

Safety and Pharmokinetics of Triamcinolone Hexacetonide in Rabbit Eyes

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ABSTRACT

Purpose: The aim of this study was to evaluate whether intravitreal triamcinolone hexacetonide (TH) is a safe, longer lasting alternative to intravitreal triamcinolone acetonide (TA) in the rabbit eye.

Methods: Three groups, each comprising of 15 Dutch-belted rabbits, received a unilateral injection of 0.1 mL of drug and 0.1 mL of physiologic salt solution in the fellow eye. Group I received TA, group II received commercially available TH, and group III received reformulated iso-osmolar triamcinolone hexacetonide (rTH). Simultaneous bilateral dark-adapted electroretinography was performed following the injection. Retinal morphology was assessed by using histopathology in each group enucleated 12 weeks after injection. High-performance liquid chromatography of vitreous isolated from the enucleated eyes was used to determine drug concentrations.

Results: A significant reduction in saturated a-wave and maximal scotopic b-wave was observed in the group II eyes relative to the fellow control eyes at both 2 and 12 weeks postinjection ($P < 0.001$ for each comparison) but not in the other groups. Histopathology showed no differences between drug-injected eyes and fellow control eyes in groups I and III, but in group II there was severe degeneration of all retina layers. In group I, the drug half-life was 17.7 ± 1.7 days, group II 44 ± 13 days, and group III 12.8 ± 2.3 days.

Conclusions: The half-life of commercially available TH in the vitreous is double that of TA, but the former is toxic to the retina in this rabbit model. Reformulated iso-osmolar TH showed no evidence of deleterious effects to retina function or structure but had a similar half-life to TA.

INTRODUCTION

INTRAVITREOUS COMMERCIALY AVAILABLE triamcinolone acetonide (TA) has been shown to lack

retinal toxicity in the doses used in clinical practice.¹⁻⁷ There is strong evidence that it is efficacious in the treatment of diffuse diabetic macular edema refractory to grid photocoagulation,⁸⁻¹¹

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The authors have no proprietary interest in any of the materials used in this study.

This research was supported, in part, by NIH EY04446, EY02520, the Retina Research Foundation (Houston, TX), and an unrestricted grant from Research to Prevent Blindness (New York, NY).

Portions of the data presented in this paper were presented at the Association for Ocular Pharmacology and Therapeutics, 8th Scientific Meeting, February 9-11, 2007, in San Diego, California.

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in age-related macular degeneration as an adjuvant to photodynamic therapy,¹² in some uveitides,^{13–15} and in macular edema associated with uveitis,¹⁶ Irvine-Gass syndrome,^{17,18} retinitis pigmentosa,¹⁹ and central or branch retinal vein occlusion.^{20–23} Often, treatment with intravitreal TA requires repeat injections. An alternative to TA with a longer half-life would be beneficial, since it would likely reduce the necessity of repeat procedures.

The half-life of intravitreal commercially available TA is 19 days in nonvitrectomized human eyes (24 days in rabbit eyes).^{24,25} Triamcinolone hexacetonide (TH) has long been used in rheumatology for intra-articular injections in the treatment of rheumatoid joints or osteoarthritis of the knee, and there is evidence of superior efficacy, compared to TA, owing to a longer half-life.^{26–30} The aim of the present study was to establish whether intravitreal TH is safe and whether its half-life is longer compared to TA in the rabbit eye. If intravitreal TH is found to be safe and to have a longer half-life than TA, it could be a promising alternative to TA reducing the requirement for repeat injections.

METHODS

Animals, anesthesia, injections, and funduscopy

All experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology (ARVO) principles of animal maintenance and care and were approved by the institutional review board at Baylor College of Medicine (Houston, TX).

Three groups, each comprising 15 Dutch-belted rabbits (2–2.5 kg) were used. Each animal received a unilateral injection of study drug, with the fellow control eye receiving an injection of physiologic salt solution (PSS). Group I received TA (Kenalog; Bristol-Myers Squibb, Princeton, NJ) 4 mg/0.1 mL, group II received commercially available TH (Aristospan; SAB-Pharma Inc., Lake Forest IL) 2 mg/0.1 mL, and group III received reformulated iso-osmolar triamcinolone hexacetonide (rTH: 2 mg/0.1 mL purified TH suspended in an especially formulated vehicle of 89.18% saline, 10.02% water, 0.75% sodium carboxymethylcellulose, and 0.04% polysorbate 80).

