Pharmacokinetics, Electrophysiological, and Morphological Effects of the Intravitreal Injection of Mycophenolic Acid in Rabbits

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Abstract

Purpose: To determine the half-life of mycophenolic acid (MPA) in the vitreous of New Zealand albino rabbits after intravitreal injection and the retinal toxicity of different doses of MPA.

Methods: Ten micrograms of MPA (Roche Bioscience, Palo Alto, CA) was injected in the vitreous of 16 rabbits, animals were sacrificed at different time-points, and vitreous samples underwent high-performance liquid chromatography. For functional and morphological studies, 5 doses of MPA (0.05, 0.5, 2, 10, and 100 μg) were injected in the vitreous of 20 rabbits. As control, contralateral eyes were injected with aqueous vehicle. Electroretinograms (ERGs) were recorded before injection and at days 7, 15, and 30. Animals were sacrificed on day 30 and retinas were analyzed under light microscopy.

Results: MPA half-life in the vitreous was 5.0 ± 0.3 days. ERG revealed photoreceptor functional impairment in eyes injected with 0.5 μg and higher on day 30, while eyes injected with 100 μg presented the same changes already from day 15. No morphological change was found.

Conclusions: MPA vitreous half-life is 5.0 days. Intravitreal injection of 0.5 μg MPA and higher causes dose- and time-related photoreceptor sensitivity decrease in rabbits. The MPA dose of 0.05 μg may be safe for intravitreal use in rabbits.

Introduction

CORTICOSTEROIDS ARE THE first-line drugs for the treatment of non-infectious uveitis. However, long-term use may be associated with severe systemic side effects and lead to treatment discontinuation.[1,2] Immunosuppressant drugs downregulate chronic inflammation and prevent recurrences but also may present significant risk of severe side effects that can lead to dose tapering and even discontinuation.[3]

Mycophenolate mofetil (MMF) is an immunosuppressant drug extensively successfully used for the prevention of organ transplant rejection and for the treatment of many autoimmune diseases, such as vasculitis, systemic lupus erythematous, psoriasis, rheumatoid arthritis, and nephritic syndrome.[4–12] MMF is a prodrug; after oral intake, it is converted in the liver to mycophenolic acid (MPA), the active substance. MPA is a selective and reversible inhibitor of inosine monophosphate dehydrogenase, an enzyme that blocks purine synthesis via a *de novo* pathway, which is preferentially used by T and B lymphocytes.[13,14]

In a preclinical study in rats, MMF inhibited the development of experimental autoimmune uveoretinitis induced by S-antigen after oral intake.[15] In clinical studies that included patients with uveitis, orally administered MMF was an efficient adjuvant therapy, either in steroid-dependent or steroid-resistant uveitis.[16–24] However, up to 21% of the patients treated with oral MMF discontinue treatment due to systemic side effects, mainly leukopenia and gastrointestinal disturbance.[17,23]

Intravitreal drug delivery has become a frequent and important approach for the treatment of posterior-segment diseases. It provides the eye with immediate exposure to therapeutic levels of the injected drug and avoids systemic side effects. Therefore, intravitreal injection of MPA may be

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a potential adjuvant therapy to autoimmune uveitis in patients who have to discontinue systemic steroid or immunosuppressive therapy due to systemic side effects. However, local side effects on the retina and other ocular structures must be considered.[25–28] No information on the morphological and functional effects of intravitreal injection of MPA is available.

The aims of this experimental study are to determine the half-life of MPA in the rabbit vitreous, as well as the functional and morphological effects of MPA in the rabbit retina following a single intravitreal injection.

Methods

Animals

Thirty-six New Zealand albino rabbits (weight 2–3 kg) were included in this study. Animals were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were approved by the Research Ethic Committee, Medical School, Universidade de São Paulo, and the Committee for Ethics in Animal Research, Psychology Institute, Universidade de São Paulo, São Paulo, Brazil.

Animals were housed in individual cages under 12/12 h light-dark cycle with free access to water and food. Before every procedure (intravitreal injection, electroretinogram [ERG], and sacrifice), animals were anesthetized with intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketamina; Agener, São Paulo, Brazil) and 6.7 mg/kg xylazine hydrochloride (Calmiun; Agener). Pupils were dilated with topical 0.5% tropicamide (Mydriacyl; Alcon, São Paulo, Brazil) and the eyes were topically anesthetized with 0.5% proxymetacaine hydrochloride (Anestalcon; Alcon). All animals were sacrificed with intravenous injection of 70 mg/kg sodium pentobarbital (Euthanyle; Brouwer, Buenos Aires, Argentina) under deep anesthesia.

