The treatment of posterior segment diseases such as age-related macular degeneration (AMD) and macular edema associated with various conditions is limited by the challenges of delivering effective doses of drugs to the vitreous, retina and choroid. To date, topical delivery has not been demonstrated to be effective due to such factors as limited intraocular penetration and multidrug resistance associated proteins. Systemic administration may be effective but long-term use may be associated with numerous side effects. Intravitreal injections may deliver therapeutic levels of the drug while minimizing the risk of systemic side effects.
Intravitreal administration of triamcinolone acetonide has been widely used for the treatment of posterior segment diseases including diabetic retinopathy, retinal vein occlusion, Irvine-Gass syndrome, uveitis, choroidal neovascularization associated with AMD, and proliferative vitreoretinopathy. Unfortunately, there have been reports of sterile endophthalmitis and vision loss thought to be related to the preservative and/or dispersion agent.

In the present study, the intravitreal pharmacokinetic profile of a triamcinolone acetonide formulation containing the preservative benzyl alcohol (TA-BA) was compared with that of a preservative-free triamcinolone acetonide formulation (TA-PF), and potential signs of retinal toxicity were also investigated.

MATERIALS AND METHODS

Sixty male New Zealand white rabbits, each weighing approximately 2.0 to 2.5 kg, were included. Throughout the observation period, the animals were maintained at the animal facility of the Faculty of Veterinary Medicine of the University of Rio Preto, São José do Rio Preto, São Paulo, Brazil. They were kept in a quiet and climatically controlled environment, with free access to standard rabbit chow and water.

Experiments were carried out in accordance with the guidelines set forth by the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. The study was approved by the Ethics Committee for Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo.

Surgical Procedures

The animals were divided into two groups. In the TA-BA group, 4 mg of a triamcinolone acetonide formulation (40 mg/ml) containing the preservative benzyl alcohol (Kenalog® Bristol-Myers, EUA) were injected into the vitreous of the right eye of 30 rabbits. In the TA-PF group, 30 rabbits received an intravitreal injection of 4 mg of a preservative-free triamcinolone acetonide formulation (40 mg/ml) (Ophthalmos® São Paulo, Brazil).

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketamin®, Cristália, Brazil– 50 mg/ml) and 4 mg/kg xylazine hydrochloride (Coopazine®, Schering-Plough Coopers, Brazil– 20 mg/ml). The ocular surface was then anesthetized by topical instillation of 1% proparacaine ophthalmic drops (Anestésico®, Allergan). The pupils were dilated with 1 drop each of 2.5% phenylephrine hydrochloride and 0.5% tropicamide. At baseline, clinical penlight eye examination and intraocular pressure (IOP) measurement (TonoPen® (Mentor, US) were performed, and after adequate anesthesia and akinesia were achieved, the right eye was injected 1.5 mm behind the surgical limbus in the superotemporal quadrant with 0.1 ml of either TA-BA or TA-PF. Anterior chamber paracentesis was performed to reduce the IOP in all rabbits.

All animals were euthanized with an overdose of intravenous thiopental (Thiopentax®, Cristália, Brazil) 100 mg/kg. Five rabbits in each group were euthanized at 3, 7, 14, 21 and 28 days following injection and their right eyes were enucleated immediately and processed for determination of intravitreal drug levels by high-performance liquid chromatography (HPLC). The vitreous of all 25 rabbits in each group was removed and frozen at −18°C until the triamcinolone concentrations were determined. The potential for toxicity associated with the intravitreal triamcinolone injections was evaluated in five animals of each group by histopathologic analyses and in three of these five animals of each group by ERG at 28 days after intravitreal injection.

Drug Level Analysis

The intravitreal drug levels were determined by high-performance liquid chromatography (HPLC) using the method described by Robinson et al. (2006). The equipment used was Waters, pump 515, automatic injector 717 plus, UV detector 2487-dual lambda absorbance, software Millenium® v.2.15.01 (Nova-Pak®, Waters, USA). A C-18 column (5 μm; 3.9 × 150 mm) was also used for separation. The flow rate used was 1.0 ml/min with a mobile phase of 60% of acetonitrile and 40% of water by volume. The experiments were performed at 20°C after the sample filtration (Durapore, 0.2 μm, Millipore). Under these experimental conditions, the retention time was 7.0 min and detection limit was 10 ng/ml. The amount of triamcinolone was expressed as triamcinolone equivalent concentration (μg/ml).

TA-BA and TA-PF Distribution of Particle Size

The distribution of particles by size within both formulations was determined with a laser particle analyzer (Hydro 2000S(A), Malvern Instruments Ltd., Malvern, UK).