Rabbits were anesthetized with intramuscular injections of 0.2 mL/100 g of body weight keta-

mine (95 mg/mL) and xylazine (5 mg/mL). Proparacaine hydrochloride 0.5% was used for additional topical anesthesia. Each pupil was then dilated with 10% phenylephrine. All eyes underwent a baseline dilated funduscopic examination. The conjunctiva was irrigated with 1 mL of 5% povidine iodine prior to injection. One (1) eye of each rabbit received an intravitreal injection of 0.1 mL of drug, using a 27-gauge needle inserted superiorly, 2 mm posterior to the limbus. The contralateral eye received an intravitreal injection of 0.1 mL of PSS, using a 27-gauge needle in the exact same fashion. The first 10 animals of each group received drug in the right eye; the remaining 5 animals received drug in the left eye. Gatifloxacin ophthalmic solution 0.3% (Allergan, Irvine, CA) was applied to the ocular surface after injection. Dilated funduscopy was performed on each occasion that an animal underwent anesthesia or euthanasia.

Electroretinography

Simultaneous bilateral electroretinography was performed 2 and 12 weeks after injection in 10 and 6 rabbits in each group, respectively, as previously described.³¹ At both time points, half the animals tested had drug injected in the right eye and half had the drug injected in the left eye. Prior to testing, rabbits were allowed to dark adapt for at least 1 hour. Under dim red light, rabbits were anesthetized, as described above, and the pupils were dilated with a single drop of 2.5% phenylephrine and 1% tropicamide. A drop of 0.5% proparacaine hydrochloride was applied for corneal anesthesia. Rabbits were placed inside a Ganzfeld dome coated with highly reflective white paint. A small amount of 2.5% methylcellulose gel was applied to the eye, and a JET contact lens electrode (LKC Technology, Gaithersburg, MD) was placed in contact with the central corneal area. Platinum reference and ground electrodes (Grass Telefactor, West Warwick, RI) were placed through the eyelid and ear, respectively. After placement in the dome, rabbits were allowed to dark adapt by being kept in complete darkness for several minutes. Signals were amplified with a Grass P122 amplifier (Grass Telefactor, West Warwick, RI, bandpass, 0.1–300 Hz). Data were acquired by using the National Instruments Lab-PC DAQ board (sampling rate, 10,000 Hz), and between 3 and 10 traces (depending on the signal-noise ratio at a given light intensity) were averaged and analyzed by using custom software written in Matlab (Mathworks,

Natick, MA). Flashes were calibrated, as previously detailed.³¹ Flashes for scotopic b-wave measurements were generated by a Grass PS33+ photostimulator. Light was spectrally filtered with a 500-nm interference filter. Flashes varied in intensity from -3.80 to -0.76 log scotopic cd-s/m². For analysis of the a-wave, we used a 1500-W (Novatron Dallas, TX) xenon flash lamp to produce a saturating light stimulus (2.97 log cd-s/m²).

Osmolality measurements

Osmolality was measured by using a Vapro Model 5520 Vapor Pressure Osmometer (Wescor Inc., Logan, UT). Five measurements were taken for each drug.

Histology

Twelve (12) weeks after injection, 3 animals in each group were euthanized with an overdose of intracardiac ketamine and xylazine. The eyes were enucleated, an incision was made in the cornea, and the eyes were fixed immediately in 10% buffered formalin. Eyes were sectioned horizontally to obtain a pupil-optic nerve section and examined macroscopically. Tissues were then processed and embedded in paraffin, sectioned at a thickness of $5\ \mu\text{m}$, and stained with hematoxylin and eosin (H&E). Light microscopy was used for histologic examinations.

Immunohistochemistry

Paraffin sections of the rabbit eye were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. All sections underwent antigen retrieval for 25 min by using Citrate Target Retrieval (DakoCytomation, Carpinteria, CA) and an adequate positive control was used. Vimentin (DakoCytomation) diluted to 1:500 and GFAP (DakoCytomation) diluted to 1:20 were used as primary antibodies for 25 min. LSAB+System-AP (DakoCytomation) was used as the detection system with diaminobenzidine (DAB; DakoCytomation) as chromogen. Slides were counterstained with hematoxylin.