MPA preparation

MPA ($C_{17}H_{20}O_6$, wt: 320.3) is a lipophilic compound slightly soluble in water (13.0 µg/mL). MPA (Roche Bioscience, Palo Alto, CA) intravitreal formulation was prepared as a sterile 10-µg suspension in single-use vials. The suspending medium (vehicle) was sterile saline solution containing the dispersion agent polysorbate 80 (0.4% w/v; Sigma-Aldrich Co., St. Louis, MO) and the suspending agent hydroxypropyl methylcellulose (0.5% w/v; Sigma-Aldrich Co.). Before injections, MPA was diluted with the vehicle to 0.05, 0.5, 2, 10, and 100 µg doses. The formulations were prepared immediately before injection.

There is no study in the literature that evaluates the levels of MPA in the vitreous after systemic use, either in humans or in rabbits that we can rely on. In addition, there are so many factors that influence drug absorption, bioavailability, and penetration into the eye (eg, age, nutritional status, time from last meal, drug solubility, and blood-retinal barrier) that theoretical calculations play a limited role and it is not possible to accurately predict the amount of drug that penetrates into the eye. Thus, we tested the highest possible concentration of MPA using the appropriate vehicle (1.0 mg/mL) and lower doses with differences from 1 to 2.3 log units (100, 10, 2, 0.5, and 0.05 µg of MPA).

Intravitreal injection

Under anesthesia and dilated pupils, 5% povidone iodine drops (Ophthalmos, São Paulo, Brazil) were instilled. Anterior chamber tap (0.1 mL of aqueous humor) was performed with a 30-gauge needle. Intravitreal injections were performed using a 30-gauge needle attached to a tuberculin syringe inserted ∼3 mm posterior to the limbus. The right eye (RE) was injected with 0.1 mL of the MPA solution and the left eye (LE) with 0.1 mL of the suspension vehicle as control.

Vitreous half-life determination

For MPA vitreous half-life determination, 16 animals received an intravitreal injection of 10 µg of MPA in the RE and 0.1 mL of vehicle in the LE. Groups of 4 animals were sacrificed at 1, 7, 14, and 28 days after intravitreal injection, eyes were enucleated, and anterior segment and lens were removed. Vitreous samples were frozen at −18°C for high-performance liquid chromatography (HPLC) assays, as well as peripheral blood samples that were drawn at the time of sacrifice to determine the serum concentration of MPA.

The ocular pharmacokinetic model was developed using previous studies as reference.[29–32] All data were fit with a single exponential curve according to Equation (1) and the estimated half-time ($t_{1/2}$) of MPA elimination was calculated with Equation (2).

$$C_{(t)} = C_0 \exp^{(-kt)} \qquad (1)$$

$$t_{1/2} = 0.693/k \qquad (2)$$

Where C (µg/mL) and C_0 (µg/mL) represent respectively, the MPA concentration at any time and at t_0, t (day) is the time after injection, and k (day^{-1}) represents the decay constant.

MPA determination

The chromatographic system consisted of a Merck-Hitachi LaChrom Elite apparatus equipped with an autosampler with sample loop of 100 µL (model L-2200; Merck-Hitachi, Darmstadt, Germany), a pump at a constant flow rate of 1.4 mL/min (model L-2130; Merck-Hitachi), and a diode array detector used at a wavelength of 215 nm (model L-2450; Merck-Hitachi). Separation chromatography was performed using an Ace 5 C18 column (250×4.6 mm id; Advanced Chromatography Technologies, Aberdeen, Scotland) maintained at 50°C (column oven model L-2300; Merck-Hitachi). A mixture of acetonitrile (Merck-Hitachi) and 40 mM phosphoric acid buffer at pH 3.0 (32:68 v/v) was used as mobile phase. Under these experimental conditions, the retention time of MPA was 14.0 min.