Clinical Examination

Clinical evaluation included ocular inspection and binocular indirect ophthalmoscopy preoperatively and at 3, 7, 14, 21, and 28 days after injection. In all rabbits, the clinical evaluation was performed by two masked observers. The IOP of experimental eyes was measured...
Electrophysiological measurements were performed at 28 days after intravitreal injection in three animals of each group. The ERG protocol is based on the international standard for electrophoretography (ISCEV). Six rabbits were kept in a dark room for at least 3 h for dark adaptation before anesthesia, which was performed by intramuscular injection of 1–2 mg/kg body weight xylazine and 10 mg/kg body weight ketamine. Pupillary dilatation was performed with 1 drop of tropicamide 15 minutes before ERG started.

ERG responses were recorded in both eyes simultaneously by means of JET contact electrodes on the corneas (Microcomponents SA). Subcutaneous needles in the skin near the lateral canthus of both eyes were used as references; a ground electrode was placed on the back. Electrode impedance was checked before and after each measurement and was less than 5 kΩ at 25 Hz. Eyes were stimulated using a Ganzfeld LED stimulator (ColorDome; Diagnosys LLC, Littleton, MA). Flashes of white light (6,500 K) with a duration of 4 ms were delivered in 5 steps of increasing luminance (0.0001, 0.0003, 0.001, 0.003, and 0.01, and 10 cd.s/m²) with 30 s inter stimulus interval (ISI) in the dark adapted stage.

A hyperbolic saturation model was fitted for the interrelation between b-wave amplitude and luminance (I) for derivation of 2 parameters: \( V_{\text{max}} \): saturated b-wave amplitude; and \( k \): luminance necessary for b-wave to reach \( 1/2 \) of \( V_{\text{max}} \) (semisaturation point). Oscillatory potentials (OP) were obtained from the response elicited by the flash of 3 cd.s/m² by means of a fast Fourier transform (FFT) implemented as a bandpass frequency filter (from 60 to 300 Hz). The absolute value of the area under the curve for all OP wavelets was determined between a- and b-wave implicit times.

After 10 min of light adaptation with a background light of 30 cd/m², light adapted ERG recordings were performed with luminance flashes 3 cd.s/m² (ISI = 2 s) followed by a 30 Hz white flicker stimulus of 3 cd.s/m².

Responses were amplified (band pass filter: 0.3–300 Hz) and stored for off-line analysis using the Espion (Diagnosys LLC, Littleton, MA) after averaging of 6 up to 40 individual measurements at each step depending on the signal/noise ratio.

**Electroretinography (ERG)**

**Histopathologic Study**

Five rabbits from each group, six of which were also used for ERG analysis, were euthanized at 28 days for histopathologic analysis. These animals were not used for dosing of intravitreal concentration of triamcinolone. The experimental eyes were enucleated and then prepared for light and electron microscopy.

The eyes were hemisected at the equator and their posterior segments were fixed in 2% formaldehyde plus 2% glutaraldehyde in a 0.1M phosphate buffer, pH 7.2, for 4 h, at 4°C. After washing in buffer, small pieces were fixed in 1% osmium tetroxide in 0.1M phosphate buffer for 2 h, at 4°C. They were then dehydrated in graded ethanol, cleared in propylene oxide and embedded in Epon 812 resin. Semi thin sections (0.5 μm) were stained with Toluidine blue for examination by light microscopy (Carl Zeiss®, Germany). If any abnormality was detected at light microscopy, electron microscopy (Philips®, EM 208, EUA) would then be performed.

**Statistical Analysis**

The Mann-Whitney non-parametric test was used to compare outcomes in both groups. For all tests, the level of significance was set at \( \alpha = 0.05 \), two tailed.

**RESULTS**

**In Vivo Release Study**

The TA concentration in the vitreous throughout 28 days of evaluation is presented in Figure 1. Median intravitreal concentrations (µg/ml) for TA-BA x TA-PF groups were 1903.1 \( \times \) 1032.9 \( (p = 0.4647) \); 1213.0 \( \times \) 570.1 \( (p = 0.0283) \); 857.8 \( \times \) 516.6 \( (p = 0.0510) \); 442.0 \( \times \) 347.9 \( (p = 0.2506) \); and 248.6 \( \times \) 102.8 \( (p = 0.1745) \); at 3, 7, 14, 21 and 28 days after injection, respectively. Median intravitreal TA concentration was significantly higher in the TA-BA compared to the TA-PF group at 7 days post-injection \( (p < 0.05) \), and at day 14 there was a trend toward significance \( (p = 0.0510) \). At the other time points investigated, there was no significant difference between the two groups in intravitreal TA concentration (Figure 1A).

**Clinical Examination and IOP Analysis**

Mild hyperemia was noted by penlight inspection immediately after injection in all rabbits. At the day 3 examination, no inflammatory signs were evident by direct visual inspection in any rabbit from both groups. Indirect ophthalmoscopy did not demonstrate evidence of drug toxicity in any rabbit from both groups during the 28-day study period. No endophthalmitis, retinal detachment or cataract developed during the study period.

