rod pathway system threshold and sensitivity. The reason that the b-wave ratio was not significant at higher intensities may reflect an intrusion or a buffering effect of the cone pathway function. As the intensity increases (greater than −0.83 log cd·s/m²), the cone pathway system contribution to the dark-adapted ERG responses increases. Because the cone pathway is less sensitive to triamcinolone treatment in the rabbit retina than in the human retina (as discussed later in this section), the intrusion of the cone pathway system would minimize the effect of triamcinolone on the rod pathway system.

The single flash recorded in the light-adapted ERG responses in the presence of rod-suppressing background light reflects activity of the cone pathway system, primarily cones and cone bipolar cells. We found only a slight increase in the amplitude ratios of the light-adapted b-wave in eyes that received triamcinolone injections (groups 1 and 3). However, these elevations were not significant (P > .05); therefore, the effect of triamcinolone injection on the rabbit cone bipolar cells seems to be minimal.

An alternative explanation for the increased rod b-wave observed in triamcinolone-injected eyes may be the presence of white vitreous corticosteroid precipitate. This precipitate may produce an optical effect, similar to that in the early stages of cataract, in which light is scattered and may enhance the ERG response. In this scenario, the increase in photons should enhance the dark-adapted ERG b-wave amplitude at the b-wave threshold where the rods are sensitive to light. Conversely, if the corticosteroid precipitate decreased the number of photons reaching the retina, the dark-adapted ERG threshold should be increased and the amplitude decreased at the low intensities. Because these patterns were not observed in eyes with corticosteroid precipitate in the vitreous (groups 1 and 3) (Figures 1 and 2), we believe that this optical scatter is not the cause of the increased b-wave amplitude observed.

Our ERG findings suggest that triamcinolone injection may enhance normal rod pathway function at middle stimulus intensities that elicit the maximum rod-driven responses. These results raise several interesting points regarding the use of corticosteroids in human retinal disease. In patients with macular edema, intravitreal injection of triamcinolone has been shown to increase visual acuity, which is primarily a cone pathway function. Glucocorticoid receptor activation results in increased ERG amplitudes and improved morphologic structure in rodent models of retinal degeneration. However, the mechanisms that improve visual function in human macular edema may be different from those in rodent models of retinal degeneration. The improvement in cone pathway function observed in patients with macular edema may be a secondary result of an anatomical improvement of the macular edema, presumably stabilization of an incompetent blood-retinal barrier. In a rodent model of retinal degeneration, preservation of retinal function (mainly the rod pathway system) and associated morphologic structure may be a primary result of apoptosis inhibition.

We also observed an increased, although statistically insignificant, cone ERG response after intravitreal corticosteroid injection. Similar to rodents, rabbits have an excitatory E-type ERG response, with a less-advanced cone pathway system than that in primates. Therefore, to understand the beneficial effects of triamcinolone treatment, further studies of the cone pathway system after intravitreal injection of triamcinolone in patients with macular edema and in healthy nonhuman primates are essential. In addition, our findings cannot be directly compared to humans because intravitreal injection of 4 mg of triamcinolone acetonide in a rabbit may result in higher concentrations of drug than in humans because of the smaller rabbit vitreous volume.

One rabbit from group 2 (supernatant injected) was excluded from analysis because of an 80% reduction in the ERG b-wave ratio as measured at day 7 following injection. Because this ratio of 0.2 was dramatically different compared with all other eyes throughout the study (ratio range, 0.9-1.4), we believe this measurement to be an artifact and not caused by the injected supernatant. Ocular examination showed no abnormalities in this eye. Histologic examination was not performed.

We evaluated the effects of intravitreal triamcinolone in rabbit eyes and found no toxic retinal effects associated with the corticosteroid or preservatives in the vehicle for up to 7 days. We demonstrated transiently increased rod ERG b-wave amplitudes in triamcinolone-injected normal rabbit eyes. Intravitreal injection of triamcinolone may be beneficial in patients with diabetes mellitus and macular edema not only by improving the anatomical edema but also by enhancing rod bipolar cell function. Further studies are needed to explore its effects on the primate cone pathway system. Our observations also suggest a potential use of triamcinolone to enhance rod pathway function in inherited retinal degeneration and dystrophy, such as retinitis pigmentosa, in which progressive peripheral rod pathway degradation is a hallmark.

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