Frozen vitreous and blood samples were thawed out at ambient temperature. After brief mixing, 500 µL of mobile phase was added to aliquots of 500 µL of vitreous and blood samples. After mixing for 1 min, samples were filtered (Durapore, 0.2 µm; Millipore, Darmstadt, Germany) and 100 µL was injected into the column. Standard stock solutions of 0.5 mg/mL MPA were prepared in methanol. This solution was added to drug-free rabbit vitreous and blood to prepare 7 non-zero concentrations in the range of 1–200 µg/mL MPA.
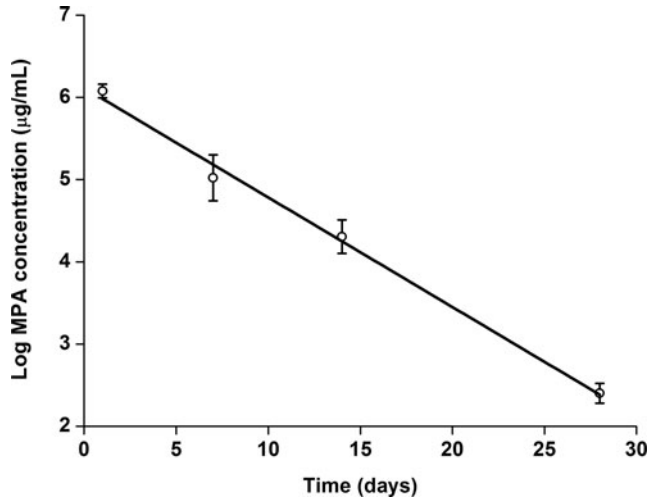
FIG. 1. Linear regression of concentrations of mycophenolic acid (MPA) in the vitreous after intravitreal injection of $10\,\mu g$ (4 eyes in each time-point).

Electroretinography

For functional study, 20 rabbits were divided in 5 groups of 4 animals each, according to the dose of MPA injected in the vitreous (0.05, 0.5, 2, 10, and $100\,\mu g$). RE was injected with MPA and LE with vehicle. Full-field ERG was performed in both eyes before intravitreal injection and 7, 15, and 30 days after injection. ERG was not performed on

the first day after intravitreal injection because caged rabbits are very sensitive to deep and prolonged anesthesia[33] (as needed for ERG recordings) and undergoing anesthesia in 2 consecutive days significantly increases the risk of death.

ERG responses were recorded using a corneal bipolar contact lens electrode (GoldLens; Doran Instruments, Inc., Littleton, MA) and a ground electrode (model E5; Grass Technologies, West Warwick, RI) placed on the ear. Light stimuli were generated by a Grass stimulator (PS33-PLUS Photic Flash Visual Stimulator; Grass Technologies) with a flash lamp bulb (PST-2100; Grass Technologies). Light stimuli were provided by a Ganzfeld Stimulator (2503B; LKC Technologies, Gaithersburg, MD) placed inside a Faraday cage ($60\times60\,cm^2$) and were controlled by a computerized system (RETI-port; Roland Consult, Brandenburg, Germany). ERG signals were amplified and digitalized (PCI-1200; National Instruments Co., Austin, TX), and analyzed by a data acquisition software (LabVIEW; National Instruments Co.). The band pass for flash ERG was set to 0.3–1,000 Hz.

The ERG protocol was modified after the International Society for Clinical Electrophysiology of Vision (ISCEV) standards.[34] Animals were dark-adapted for 30 min and anesthetized, pupils were dilated, and eyes were topically anesthetized. The ISCEV-modified ERG protocol included 5 flash luminances in dark-adapted state (0.003, 0.03, 0.3, 3, and 30 $cd\cdot s/m^2$), light-adapted state (after light adaptation with background luminance of 25 cd/m^2 for 10 min,
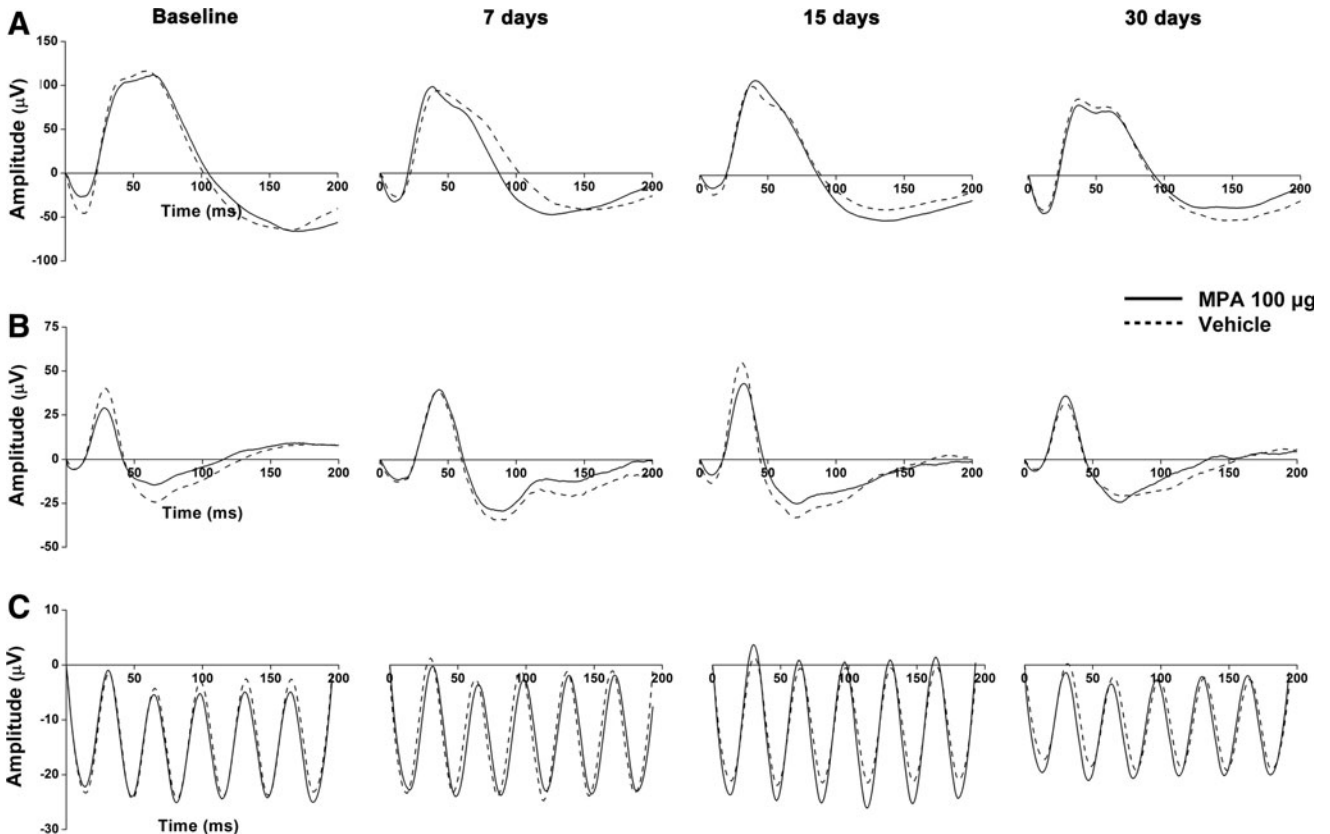