High-performance liquid chromatography (HPLC)

Two (2), 4, 8, and 12 weeks following injection, 3 animals in each group were euthanized with an overdose of intracardiac ketamine and xylazine. The eyes were enucleated, the corneal cap was removed, and the vitreous was extracted and placed

into a test tube. Methanol 10:1 by volume was added to the vitreous sample, and the mixture was vortexed at room temperature for 2 min; 1 mL of the mixture was centrifuged in a TL-100 ultracentrifuge (Beckman Coulter, Fullerton, CA) at 40,000 rpm for 20 min at a temperature of 20°C. Of the resultant supernatant, 0.5 mL was mixed with 0.5 mL of 40% methanol, and this mixture was filtered with $0.45\ \mu\text{m}$ Acrodisc® 3 CR PTFE filter before injection to an HPLC C18 column (Agilent, 4.6×250 mm, 5-micron, ZORBAX SB C-18).

The samples were analyzed using Shimadzu gradient HPLC (Shimadzu Co., Kyoto, Japan) and a system of 0.1% trifluoroacetic acid (TFA) (buffer A) versus 0.1% TFA in acetonitril (buffer B) with a flow rate of 1 mL/min. A $10\ \mu\text{L}$ volume of each sample was injected on to the C18 column that was pre-equilibrated with 30% buffer B, then washed with 5 min of 30% buffer B. TA was eluted with a linear gradient of acetonitril (30%–100% containing 0.1% TFA). TA was monitored by the absorbance at 239 nm, using a Shimadzu SPD-M20A UV/VIS (Shimadzu Co.) photodiode array detector interfaced to a computer running Shimadzu LCsolution software. The TA and rTH concentration in the peak was calculated by using appropriate standard curves by LCsolution software. These standard curves were linear from 0.2 to $10\ \mu\text{g}$ (correlative >0.99), using seven different amounts of TA and rTH standards.

Data analysis and statistics

Scotopic b-wave analysis was performed by fitting light stimulus and response to a saturating hyperbolic function (Naka-Rushton) of the form shown in Equation 1:

$$b = \frac{b_{\max, \text{scot}} I}{I_{0.5} + I} \quad (1)$$

where b is the maximum filtered b-wave, $b_{\max, \text{scot}}$ is the saturating dark adapted b-wave amplitude, I is the intensity of stimulus, and $I_{0.5}$ is the half-saturating stimulus. From such an analysis, $b_{\max, \text{scot}}$ and $I_{0.5}$ were extracted. For a-wave analysis, the maximum a-wave amplitude was recorded at each light stimulus. The a-wave amplitude recorded from a saturating light stimulus (2.97 log candela-sec/m²) was denoted as a_{sat} .

We used the paired Student t test to test for a difference in the a_{sat} or $b_{\max, \text{scot}}$ between drug injected-eyes and control eyes in each group at 2

and 12 weeks. Statistical significance was defined as $\alpha = 0.05$. Finally, we qualitatively compared fundusoscopic and histopathologic findings between drug-injected eyes with control eyes.

For calculating intravitreal half-lives, the vitreal concentrations of the drugs were measured as above and fit to Equation 2:

$$A(t) = A_0 e^{-t/\tau} \quad (2)$$

where $A(t)$ equals the concentration of drug A at time t , A_0 is the initial concentration of drug A, and τ is a time constant. In this equation, the half-life $t_{1/2}$ is equal to $0.693 \cdot \tau$.

RESULTS

Fundusoscopic findings

Ten (10) animals in each group underwent a bilateral dilated fundus examination 2 weeks after injection, 3 animals in each group 4 and 8 weeks after injection, and 6 in each group 12 weeks after injection. Animals in groups I and III showed no differences on clinical examination between injected and control eyes; notably, no eye developed evidence of uveitis, cataract, retinal detachment, or retinal pigment epithelium (RPE) changes, and the depot of intravitreal medication could be seen in all injected eyes. Injected

eyes in group II showed coarse RPE mottling with nongeographic RPE atrophic changes, without evidence of uveitis or cataract; no retinal detachment could be observed fundusoscopically.

Electroretinography

A significant reduction in saturated a-wave was observed in the group II drug-injected eyes relative to the fellow control eyes at both 2 and 12 weeks ($P = 0.0008$ and 0.0005 , respectively) but not in the other groups (Figs. 1 and 2). A similar decrease was found in the maximal scotopic b-wave amplitude in the group II drug-injected eye relative to the fellow control eyes at both 2 and 12 weeks ($P = 0.0002$ and 0.0004 , respectively) (Figs. 1 and 3).

