RETINAL TOXICITY OF INTRAVITREAL KENALOG IN ALBINO RABBITS

YARON LANG, MD,* ESTHER ZEMEL, PHD,† BENJAMIN MILLER, MD,‡ IDO PERLMAN, PHD†

Purpose: To evaluate possible toxicity of intravitreal Kenalog (commercial triamcinolone acetonide) to the retina of albino rabbits.

Methods: Forty-three albino rabbits were injected intravitreally with 0.1 mL of experimental solution to the right eye and 0.1 mL of saline to the left eye (control). Rabbits in Group A (n = 28) were injected with 4 mg/0.1 mL of Kenalog suspension; rabbits in Group B (n = 8) were injected with 0.1 mL of Kenalog vehicle; and rabbits in Group C (n = 7) were injected with 4 mg/0.1 mL of triamcinolone acetonide. Rabbits were examined ophthalmoscopically and by electroretinogram (ERG) recordings before and at different time intervals after injection. At the end of follow-up, animals were killed and the retinas were prepared for light microscopy.

Results: Thirty-eight rabbits completed 4 weeks of follow-up. Follow-up for 8 and 17 weeks was completed by 29 and 3 rabbits, respectively. Intravitreal commercial Kenalog or its vehicle alone caused approximately 50% reduction in the ERG b-wave amplitude at the end of follow-up. Pure triamcinolone acetonide caused only mild (up to 14%) reduction of the ERG b-wave amplitude. Histologic examination of retinas exposed to Kenalog or its vehicle showed severe damage to all retinal layers in areas close to the site of Kenalog injection.

Conclusions: Intravitreal injection of 4 mg Kenalog suspension is retinotoxic to albino rabbit eyes. The vehicle of Kenalog is probably the main cause of this toxicity.

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Kenalog (commercial triamcinolone acetonide [TA]) is a steroidal preparation that is commonly used in the ophthalmic clinic for treating a variety of sight-threatening eye conditions. It is used in patients with unresponsive diabetic macular edema,[1–4] age-related macular degeneration (AMD),[5–8] cystoid macular edema related to uveitis,[9] birdshot retinochoroidopathy,[10] macular edema following central[11–13] or branch[14,15] retinal vein occlusion, and as an intraoperative tool during vitrectomy procedures.[16–18] In animal models, TA was tested to prevent the development of experimental proliferative vitreoretinopathy (PVR)[19–21] and of preretinal neovascularization.[22]

TA alone suspended in balanced saline solution was injected intravitreally in pigmented rabbits, and was shown to be nontoxic by electroretinography (ERG) and histology during 3 months of follow-up.[23] However, in addition to TA, commercial Kenalog contains benzyl alcohol, carboxymethyl cellulose, polysorbate (Tween 80), and saline. Benzyl alcohol has been

From the *Department of Ophthalmology, Ha'Emek Medical Center, Afula; †The Ruth and Bruce Rappaport Faculty of Medicine, Technion–Israel Institute of Technology and Rappaport Institute, Haifa; and ‡Department of Ophthalmology, Rambam Medical Center, Haifa, Israel.

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Reprint requests: Yaron Lang, MD, Dept. of Ophthalmology, Ha'Emek Medical Center, Afula 18000, Israel; e-mail: ylang@012.net.il

found lately to be toxic to the retina of albino rabbits.[24] Similarly, the chemical Myristyl-γ-picolinium, serving as preservative in the steroidal preparation depo-Medrol, was found to be toxic to albino rabbit retina.[25,26] Therefore, the toxicity of commercial drugs always should be tested. Kenalog, injected intravitreally in pigs and rats, did not cause apparent retinal toxicity as assessed from clinical inspection, photography, and histologic examination.[27,28] The potential toxic effects of vehicles for some commercial steroid preparations, including Kenalog, were tested in pigmented rabbits and were proved to be nonretinotoxic during 14 days of follow-up by clinical inspection and histologic evaluation.[29] However, TA was shown to remain inside the nonvitrectomized rabbit eye for as long as 41 days after intravitreal injection,[30] and therefore, long-term follow-up is needed. Furthermore, retinal toxicity of drugs should also be tested by sensitive functional tests that can be affected before any clinical or morphologic changes become evident.[26]

The purpose of the present study was to evaluate long-term effects of intravitreal Kenalog in New Zealand albino rabbits (NZW rabbits) by clinical, electrophysiologic, and histologic examination to test possible toxicity of the drug.

Materials and Methods

Animals

Adult (12–14 weeks old) NZW rabbits (n = 43), weighing 2–2.5 kg, were housed in separate cages, under a 12/12 hours light/dark cycle. Water and food were given ad libidum. Before inclusion in the study, animals were tested for clinical abnormality (e.g., cataract, retinal malformation) or ERG asymmetry (differences of >10% in dark- and light-adapted a-wave or b-wave amplitudes) between the two eyes.

Before clinical examination, ERG recordings, and intravitreal injection, the rabbits were anesthetized by intramuscular injection (0.5 mL/Kg body weight) of a mixture composed of 1.0 mL ketamine hydrochloride (10 mg/mL), 0.2 mL acepromazine maleate solution (10%), and 0.3 mL xylazine solution (2%). The pupils were fully dilated with cyclopentolate hydrochloride 1%. At different time intervals during the follow-up period, rabbits were killed by intravenous injection of sodium pentobarbital (80 mg/Kg body weight). The eyes were enucleated and the retinas were prepared for light microscopy.

Principles of laboratory care (NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Human Care and Use of Laboratory Animals (revised 1986), and the US Animal Welfare Act, as amended, were followed, as well as national laws and institutional guidelines.