FIG. 2. Representative ERG records of 1 animal at different time-points after intravitreal injection of $100\,\mu g$ MPA. **(A)** Dark-adapted state (stimulus of 30 $cd\cdot s/m^2$); **(B)** light-adapted state (stimulus of 3 $cd\cdot s/m^2$); **(C)** light-adapted flicker (stimulus of 3 $cd\cdot s/m^2$ at 30 Hz).
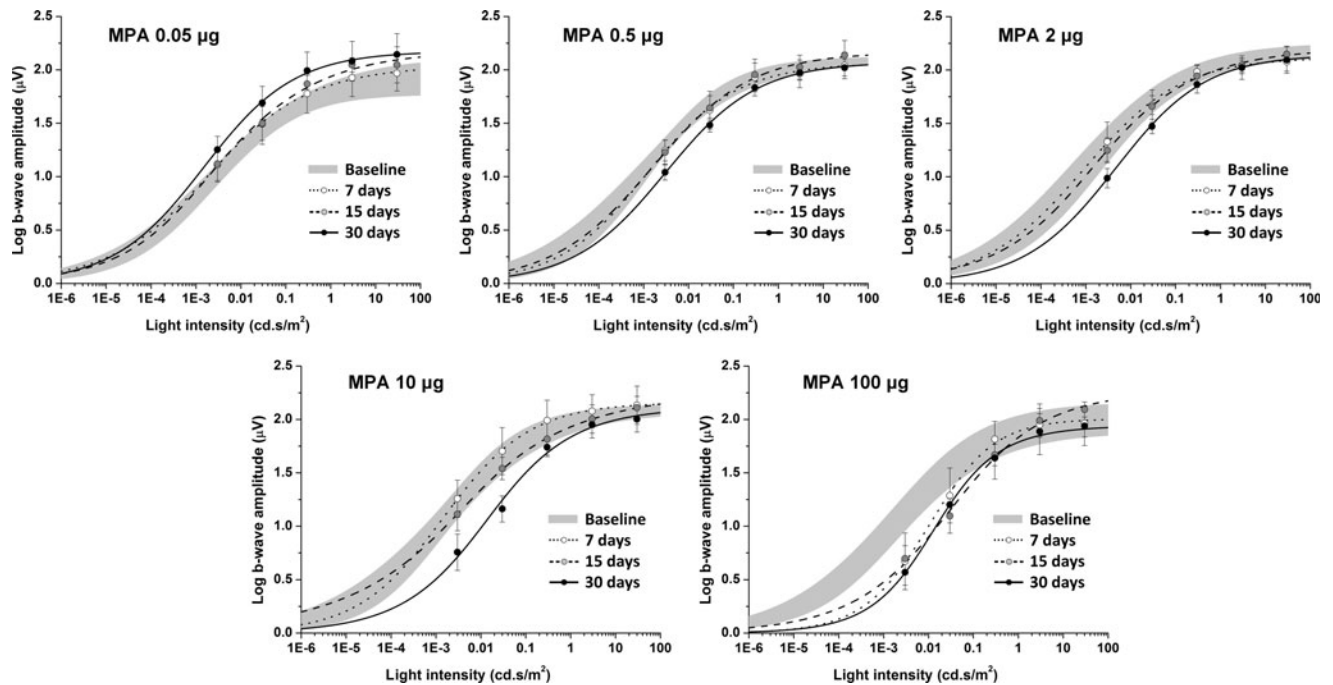
FIG. 3. Log b-wave amplitude versus flash intensity curves of eyes injected with different doses of MPA. *Gray areas* represent the mean ± standard deviation of 4 eyes before intravitreal injection. *Dots* and *error bars* represent mean ± standard deviation of the 4 eyes after intravitreal injection. Contralateral eyes, injected with aqueous vehicle, did not present differences from baseline.