There was a significant increase in IOP at 1 min and at 3 days after the procedure in both groups \( (p < 0.05) \). There was no significant difference between the two groups.
groups in IOP at any of the study evaluation time points (Figure 1B).

**TA-BA and TA-PF Distribution of Particle Size**

Analysis of the mean distribution of TA particle size demonstrates that the TA-PF particles were significantly smaller compared to the TA-BA particles (Figure 2).

**ERG**

There were no significant differences in ERG responses when results from treated and non-treated eyes were compared.

The parameters from the hyperbolic function (Naka-Rushon) $V_{max}$ and $k$ are represented in Tables 1 and 2 for all animals, and an example is shown in Figures 3 and 4. Similar results were observed in the two groups for the a- and b-wave amplitude and implicit time measured on the high intensity flash ERG response (Table 2).

There were also no significant differences between treated and non-treated eyes with respect to light adapted responses.

**Histopathologic Study**

Histopathologic examination demonstrated no signs of retinal toxicity or inflammatory cell infiltration. No structural abnormalities were noted at day 28 by light microscopy and the normal anatomy of the retina was preserved in both groups (Figure 5).
DISCUSSION

The present study suggests a trend of higher intravitreal TA concentrations through 14 days post-injection if TA was associated with the benzyl alcohol preservative (TA-BA). Due to the variability in TA concentration measurements, a larger number of rabbits would be needed to determine whether there is a difference between the formulations with respect to intravitreal TA concentration. One may hypothesize that the faster initial clearance of triamcinolone in the TA-PF group is related to the smaller triamcinolone particles in this group compared to the TA-BA group, although a previous study using a different TA-PF formulation reported no significant difference in vitreous levels of TA-BA compared to TA-PF. In fact, this subject is controversial. According to Oishi et al. (2008), TA preparations with smaller and well dispersed intravitreal particles may have a shorter elimination half-life, and the suspension medium in which they are prepared may contribute to a quick dispersion without agglutination in the vitreous body. Accordingly, Moshfeghi et al. (2009) found that larger particles and greater aggregation may have less migration of particles throughout the vitreous cavity and longer duration of degradation. However, Missel et al. (2010) suggest the dissolution rates of TA preparations with different particle sizes cease to increase at particle diameters much larger than the particle size for either Kenalog® (14–21 μM) or Triesence™ (5–6 μM), and the dissolution rates for these two products would be comparable under conditions in which they form well-defined depots within the vitreous.

Intravitreal triamcinolone levels from both the TA-BA and the TA-PF formulations show variability within each group and study period at each of the time points investigated. This variation has been
reported previously and is related to drug shaking and aspiration variation intrinsic to the intravitreal injection procedure.\textsuperscript{19} This may also explain the 3-day triamcinolone dosage of less than 2 mg in both groups, when compared to the approximately 3.5 mg reported by Kim et al. for both formulations at the same study period (estimated data according to elimination rate constant).\textsuperscript{15} As a consequence, extrapolated TA half-lives from both formulations in the current study (TA-PF: 7.995 ± 2.793 days; TA-BA: 8.166 ± 1.287 days) are also lower than the ones reported by Kim (TA-PF: 24 days; TA-BA: 23 days). However, since the procedures in both groups were performed by the same experienced retinal surgeon, using an adequate number of animals,\textsuperscript{15,20} bias regarding the intravitreal injection was reduced, supporting the comparative analysis.

There was no significant difference in IOP between the two groups throughout the 28-day study period. However, a significant increase in IOP occurred in both groups at 1 min and at 3 days post-injection when compared to pre-injection IOP. The initial IOP increase after injection was relieved by anterior chamber paracentesis; however, median IOP remained above pre-injection levels in both groups at 3 days after TA injection. Similar corticosteroid-related IOP increase in rabbits was also reported by Kai et al.\textsuperscript{21} In addition to ocular hypertension, cataract is also an important complication of intraocular steroids\textsuperscript{21} and was not observed in the present study, probably due to the short follow-up period.

The potential for triamcinolone acetonide-related retinal toxicity remains a controversial issue. Yu et al.\textsuperscript{22} observed destruction of photoreceptor outer segments and migration of macrophage-like cells in the subretinal space after intravitreal injection of 4 and 8 mg of TA-BA in rabbits eyes. On the other hand, and consistent with the results of the current study, Kim et al.\textsuperscript{15} observed no histopathologic or ERG evidence of retinal toxicity following intravitreal injection of TA-BA or TA-PF in rabbit eyes.

In summary, TA-BA and TA-PF show similar vitreous pharmacokinetics in normal rabbit eyes, except for a faster initial clearance of triamcinolone in the TA-PF group. Both TA-BA and TA-PF were well tolerated and
no associated retinal toxicity was evident in this short-term study. The observed retinal safety profile of both intravitreal TA-PF and TA-BA, at a 4 mg dose, reinforces that these drugs may be effective alternative therapeutic strategies in human eyes.

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REFERENCES