Drug

Kenalog (Bristol-Myers Squibb, Dublin, Ireland) is available in Israel as a 1 mL vial of suspension, containing 40 mg triamcinolone acetonide. The vehicle contains 1.5% benzyl alcohol (0.99% in the United States), sodium carboxymethylcellulose, polysorbate (Tween 80), sodium chloride, and water. To separate the active component (TA) from the vehicle, we left the Kenalog syringe standing upside down for 15 minutes until white sediment of TA was separated from an upper layer of clear vehicle. The vehicle then was aspirated using a sterile syringe and 21 G needle and was kept separately. An equal volume of saline was added to the TA and the new mixture was stirred to ensure homogeneity of the suspension. The volume of injected Kenalog suspension, TA suspension, vehicle, or saline was 0.1 mL for all rabbits.

Rabbit Groups

Twenty-eight out of the 43 rabbits were treated with 0.1 mL of whole Kenalog suspension (4 mg) injected intravitreally to the right (study) eye while saline was injected intravitreally to the left, control eye (Group A). Eight rabbits (Group B) were treated with vehicle and saline, injected intravitreally into the right and left eyes, respectively. An additional 7 rabbits (Group C) were treated similarly with 4 mg/0.1 mL of TA suspension and saline solution that were injected intravitreally into the right and left eyes, respectively.

Thirty-eight rabbits completed 4 weeks of follow-up. Eight weeks of follow-up were completed by 29 rabbits, belonging to Groups A (n = 15), B (n = 7), and C (n = 7). Three rabbits of Group A were followed for 17 weeks.

Intravitreal Injection

All intravitreal injections were performed similarly. A 25-gauge needle was inserted into the vitreous, approximately 1 mm posterior to the limbus, and directed under visual control (indirect ophthalmoscope) towards the retina, just above the optic disk. A volume of 0.1 mL was slowly injected with the bevel of the needle pointing away from the retina. Three rabbits out of 28 that were injected with Kenalog (Group A) were excluded from the study due to perioperative complications: inadvertent touch of the retina by the injection needle occurred in two rabbits and traumatic cataract obscuring the retina developed postoperatively in one additional rabbit. Overall, 40

rabbits (25 of Group A, 8 of Group B, and 7 of Group C) were included in the study.

Intraocular Pressure Measurements

In seven of the rabbits injected with Kenalog (Group A), applanation tonometry (Tonopen XL, Medtronic, Jacksonville, FL) was performed in both eyes, while the animals were under general anesthesia. The Tono-Pen self-calibration was performed before each use. Readings with variability greater than 5% were rejected. The measurements were taken from both eyes of the animals before, 1 minute after, 1 hour later, and then 2, 4, and 8 weeks after intravitreal injection.

Electroretinogram

The ERG responses were recorded simultaneously from both eyes, using corneal electrodes (Medical Workshop, Groningen, Holland) as active electrodes. Surgical needles, inserted into the ears, served for reference and ground electrodes. The ERG signals were amplified (x20,000) and filtered (0.3–300 Hz) by differential amplifiers (Grass, West Warick, RI), and then stored in a computer equipped with a data acquisition board (National Instruments, Austin, TX). To improve signal/noise ratio, 6 ERG responses (10-second intervals) were averaged in the dark-adapted state, while 15 responses (1 Hz) were averaged in the light-adapted state. Light stimuli were obtained from a Ganzfeld light source (LKC Technologies, Gaithersburg, MD), having an unattenuated intensity of 5.76 cd-s/m^2.

ERG analysis was based on amplitude measurements. The a-wave was measured from the baseline to the trough of the negative wave, and the b-wave amplitude was defined from the trough of the a-wave to the peak of the b-wave. The light-adapted ERG responses of rabbits were of small amplitude (<100 μV) to allow reliable quantitative analysis.

The response-intensity curve of the dark-adapted ERG b-wave was fitted to a hyperbolic function[31]:

$$V/V_{max} = I/(I + \sigma) \quad (1)$$

where V is the amplitude of the ERG b-wave that was elicited by a stimulus of intensity I. Vmax is the maximal amplitude and σ is the semisaturation constant.

This study was designed for prolonged ERG follow-up. Therefore, we induced relatively light anesthesia as described before and lowered animal discomfort by applying topical anesthetic drops (benoxinate HCL 0.4%) to both eyes. Thus, we encountered ERG variability for a given rabbit between recording sessions. To circumvent this variability, we assessed the functional integrity of the experimental eye in a given postinjection time interval from the Vmax ratio (experimental/control) and the difference of log σ (experimental-control).[36]

All ERG measurements and clinical examinations were performed before and at different time intervals after intravitreal injection (1 day, 1, 2, 4, 8, and 17 weeks). The effects of the experimental drugs on the functional integrity of the retina were evaluated by comparing the ERG data of the experimental and control eyes at each time interval.

To separate the Kenalog effect on photoreceptors from that on bipolar cells, we isolated the P-III component of the ERG by a mixture of 2-amino-4-phosphonobutyric acid (APB) and 2-cis-piperidine-2, 3-dicarboxylic acid (PDA) that was injected intravitreally in a dose of 0.67 mmol/L and 1.33 mmol/L, respectively. This mixture has been shown to block synaptic transmission from the photoreceptors to second order neurons, isolating the receptor component (P-III) of the ERG.[32]

Histology

Light microscopy was conducted to assess the extent of structural damage and to identify the retinal layers that were involved. After enucleation, the eye was soaked for 10 minutes in 2% paraformaldehyde solution and 2.5% glutaraldehyde in 0.1 moles/L phosphate buffer saline (PBS) of pH 7.4. Then, a small slit was made, 2 mm posterior to the limbus, to facilitate fixation. After 72 hours, the anterior segment of the eye was removed by a circumferential incision 2 mm posterior to the limbus. The posterior eyecup was bisected at the level of the optic disk. One half of each eyecup was rinsed in BPS and then dehydrated twice in 70% alcohol for 3 hours and twice in 96% alcohol for another 3 hours. Embedding was done in a JB-4 resin (Bio-Rad, Waterford, England). Tissue sections were cut by a microtome (Reichert Jung, Heidelberg, Germany) at a thickness of 2 μm, and mounted onto slides that were stained with Richardson's stain.