3 cd·s/m² flashes were presented), and light-adapted flicker (3 cd·s/m² flashes presented at 12, 18, 24, and 30 Hz).

ERG data analysis. The a- and b-wave amplitudes and implicit times were quantified. The a-wave amplitude was measured from the baseline to the minimum amplitude after light stimulus onset. The a-wave time-to-peak, or implicit time, was measured from flash onset to the a-wave peak. The b-wave amplitude was measured from the a-wave through the b-wave peak amplitude. Similarly, the b-wave implicit time corresponded to the time of occurrence of its peak amplitude.

Dark-adapted b-wave mean amplitudes were plotted as log response versus light intensity curves and fitted with Naka–Rushton equation:

$$V = V_{max} \frac{I^n}{k^n + I^n}$$

Where V_{max} is the b-wave saturating amplitude, l is the light intensity, k is the intensity needed for 50% of V_{max}, and n is the slope of the function, representing the dynamic range of the measured wave.

Correlations between b-wave and a-wave amplitudes in eyes injected with different doses of MPA and vehicle at different time-points for the dark-adapted state were plotted.

The 12, 18, 24, and 30 Hz flicker recordings were analyzed using the Fourier fast transform algorithm with 100 Hz low-pass filtering. The amplitudes and phases of the first, second, and third harmonics were calculated and plotted for

TABLE 1. PARAMETERS OBTAINED FROM B-WAVE MEAN AMPLITUDE VERSUS FLASH INTENSITY CURVES USING THE NAKA–RUSHTON EQUATION (DARK-ADAPTED STATE)

	Vmax *(log, μV)*				k *(cd·s/m²)*				n			
MPA (mg/mL)	*Baseline*	*7 days*	*15 days*	*30 days*	*Baseline*	*7 days*	*15 days*	*30 days*	*Baseline*	*7 days*	*15 days*	*30 days*
0.05	1.95	2.04	2.18	2.17	0.002	0.002	0.005	0.002	0.40	0.39	0.42	0.43
0.5	1.95	2.07	2.17	2.07	0.002	0.001	0.002	0.004[a]	0.40	0.44	0.40	0.43
2	2.14	2.12	2.20	2.14	0.001	0.001	0.002	0.007[b]	0.43	0.41	0.40	0.43
10	2.11	2.16	2.19	2.08	0.002	0.002	0.003	0.016[c]	0.41	0.45	0.39	0.50
100	2.02	2.01	2.27	1.94	0.002	0.007	0.031[d]	0.020[e]	0.40	0.63[f]	0.41	0.61[g]