Histopathology and immunohistochemistry

Hematoxylin and eosin staining of Group 1 (control) eyes showed normal morphology (Fig. 4A). Eyes in group II showed degeneration of all retinal layers, with focal chorioretinal scars and multifocal flat retinal detachments (Fig. 4B). There were no apparent differences between control (Fig. 4A) and group III eyes (Fig. 4C) by H&E staining. Immunohistochemistry, using vimentin showed normal Müller foot processes (Fig. 5A). Immunohistochemistry, using vimentin, showed a loss of polarity of the Müller cells, proliferation

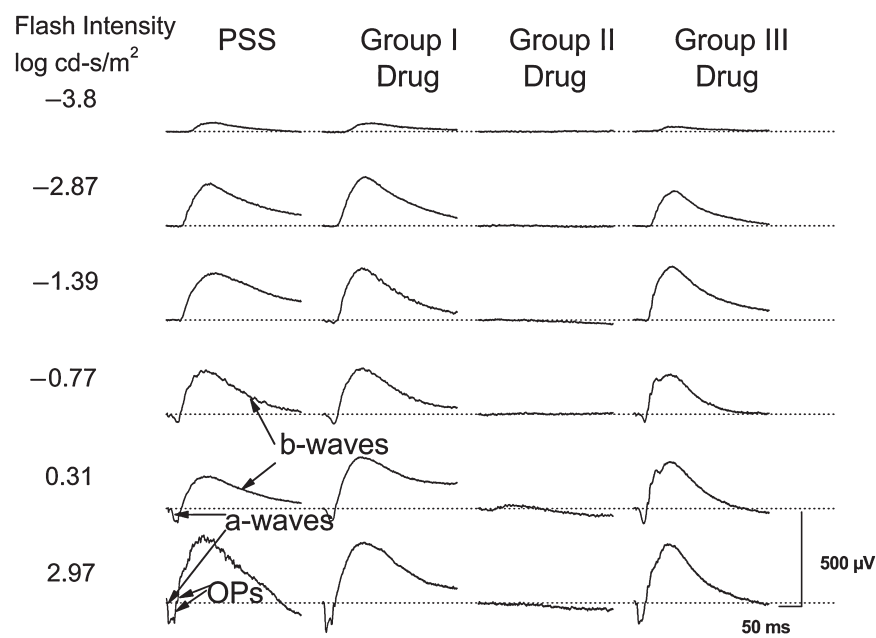


FIG. 1. Representative electroretinographs (ERGs) from physiologic salt solution-injected eyes, as well as three drug groups—groups I, II, and III at different stimulus intensities. Note the relatively normal ERGs in groups I and III and the drastically reduced responses in group II. OPs, oscillatory potentials.