Data Analysis

A primary outcome was considered any deterioration in ERG recording in the study eyes along the follow-up. A change in an ERG parameter of the experimental eye relative to the control eye during the follow-up period with a p value smaller than 0.05 was regarded as significant. A secondary outcome was regarded as elevation of the rabbit intraocular pressure (IOP)

above 21 mm Hg or any severe ocular complication during follow-up or any anatomic damage found on histology after euthanasia of animals.

All data obtained in each clinical and ERG examination as well as on histologic work-up were noted for later evaluation.

Statistical analysis was done using the SPSS v11.5 software (SPSS Inc., Chicago, IL). Comparative statistical analysis of ERG parameters values were evaluated using the analysis of variance (ANOVA) test, the nonparametric Friedman test, the nonparametric Mann-Whitney test, and the Wilcoxon signed ranks test. For IOP statistical analysis we used the Mann-Whitney test. Probability (P) values of <0.05 were considered statistically significant.

Results

Clinical Observations

After intravitreal injection, TA appeared as a white opaque mass floating in the vitreous in front of the retina. The drug suspension mass became smaller during follow-up, and disappeared completely in most rabbits (35 out of 38) after 4 weeks. In three rabbits, remnants of Kenalog could be noticed for as long as 8 weeks after injection. None of the rabbits included in the study developed significant complications following intravitreal injection. Local lens opacities were noticed in two rabbits 1 week after injection without any further aggravation. Small peripheral retinal hemorrhages were noticed in another rabbit 1 week after intravitreal injection and disappeared uneventfully 2–3 weeks later.

IOP was measured in seven rabbits of Group A, and the results are shown in Figure 1. Immediately after intravitreal injection, the IOP increased in all 14 eyes. It rapidly decreased to normal ranges 1 hour following injection. However, in eyes injected with Kenalog, a second increase of the IOP appeared in three of the seven eyes, 2–8 weeks after Kenalog injection. However, in two rabbits the IOP normalized at 8 weeks after injection. No secondary IOP increase was observed in eyes injected with saline alone. The difference in mean IOP between study and control eyes was statistically significant only at 4 and 8 weeks after injection ($P = 0.027$ and $P = 0.039$, respectively, Mann-Whitney test). The transient increase in IOP immediately following intravitreal injection was accompanied by a mild corneal edema that resolved completely within 2–3 hours in all 80 eyes of the study.

Electroretinogram

Figure 2 shows representative light- and dark-adapted ERG responses of one rabbit from Group A.

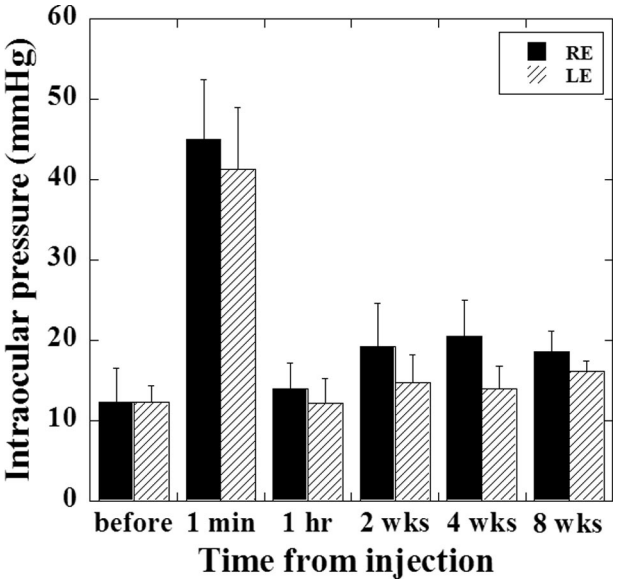

Fig. 1. The effect of intravitreal Kenalog on the intraocular pressure (IOP) in seven rabbits. IOP measurements were taken before and then 1 minute, 1 hour, 2, 4, and 8 weeks following intravitreal injection of Kenalog to the study eyes (filled bars) and saline to the control eyes (striped bars).

The rabbit was injected with Kenalog in the right eye and with saline in the left eye (upper and lower traces in each pair of responses, respectively). Severe ERG deficit, manifested mainly by a decrease in the b-wave amplitude, is noticed in the study eye compared to the control eye as early as 1 day after injection, and is evident for the entire follow-up period. We constructed the response-intensity curves and fitted them

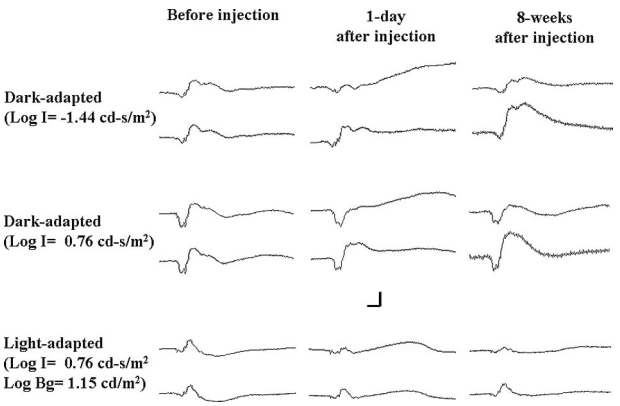

Fig. 2. The effects of Kenalog upon the dark- and light-adapted electroretinogram (ERG) responses of one rabbit. The ERG responses were recorded before (left column), 1 day (middle column), and 8 weeks (right column) after intravitreal injection of Kenalog into the right eye and saline into the left eye (upper and lower traces in each pair of responses, respectively). Background intensity (in cd/m^2) and intensities of light stimuli (in cd-s/m^2) are given in log units to the left of each row of responses. Calibration bars: vertical, 100 μV, horizontal 50 msec.