[a]$P = 0.028$.
[b]$P = 0.015$.
[c]$P = 0.005$.
[d]$P < 0.001$.
[e]$P = 0.006$.
[f]$P = 0.013$.
[g]$P = 0.031$.
V_{max}, log b-wave saturating amplitude; k, light intensity at 50% of V_{max}; n, curve slope; MPA, mycophenolic acid.
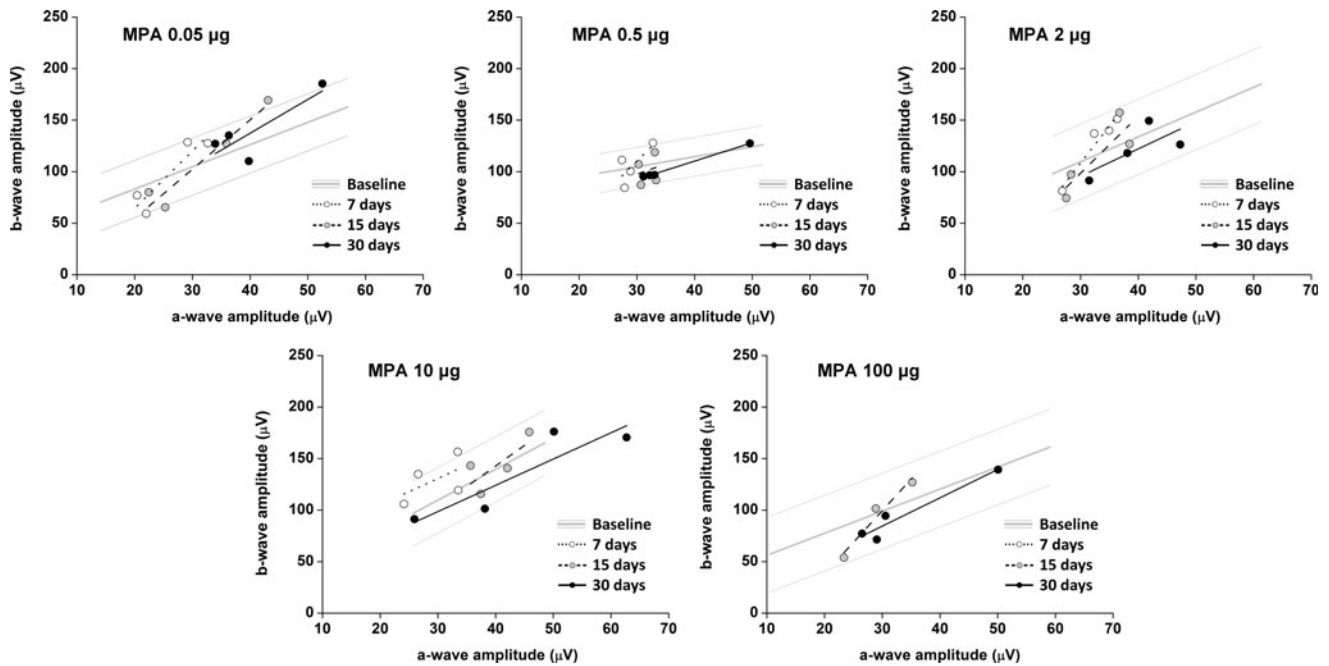
FIG. 4. b-Wave to a-wave amplitude correlations in eyes injected with different doses of MPA at different time-points (dark-adapted state, stimulus of 30 cd·s/m^2). Baseline data are represented as linear regression ± standard deviation. Dots represent the a- and b-wave amplitudes of each animal, and lines represent the linear regression for each group. Contralateral eyes, injected with aqueous vehicle, did not present differences from baseline.

all test conditions. The results of the eyes injected with MPA and vehicle were compared for each MPA dose, stimulus frequency, and time-point after treatment.

Morphological analysis

After the last ERG recording on day 30, animals were sacrificed and eyes were processed for light microscopy. Immediately after sacrifice, eyes were enucleated, the anterior segment and lens were removed, and the posterior segment was fixed in ALFAC fixative solution. Samples were included in paraffin and 7-μm-thick sections of the inferior midperipheral posterior segment (retina/choroid/sclera) were stained with hematoxylin and eosin (Sigma-Aldrich Co.) and were analyzed under light microscopy. Eyes injected with MPA were compared with vehicle-injected fellow eye of the same animal. Thickness and gross organization of each retinal layer were analyzed.

Statistical analysis

Amplitudes and implicit times are expressed as mean ± standard deviation. Statistical analyses were carried out using repeated measures ANOVA and Fisher's least significant difference test as *post hoc* test. Parameters from Naka–Rushton equation of b-wave amplitude versus light intensity were analyzed with 1-way ANOVA and paired 2-tailed *t*-test with Bonferroni correction for the number of comparisons among treatment groups and intervals. $P < 0.05$ was considered statistically significant.

Results

Determination of MPA half-life in the vitreous

The HPLC method was validated and successfully used for the determination of MPA vitreous concentration. The chromatograms obtained during the analysis were very clean and no significant interference could be detected at the retention time of the blank samples (data not shown). The calibration curve was obtained by linear regression analysis plotting of peak-area ratio versus the MPA concentration ($y = 432700x - 3472.9$; $R^2 = 0.9996$). These data suggest reproducible results and that the assay was accurate and reliable. MPA vitreous concentrations at different time-points are presented in Figure 1. Estimated half-time ($t_{1/2}$) of MPA elimination from rabbit vitreous was 5.0 ± 0.3 days ($R^2 = 0.993$; $P = 0.004$) and the drug was detectable for 29 days (sensitivity = 1 μg/mL).

MPA was not detectable in either eye injected with saline or peripheral blood samples.