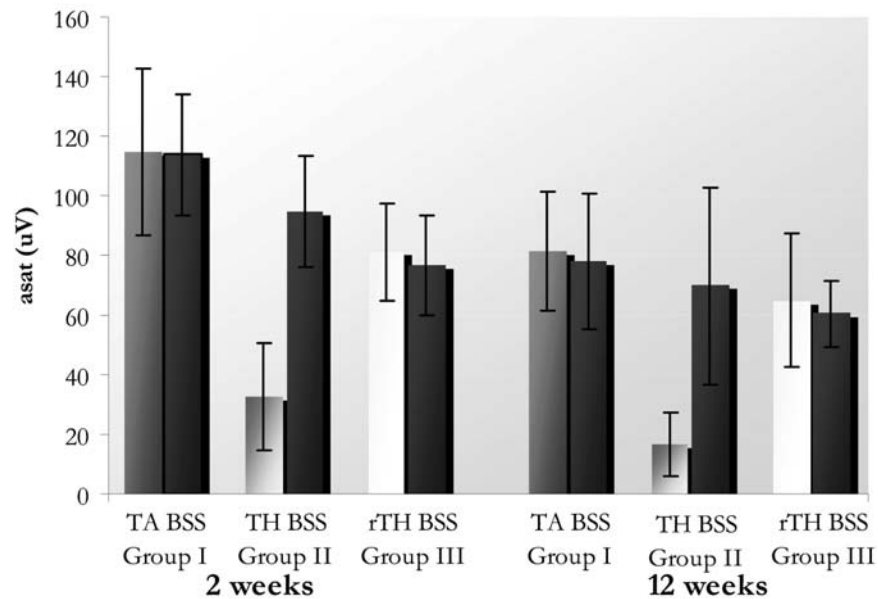


FIG. 2. Mean saturated a-wave amplitudes in each group with bars indicating $t_{0.05}$ times standard errors. The difference in mean amplitude between drug injected and control eyes is significant in group II at both 2 and 12 weeks ($P = 0.0008$ and 0.0005 , respectively). Differences in groups I and III are not statistically significant. TA, triamcinolone acetate; PSS, physiologic salt solution; TH, triamcinolone hexacetate; rTH, reformulated triamcinolone hexacetate.

of astrocytes, and highlights the chorio-retinal scars in group II eyes (Fig. 5B). Immunohistochemistry for vimentin showed diffuse expression from the internal limiting membrane to the outer nuclear layer, suggesting normal length of Müller cells with normal foot processes for control (Fig. 5A) and group III (Fig. 5C) eyes. Im-

munohistochemistry, using glial fibrillary acidic protein in groups I and III, showed typical faint positivity of the foot processes of the Müller cells (Figs. 6A and 6C); however, in group II, the distorted Müller cells are positive and the chorio-retinal scar shows the involvement of Müller cells (Fig. 6B).

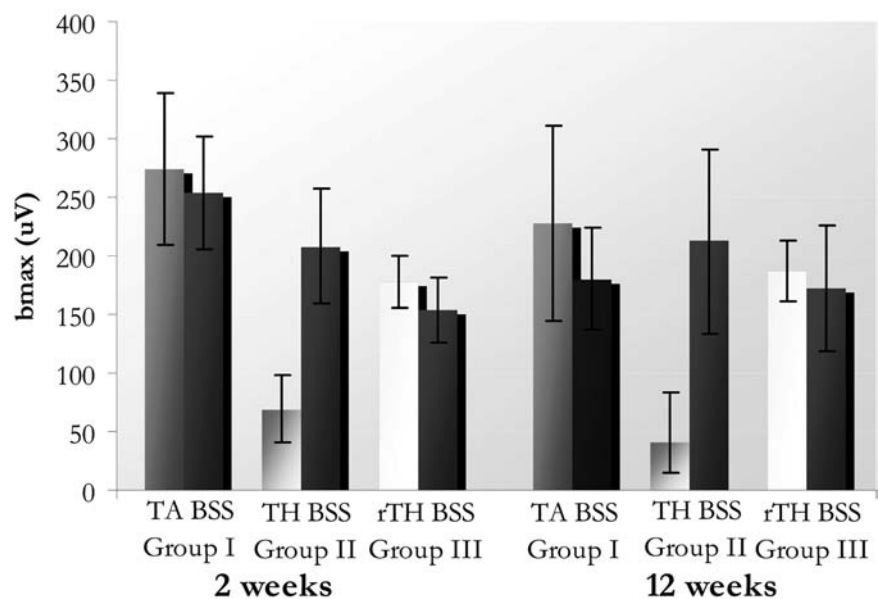


FIG. 3. Mean of the maximal scotopic b-wave amplitudes in each group (bars indicate $t_{0.05}$ times standard error). The difference in mean amplitude between drug injected and control eyes is significant in group II at both 2 and 12 weeks ($P = 0.0002$ and 0.0004 , respectively). Differences in groups I and III are not statistically significant. TA, triamcinolone acetate; PSS, physiologic salt solution; TH, triamcinolone hexacetate; rTH, reformulated triamcinolone hexacetate.