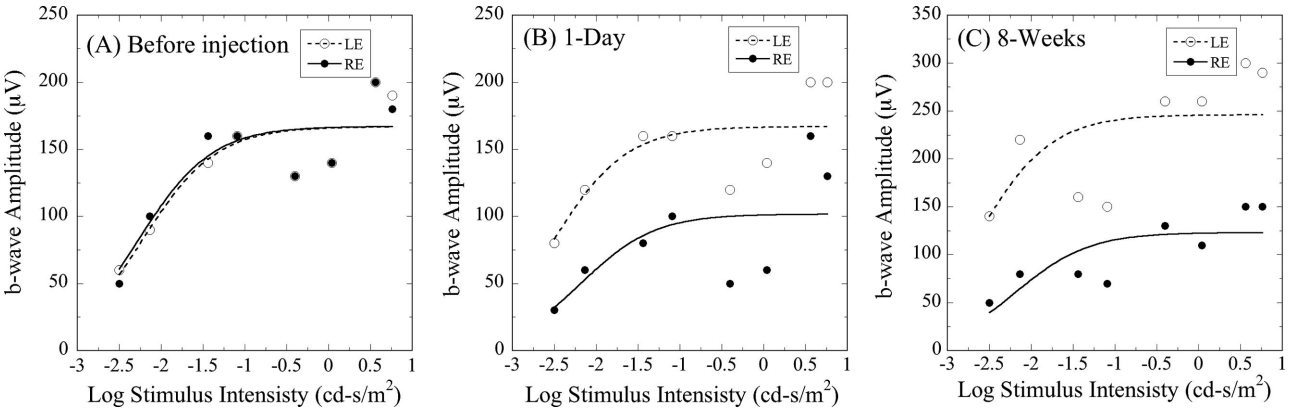

Fig. 3. Effects of Kenalog on the response-intensity curves of the rabbit whose electroretinogram (ERG) responses are shown in Figure 2. ERG data were obtained before intravitreal injection (**A**), 1 day (**B**), and 8 weeks (**C**) after injection. Data were fitted to equation (1) to calculate the amplitude of the maximal response (Vmax) and the semisaturation constant (σ). Response-intensity curves are compared for the right, experimental eye and the left, control eye (filled and open circles, respectively).

to equation (1) to obtain the maximal amplitude (Vmax) and the semisaturation constant (σ). As shown in Figure 3, the two eyes were characterized by a similar response-intensity curve before injection. A substantial damage (39% reduction in b-wave Vmax) in the study eye compared to the control eye is seen already at 1 day postinjection (Figure 3B, 101.8 μV compared to 167.1 μV). The semisaturation constant of the study eye increased by 0.33 log unit compared to the control eye (–2.17 compared to –2.50 log cd-s/m^2). At 8 weeks after injection (Figure 3C), the b-wave Vmax of the study eye was reduced by 50% compared to the control eye (123.3 μV compared to 246.2 μV), and the semisaturation constant decreased by 0.45 log units (–2.62 compared to –2.17 log cd-s/m^2). Although Figures 2 and 3 clearly show the decrease of the b-wave amplitude in the study eye after intravitreal Kenalog injection, there is an apparent improvement in both eyes noticed 8 weeks after injection. However, the ratio study/control of the b-wave Vmax obtained 1 day and 8 weeks after intravitreal injection (0.61 and 0.50, respectively) are relatively constant with slight deterioration of the study eye with time. The change with time of the ERG pattern and amplitudes may represent different physiologic states at different sessions. However, such changes affect both eyes similarly, and therefore the Vmax ratio was less affected.

Findings similar to those shown in Figures 2 and 3 were obtained in all rabbits of Group A. For all eyes tested in a given time interval after Kenalog injection, the b-wave Vmax, Vmax ratio, the semisaturation constant (σ), and the differences in log σ were calculated and averaged (Table 1).

The mean Vmax value obtained from the study eyes before intravitreal Kenalog injection (207.8 μV) deceased at 1, 2, 4, and 8 weeks after the injection (133, 110.9, 135.5, and 122.1 μV, respectively). The differences between the preinjection value and those obtained 1, 2, 4, and 8 weeks after the injection were statistically significant ($P = 0.008$, 0.02, 0.02, and $P < 0.001$, respectively, analysis of variance test). In contrast, no statistically significant differences were found between the same parameters in the control eyes.

The mean Vmax ratio along the entire follow-up period of Group A rabbits is shown in Figure 4A. The mean preinjection Vmax ratio (1.05 $\pm$ 0.12) was well within our laboratory data of normal rabbits,[25,36] indicating no differences in retinal function between the two eyes of each rabbit before injection. The mean Vmax ratio decreased to 0.49 ($\pm$0.13, n = 16) 1 day after injection. Following a slight recovery at 1 week (mean Vmax ratio = 0.59 $\pm$ 0.15, n = 25), the degree of Kenalog-induced functional damage stabilized, and at 2, 4, and 8 weeks after injection, the mean Vmax ratios were 0.56 $\pm$ 0.12 (n = 24), 0.48 $\pm$ 0.13, and 0.52 $\pm$ 0.13 (n = 15), respectively. The differences between the mean Vmax ratio obtained before and those obtained 1, 2, 4, and 8-weeks after intravitreal Kenalog injection were all statistically significant ($P < 0.001$, ANOVA test). The additional deterioration shown in Figure 4A at 17 weeks of follow-up (Vmax ratio = 0.33) is probably not significant since only three rabbits were tested at this time interval.