Electrophysiological analysis

Dark- and light-adapted representative ERG records of 1 animal injected with the highest dose of MPA (100 μg) are shown in Figure 2.

The light- and dark-adapted a- and b-wave mean amplitude and implicit time were analyzed. No statistically significant differences were found between baseline values and postinjection values on days 7, 15, and 30 at any MPA tested dose.

Dark-adapted log b-wave mean amplitudes versus flash intensity curves of eyes injected with MPA are presented in Figure 3. Curves of 7, 14, and 28 days postinjection were compared with preinjection curve. Parameters V_{max}, k, and n are presented in Table 1. V_{max} did not change over time after intravitreal injection of any dose of MPA. However, k significantly increased 30 days after intravitreal injection of 0.5 μg MPA and higher doses (0.5 μg, 305% increase, $P < 0.001$; 2 μg, 423% increase, $P = 0.028$; 10 μg, 882% increase, $P = 0.015$; and 100 μg, 1,565% increase, $P = 0.005$).
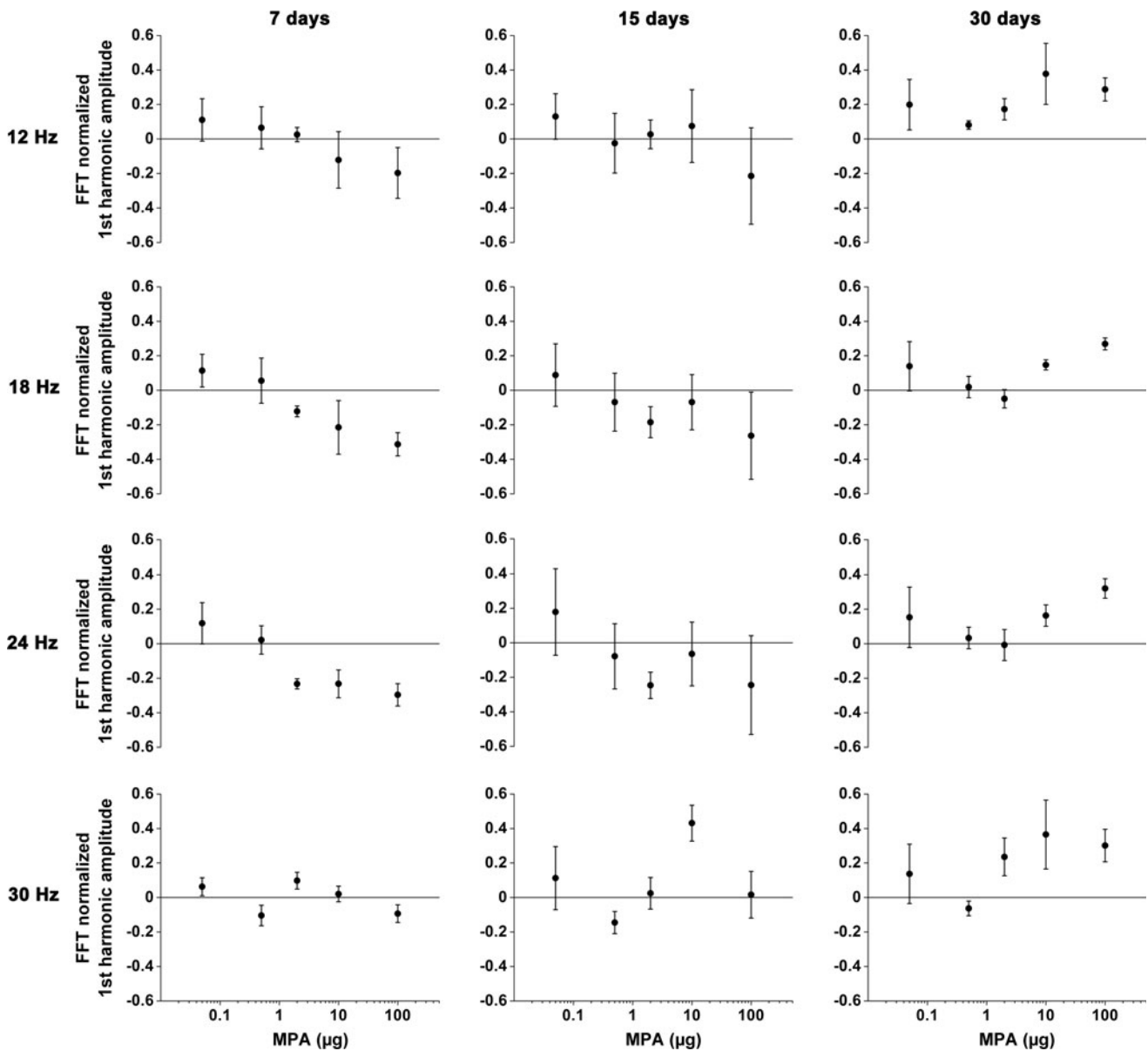
FIG. 5. Flicker results of eyes injected with 5 doses of MPA for stimulus frequency and time-point after intravitreal injection. Differences between the MPA and vehicle normalized FFT first-harmonic amplitude values are plotted.