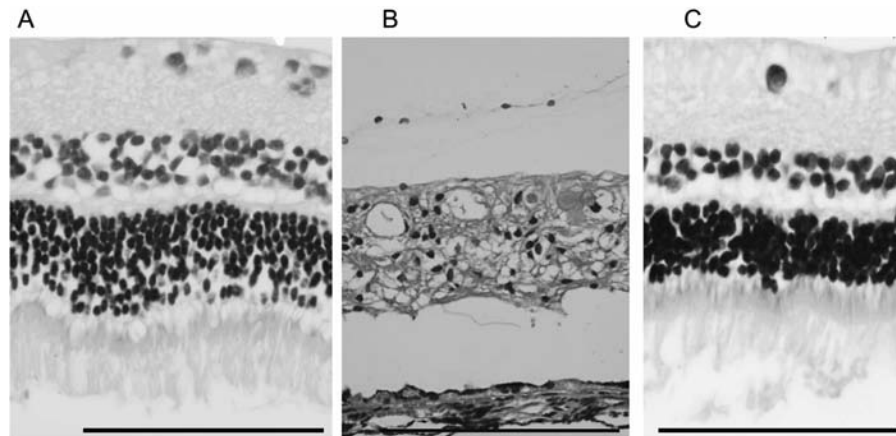


FIG. 4. Hematoxylin and eosin. There are no observed differences between group III (C) and control eyes (A), whereas there is degeneration of all retina layers and atrophy of the choriocapillaris in group II eyes (B). Scale bars, 100 μm .

Osmolality measurements

Osmolality measurements revealed that TA has an osmolality of 225 ± 3 mmol/kg, TH an osmolality of 3917 ± 31 mmol/kg, while reformulated TH (used in group III) had an osmolality of 274 ± 4 mmol/kg.

HPLC

The half-life of TA was 17.7 ± 1.7 days, of commercial TH 44 ± 13 days and of reformulated TH (used in group III) 12.8 ± 2.3 days (Fig. 7A, 7B, and 7C).

DISCUSSION

Our findings regarding group I eyes have been previously published and discussed in detail.²

Our new results suggest that commercially available TH is detrimental to retinal function and caused widespread retinal degeneration. This may have been related to the hyperosmolar vehicle in which commercially available TH is suspended or to pharmacologic toxicity. Indeed, intravitreal injection of hyperosmolar solutions (0.05 mL of 500 mOsm or greater) are known to cause retinal degeneration,³² however, whitening and detachment of the retina were the findings originally described, which we did not observe. Further, whereas electron microscopy showed pathologic changes in the RPE following injection of hyperosmolar solutions,³² prominent RPE changes, such as the ones we observed, were not described. Therefore, it is possible that the deleterious effects observed were secondary to toxicity of the vehicle, in addition to, or instead of, the hypersomolarity of the injected drug.

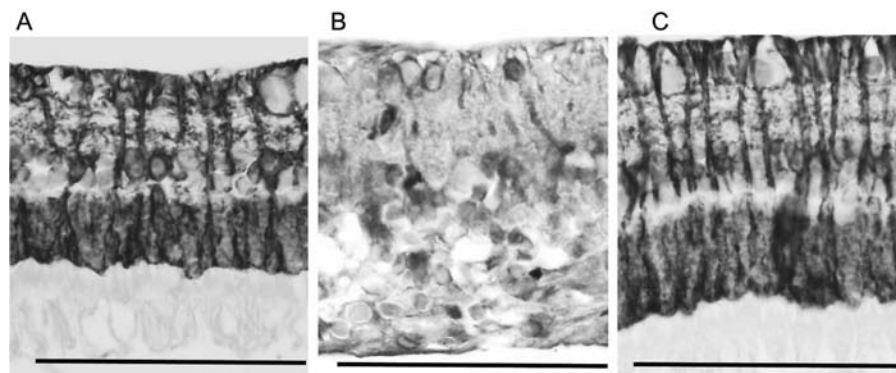


FIG. 5. Vimentin immunohistochemistry of pupil-optic nerve sections of a control eye (A) and group III (C) eye shows very similar staining of the Müller foot processes from the inner limiting membrane to the outer nuclear layer. Group II (B) eyes show loss of the polarity of the Müller cells and proliferation of astrocytes (arrow). Scale bars, 100 μm .