The semisaturation constant varied considerably between the eyes of Group A rabbits throughout the follow-up period. The mean difference in log σ varied from -0.35 to 0.6, and therefore the standard deviations were relatively large (between 0.19 and 0.34 for all time intervals). Log σ differences between study and control eyes throughout the follow-up had no

Table 1. Mean Vmax Values, Calculated Vmax Ratios, Mean Semisaturation Constant (σ, Expressed in Log Values) and the Difference in Log σ (Δ Log σ) Obtained From Both Eyes of All Three Groups During the Follow-up

Group	Parameter Evaluated	Before	1 Day	1 Week	2 Weeks	4 Weeks	8 Weeks	12 Weeks
A	Vmax study eye	207.8	129.9	133	110.9	135.5	122.1	N/A
	Vmax control eye	199.3	156	209.5	206.7	233	226	N/A
	Vmax ratio (R/L)	1.05	0.49	0.59	0.56	0.48	0.52	N/A
	Log σ study eye	–1.7	–1	–2.2	–1.96	–2.36	–1.98	N/A
	Log σ control eye	–1.8	–1.6	–1.85	–2.1	–2.1	–0.32	N/A
	ΔLog σ	0.1	0.6	–0.35	0.14	–0.26	–1.66	N/A
B	Vmax study eye	200.5	48	91.51	62.8	59.9	69.3	84.55
	Vmax control eye	187.4	123.8	154.26	143.4	147.17	159.8	173
	Vmax ratio (R/L)	1.06	0.4	0.69	0.55	0.53	0.52	0.52
	Log σ study eye	–1.94	–2.35	–1.68	–2.02	–2.38	–1.9	–1.98
	Log $\sigma\Delta$ control eye	–2.06	–2.02	–1.75	–1.93	–2.15	–1.6	–1.45
	ΔLog σ	0.12	–0.33	0.07	–0.09	–0.23	–0.3	–0.53
C	Vmax study eye	188.8	166.2	194.5	189.8	173.8	128.2	231
	Vmax control eye	225.9	188.3	186.3	217.57	159	179.2	219.8
	Vmax ratio (R/L)	0.99	0.84	0.97	0.99	0.91	0.83	0.86
	Log σ study eye	–1.72	–1.77	–1.79	–1.43	–1.21	–1.7	–1.19
	Log σ control eye	–1.57	–1.68	–1.75	–1.21	–1.03	–1.57	–1.17
	ΔLog σ	–0.15	–0.09	–0.04	–0.22	–0.18	–0.13	–0.02

N/A = not available.

statistical significance. Therefore, these data are not shown.

Fifteen rabbits were treated with either the vehicle extracted from Kenalog suspension (Group B, n = 8) or with pure TA (Group C, n = 7). Also for these rabbits, the b-wave Vmax, the Vmax ratio, the semisaturation constants (σ), and the log σ difference were derived for each ERG recording session (Table 1). In Group B, the mean Vmax value obtained from the study eyes before injection was 200.5 μV. This value decreased to 91.5, 62.8, 59.9, and 69.3 μV at 1, 2, 4, and 8 weeks after intravitreal vehicle injection, respectively. The differences between the mean Vmax value obtained before and those obtained 1, 2, 4, and 8 weeks after intravitreal Kenalog injection were all statistically significant ($P = 0.007$, Friedman test). In Group C, the mean Vmax value obtained from the study eyes before injection (188.8 μV) demonstrated only slight change at 1, 2, 4, and 8 weeks after intravitreal TA injection (194.5, 189.8, 173.8, and 128.2 μV, respectively). These differences were not statistically significant. No statistical significance was found for the differences between the mean Vmax values obtained before injection and those obtained at

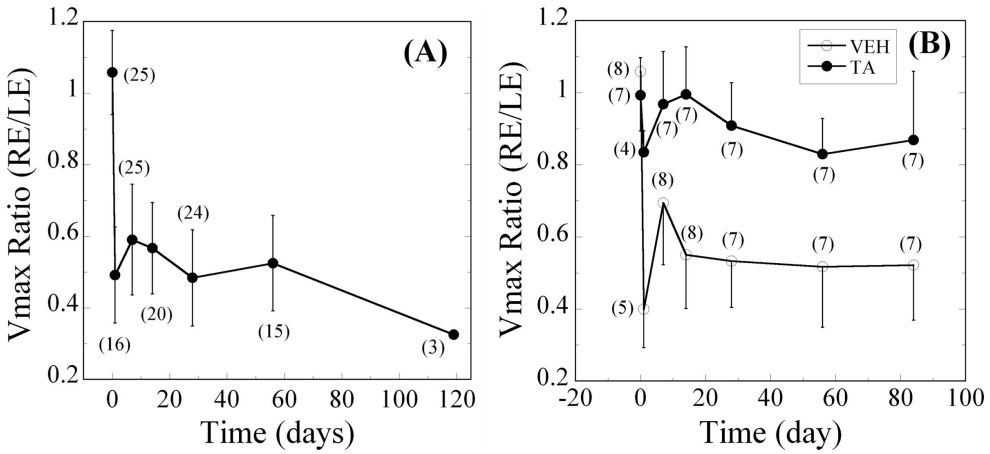

Fig. 4. Progression of Kenalog-induced functional damage to the retinas of rabbits of Group A (**A**), and the effects of Kenalog vehicle and triamcinolone acetonide (TA) extracted from whole Kenalog suspension on the retinas of rabbits of Groups B and C, respectively (**B**). Vmax ratios (study eye/control eye) were derived for each rabbit at each ERG recording session. Mean (±SD) values of Vmax ratios are plotted as a function of time after intravitreal injection of Kenalog. In parenthesis are shown the numbers of rabbits examined at each time interval. In (**B**), only one side of SD is shown for clarity of the figure.

1, 2, 4, and 8 weeks after saline injection to the control eyes in Groups B and C.

Figure 4B demonstrates the mean b-wave Vmax ratios of Group B and Group C (open and filled circles, respectively) along the entire follow-up. The mean preinjection Vmax ratio was 1.06 ± 0.16 for the eight rabbits of Group B and 0.99 ± 0.1 for the seven rabbits of Group C. These rabbits were followed clinically and by ERG recordings up to 12 weeks following intravitreal injections. One day after injection the mean Vmax ratios were 0.4 ± 0.1 (n = 5) and 0.84 ± 0.06 (n = 4) for Groups B and C, respectively. At 4 weeks time interval, mean Vmax ratios of 0.53 ± 0.13 (n = 7) and 0.91 ± 0.11 (n = 7) were measured for Groups B and C, respectively. These values remained stable also at 12 weeks after injection: 0.52 ± 0.15 (n = 7) for Group B and 0.86 ± 0.19 (n = 7) for rabbits of Group C.

The data presented in Figure 4B demonstrate that the main toxic effect of Kenalog can be attributed to the vehicle of the drug and not to the active ingredient (TA). The differences between the mean Vmax ratio obtained before injection and the mean Vmax ratios obtained along the follow-up were statistically significant in Group B rabbits ($P = 0.002$, Friedman test). However, no statistical significance was found between the mean preinjection Vmax ratio and those obtained along the follow-up in Group C ($P = 0.166$, Friedman test).

The semisaturation constant varied slightly between the eyes in the two groups throughout the follow-up period. The mean difference in log σ varied from −0.33 to 0.07 in Group B and from −0.22 to −0.04 in Group C. Since the standard deviations were relatively large (0.13–0.27 for Group B and 0.1–0.26 for Group C) for all time intervals, the change in σ had no statistical significance (data not shown).

Comparing the Vmax results between the groups showed a statistically significant difference between the mean Vmax values obtained from study eyes of Group A and study eyes of Group C at 1, 2, 4, and 8 weeks after intravitreal injection of Kenalog and TA, respectively ($P > 0.001$, Mann-Whitney test). We also found statistically significant differences between the mean Vmax ratios obtained from Group A rabbits and Group C rabbits along the follow-up ($P < 0.001$ at 1, 2, 4, and 8 weeks after injection, Mann-Whitney test). No statistically significant difference was found between the mean Vmax values obtained from study eyes of Group A and study eyes of Group B along the follow-up.

To compare the relative effect of Kenalog, its vehicle, and TA on the inner and outer retina, we calculated a/b ratios elicited by the brightest stimulus, obtained before and 8 weeks after intravitreal injection for each group in both eyes. These data were available in 16, 5, and 5 rabbits of Groups A, B, and C, respectively, and are presented in Table 2. The mean preinjection a/b ratios were similar in both eyes in all three groups and the differences were not statistically significant ($P = 0.258$, $P = 1.000$, and $P = 0.686$ for Groups A, B, and C, respectively, Wilcoxon signed rank test). However, 8 weeks after injection, the mean a/b ratio obtained from the right eyes increased by 187%, 162%, and 127% in Groups A, B, and C, respectively, compared to that obtained from the left eyes. The differences between the mean a/b ratios obtained before and those obtained 8 weeks after injection were statistically significant in Groups A and B ($P < 0.001$, $P = 0.043$, respectively, Wilcoxon signed rank test). The difference between the mean a/b ratios obtained before and those obtained 8 weeks after injection was not statistically significant ($P = 0.08$) in Group C. Therefore, in Groups A and B, the b-waves were affected more than the a-waves, indicating a greater effect of intravitreal Kenalog and its vehicle on the inner retina compared to their effect on the outer retina, where the a-waves are generated by the photoreceptors.[33]

To test the above conclusion, we isolated the photoreceptor component of the ERG responses in two rabbits, 4 weeks after intravitreal injection of Kenalog, as shown in Figure 5 for one of them. The ERG responses were first recorded to assess Kenalog-induced deficit to the b-wave (Figure 5, left column).

Table 2. Mean a/b Ratios Derived From the Right (RE) and Left (LE) Eyes of All Three Groups Before and 8 Weeks After Intravitreal Injection

Group	N	Time From Injection	Mean a/b Ratio RE	Mean a/b Ratio LE	*P* Value	RE–LE Change, %
A	16	Before	0.38 ± 0.133	0.38 ± 0.133	0.258	0
		8 Weeks	0.62 ± 0.17	0.33 ± 0.08	<0.001	187
B	5	Before	0.34 ± 0.11	0.34 ± 0.13	1.000	0
		8 Weeks	0.52 ± 0.22	0.32 ± 0.11	0.043	162
C	5	Before	0.31 ± 0.14	0.31 ± 0.1	0.686	0
		8 Weeks	0.33 ± 0.09	0.26 ± 0.06	0.08	127

N = number of rabbits in each session.

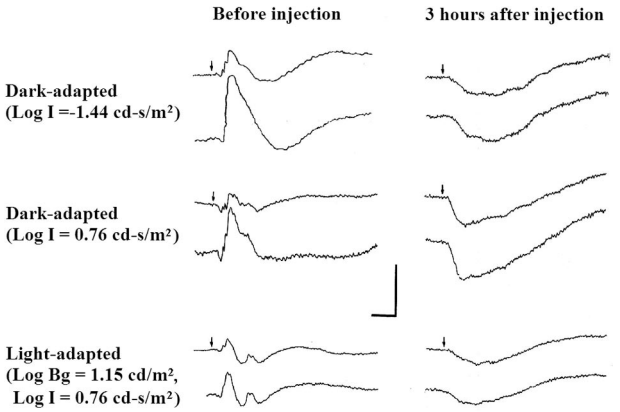

Fig. 5. Dark-adapted electroretinogram (ERG) responses of one rabbit that were recorded 4 weeks after intravitreal injection of Kenalog into the right eye and saline into the left eye. Responses are shown before (left column) and 3 hours after (right column) intravitreal injection of a mixture of 2-amino-4-phosphonobutyric acid (APB) and 2-cis-piperidine-2, 3-dicarboxylic acid (PDA) into both eyes. The mixture blocked synaptic transmission in the OPL, and effectively isolated the receptor component of the ERG. The ERG responses of the right and left eyes are compared in each pair of responses (upper and lower traces, respectively). Calibration bars: vertical, 200 μV, horizontal, 50 msec. Time of light stimulus is indicated with small arrows.