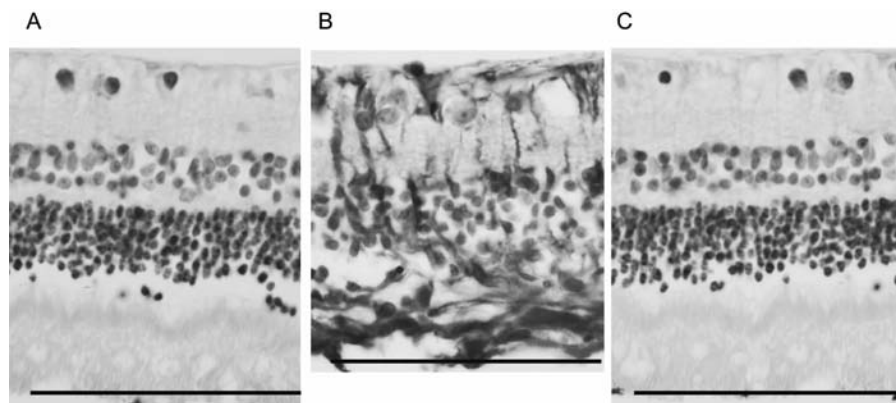


FIG. 6. Glial fibrillary acidic protein immunohistochemistry of retina of a control eye (A) and group III (C) eye shows normal faint staining of the Müller foot processes. Group II (B) eyes show distorted Müller cells, and that the chorioretinal scar involves Müller cells. Scale bars, 100 μm .

In order to circumvent toxicity of the vehicle, TH was purified and suspended in a reformulated iso-osmolar vehicle, which was used in group III eyes. Although there was a lack of deleterious effect of reformulated TH to retinal function and structure, reformulation with a new vehicle rendered the half-life of the medication in the vitreous shorter than, albeit comparable to, that of commercially available TA (Kenalog). Our estimate of the latter's half-life in the vitreous was 17.7 ± 1.7 days, which is similar to previous estimates of its half-life in human vitreous (19 days)²⁵ and comparable to a previous estimate of its half-life in rabbit vitreous (24 days).²⁴

Certain limitations of our study need be considered. The small number of animals used in each group (10 for week 2 electroretinography, 6 for week 12 electroretinography, and 3 for histology) makes it possible that a real difference was not detected (type II statistical error). Since

no statistically significant difference in electroretinography between reformulated TH and control eyes was detected, careful attention to the power of the study is appropriate: Assuming that a clinically insignificant change in electroretinography would be less than a 20% difference, we would have needed at least 23 animals in group III, given the observed measurement variance in order to achieve an 0.9 power of detecting a difference at the $\alpha = 0.05$ level. Additionally, the effects of ocular pigmentation were not specifically addressed by our study. In contrast to the clinical situation, these animals had retinae unaffected by disease (it may be that diseased retina and RPE are more susceptible to cytotoxicity). Dark-adapted electroretinography does not assess cone function, and rabbits do not have a fovea. Finally, histology was performed on pupilloptic nerve sections; therefore, the presence of localized photoreceptor damage outside of these sections cannot be excluded.

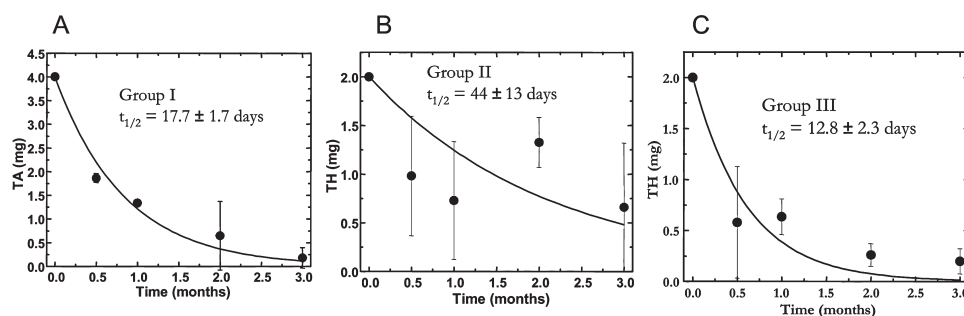


FIG. 7. Drug concentrations as measured by high-performance liquid chromatography from vitreous of group I (A), group II (B), and group III (C) eyes. Single exponential decay curves are fit (see Methods) to estimate vitreal half-lives ($t_{1/2}$).

CONCLUSIONS

This paper outlines an attempt to find an alternative to intravitreal TA, which would require fewer repeat injections. In conclusion, it appears that commercially available TH is toxic to the retina. Reformulation of TH with an iso-osmolar vehicle leads to a preparation without demonstrable toxicity to the retina, but which also has a half-life that is not longer than that of commercially available TA. It is, therefore, unlikely that intravitreal TH has any significant advantages over intravitreal TA in clinical practice.

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Received: September 4, 2007

Accepted: November 16, 2007

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