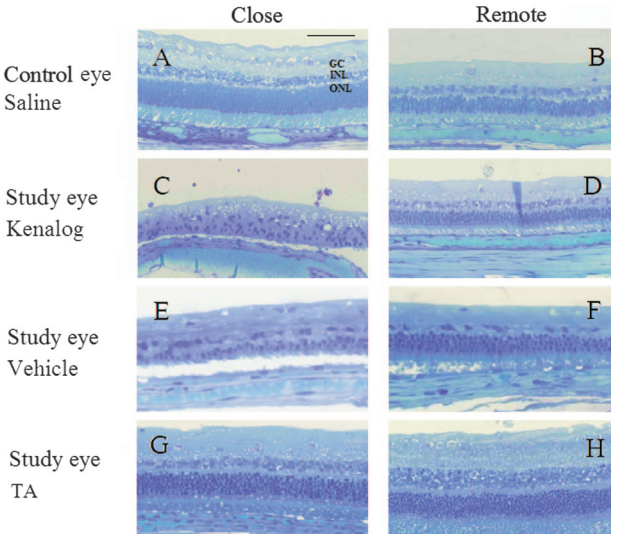

Fig. 6. Transverse retinal sections from control (**A** and **B**) and experimental (**C** to **H**) retinas of rabbits killed 8 weeks (**E**, **F**, **G**, and **H**) and 17 weeks (**A** to **D**) after intravitreal injection of Kenalog (**C**, **D**), its vehicle (**E**, **F**), and triamcinolone acetonide (**G**, **H**). Retinal sections from areas close (just inferior to the medullary rays) to the injection site (**A**, **C**, **E**, and **G**) and from a remote (10 mm inferior to the medullary rays) area (**B**, **D**, **F**, and **H**) are shown. ONL = outer nuclear layer; INL = inner nuclear layer; GC = ganglion cell layer. Calibration bar: 100 μm (applies to all micrographs).

Then, we blocked pharmacologically synaptic transmission from the photoreceptors to the second order neurons using a mixture of APB and PDA (see Materials and Methods). Three hours later, the ERG responses were recorded again (Figure 5, right column). The pharmacologic block almost completely eliminated the b-wave, while the a-wave was augmented in amplitude. Measurements of the a-wave amplitude revealed a 21.4% decrease in Vmax of the study eye compared to a 58.6% reduction in the maximal amplitude of the b-wave of the same eye. Very similar results were obtained in the other rabbit that was tested for the effects of Kenalog on the isolated receptor component of the ERG.

Histology

Histologic examination of the retina was performed on eight of the rabbits after different periods of follow-up. Transverse sections of retinas obtained from four eyes of three rabbits are shown in Figure 6. These sections were taken from rabbits that had already exhibited stable deficit in retinal function. Micrographs from control and experimental eye of a rabbit from Group A (injected with Kenalog) and killed after 17 weeks of follow-up (A–D) are compared to these of rabbits from Group B (E, F) and Group C (G, H). The latter two were killed after 8 weeks of follow-up. Two micrographs are shown for the control retina (A and B) and two for each of the study retinas (C–H). From each retina, one micrograph is from an area close to the site of injection (A, C, E, and G) and one from an area located in the inferior retina approximately 10 mm from the site of injection (B, D, F, and H). Structural damage manifested by thinning of the retina and disappearance of normal stratification of retinal layers and disappearance of photoreceptors is seen in the study retinas obtained from areas close to the site of injection of Groups A and B (Figure 6, C and E). In a more remote retinal area (Figure 6, D and F), very slight damage is seen at the inner nuclear layer (INL) of the retina, manifested mainly by decreased number of cells compared to the number of these cells in the control figure (B). The micrographs obtained from Group C present an apparently normal retinal structure. Similar findings were found in all other retinas that were prepared for light microscopy.

Discussion

The electrophysiologic and anatomic findings described here demonstrate substantial functional and structural damage to the retina of albino rabbits by intravitreal injection of Kenalog. Kenalog induced a significant reduction in the maximum response amplitude (Vmax) and a negligible change in the semisaturation constant σ indicating diffuse retinal damage but relatively normal function of undamaged regions.[31]

The mean preinjection Vmax ratio (Figure 4) was close to unity (1.05, 1.06, and 0.99 for rabbits of Groups A, B, and C, respectively), indicating no significant functional difference between the two eyes of each rabbit. One day after injection, the mean Vmax ratios were 0.49, 0.4, and 0.84 for Groups A, B, and C, respectively. At 4 weeks time interval, averaged Vmax ratios of 0.48, 0.53, and 0.91 were measured for Groups A, B, and C, respectively. These values, which remained stable also at 8 and 12 weeks after injection, demonstrate that Kenalog caused damage to the retina at a fast rate that could be detected by the ERG responses as soon as 1 day postinjection. Since the degree of functional damage in the rabbits treated with the vehicle alone (Group B) was similar to that seen in rabbits treated with Kenalog, we suggest that the main toxic effect of Kenalog arises from the vehicle of the drug and not the active ingredient (TA).

Analysis of Kenalog effects upon the ERG components can localize the sites of damage. The a-wave of the flash ERG reflects photoreceptor activity, while the b-wave is generated in the inner nuclear layer (INL)[33] by ON-center bipolar cells[34] with involvement of Muller cells.[33] Our data indicate that Kenalog is toxic to all retinal elements contributing to the ERG responses, but the neurons of the inner retina and Müller cells are more susceptible to Kenalog toxicity relative to the photoreceptors (Figure 5). This conclusion is also supported by the increase in a/b ratios of the right, study eyes compared to the corresponding ratios of the left, control eyes. Since the b-wave was affected more severely than the a-wave, a/b ratio increased 8 weeks after injection (Table 2). The histologic observations also indicate higher susceptibility of the inner retina (Figure 6). Damage to all retinal layers was seen in areas close to the site of injection in all rabbits (Figure 6, C and E), and involvement only of the inner retina (Figure 6, D and F).

The issue of Kenalog toxicity to the retina and the identity of the toxic components are still under debate. In a previous work, both ERG and histology showed no retinotoxic effects in rabbits following intravitreal injection of Kenalog into silicone-filled vitrectomized eyes.[35] These results could be explained by compartmentalization of Kenalog by the silicone, preventing the drug from diffusing away to contact large retinal areas. Another study showed that the vehicle of Kenalog was nontoxic when injected intravitreally into pigmented rabbits.[29] In this article, however, only histologic evaluation for retinal toxicity was performed, while ERG was not recorded. It is also possible that ocular pigmentation may provide a degree of protection against Kenalog and its vehicle, as demonstrated before for gentamicin toxicity.[36] In a recent report, intravitreal Kenalog, the supernatant alone, and TA diluted in saline were found nontoxic to albino rabbits electroretinographically and by histology following short (1 week) follow-up period.[37] However, in another report, increasing concentrations of TA (4%, 16%, and 25%) were found not toxic to albino rabbit retina even after long (1 month) follow-up.[38]

When steroidal preparations were tested for retinal toxicity, the vehicles were found to play an important role.[25,26,29] Our findings also point to the vehicle of Kenalog as the toxic component (Figures 4B and 6E). The observation that the vehicle and not TA contains the toxic component is supported by a previous study showing by electroretinography and histology that intravitreal triamcinolone acetonide alone was nonretinotoxic to pigmented rabbits even after 3 months of follow-up.[23] These conclusions are in contrast with a recent study reporting that triamcinolone and not benzyl alcohol, a component of Kenalog vehicle, was toxic to cultured human retinal pigment epithelium (RPE) cells and human glial cells.[39] However, in this in vitro study, the high concentration of free TA crystals in direct contact with the cultured RPE cells may have reduced the viability of the cells, while such direct contact of TA with RPE cells is unlikely to occur in in vivo studies such as ours. In a recent publication, 4 and 25 mg of Kenalog with its vehicle or the vehicle alone were found retinotoxic to albino rabbit retina by both ERG evaluation and pathology after 2 months of follow-up.[40] These results are similar to ours.

Our data cannot point to the component of the vehicle that is responsible for the toxic effects of Kenalog. However, benzyl alcohol has been blamed to cause death in neonates,[41] and therefore its use in neonatal intravenous solutions has been banned by the US Food and Drug Administration.[42] In addition, benzyl alcohol was found to be toxic to the central nervous system of dogs when injected intrathecally.[43] These data may point to benzyl alcohol as the main toxic component in Kenalog. This is supported also by a recent report demonstrating histologic changes in the outer retina of rabbits caused by benzyl alcohol.[24]

It has also been shown that following filtered or nonfiltered separation of triamcinolone from the vehicle, the concentration of benzyl alcohol increased to 3.6%–4.3% (compared to 0.99% in commercial Kenalog suspension distributed in the United States) due to the high affinity of benzyl alcohol to lipophilic environment such as TA crystals.[44] In our study, TA was separated manually from the vehicle using no filter. Therefore, the TA fraction was probably contaminated by vehicle which may have caused the mild ERG deficit found in the rabbits of Group C (Figure

4B). The fact that the TA fraction in our work was contaminated by the vehicle may also explain the small increase (127%) in the mean a/b ratio of the right eyes compared to that obtained from the left eyes encountered in Group C. However, the relatively mild effect of the contaminating vehicle presented in the TA fraction injected to the right eyes of rabbits in Group C did not cause morphologic changes seen by histology (Figure 6, G and H). It should be stressed again that our results were based upon the Kenalog suspension that is distributed in Israel, which contains a 1.5-fold higher concentration of benzyl alcohol compared to that in the United States (1.5% versus 0.99%).

A possible side effect of intravitreal Kenalog that has been previously reported in patients is an increase in IOP.[1,5,12,45–47] Although NZW rabbits are not an ideal species for assessing steroid-induced glaucoma, an increase in IOP may alter the ERG responses since blood circulation through the retinal vessels can be hampered, affecting the functional integrity of neurons in the inner retina and probably bipolar cells. To test this possibility, we measured IOP in seven of the rabbits and found an immediate IOP increase in both eyes following intravitreal injection of either Kenalog suspension or saline (Figure 1). The IOP recovered towards the preinjection level within 1 hour (Figure 1). This cannot explain the early (1-day) decrease in the b-wave amplitudes of the study eyes compared to the control eyes since both were similarly affected by the IOP. A second and significant elevation of the mean IOP was found at 4 and 8 weeks following intravitreal Kenalog injection. However, since elevated IOP was noticed in only one study eye at 8 weeks after injection, it seems that Kenalog had only mild effect on IOP of rabbits, but was highly toxic to retina.

The data presented here clearly demonstrate retinal toxicity by Kenalog to albino rabbits. Generalizing the findings described here for albino rabbits to humans should be done with extreme caution since 1) the vitreous volume of rabbits is approximately 1.5 mL[48] compared to approximately 4 mL in humans. Therefore, if the same dose of Kenalog is injected, its concentration in the rabbit vitreous is almost threefold higher than in human vitreous; 2) clearance of Kenalog from human vitreous may be faster than from rabbit vitreous because of the absence of retinal vascular system in rabbits. This possibility may also explain the fact that intravitreal Kenalog was not reported to induce obvious retinal toxicity in pigs and rats,[27,28] whose retina is vascularized; 3) there are species differences in retinal susceptibility to drugs.

Despite these considerations, the possibility that preservatives of commercial steroids are toxic to the retina prompted some ophthalmic clinics to remove most of the vehicle from the Kenalog suspension before intravitreal injection.[2,12] However, our results show that a simple separation of the vehicle from the TA is not absolutely safe, and even a small amount of the vehicle may still be toxic. Therefore, the use of preservative-free triamcinolone for ophthalmic treatment should be tested, as suggested previously.[49]

Key words: intravitreal injection, Kenalog, retinal toxicity, triamcinolone acetonide.

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