As for $100\,\mu g$, k increased 931% already 15 days after intravitreal injection of MPA ($P=0.006$). The n parameter significantly increased 7 days (55% increase, $P=0.013$) and 30 days (51% increase, $P=0.031$) after $100\,\mu g$ MPA injection. Eyes injected with aqueous vehicle did not present significant differences.

The b-wave to a-wave amplitude correlations for dark-adapted state before and after injection of the 5 tested MPA doses did not show significant differences at any time-point or flash intensity. Graphs of b-wave to a-wave amplitude correlations for $30\ cd\cdot s/m^2$ are shown in Figure 4.

Flicker analysis showed that the amplitudes and phases of the different harmonics presented similar tendencies among eyes injected with MPA and vehicle. Therefore, we used the first-harmonic amplitudes for comparisons. Data were individually normalized to the mean amplitude of the eyes injected with vehicle, and the MPA and vehicle differences were calculated. Mean flicker amplitudes of eyes injected

with MPA did not present statistical difference during follow-up (Fig. 5). However, graph analysis suggests dose- and time-related tendencies of flicker amplitude decrease with MPA dose increase at 12, 18, and 24 Hz on days 7 and 15 after intravitreal injection.

Morphological analysis

No major histological changes were observed in light microscopy of eyes injected with MPA and vehicle 30 days after intravitreal injection. Histological sections of representative retinas injected with the 5 tested MPA doses are shown in Figure 6.

Discussion

In this study, we investigated the pharmacokinetics of MPA in the rabbit vitreous and safety of different doses of
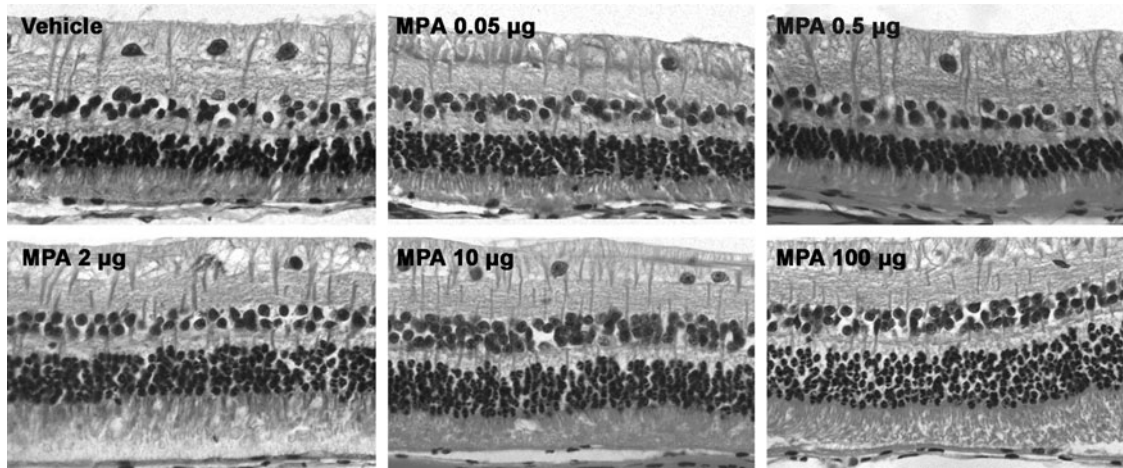
FIG. 6. Light micrographs 30 days after intravitreal injection of different doses of MPA showing well-preserved retinal morphology.

MPA after intravitreal injection. MPA half-life in the vitreous of non-inflamed and phakic eyes is 5 days and the drug remains detectable for 29 days. MPA is not detectable in the vitreous of the fellow eye and in the serum, suggesting that systemic absorption, if any, may not be significant. With regard to safety, electrophysiological results suggest that MPA causes dose- and time-related photoreceptor functional impairment. MPA doses of 0.5 μg and higher cause photoreceptor sensitivity decrease on day 30, while eyes injected with 100 μg present the same changes already from day 15.

Although MPA has been successfully used to treat uveitis after oral administration of its prodrug MMF, systemic treatment has to be discontinued in up to 21% of the patients due to systemic side effects.[17,23,24] Intravitreal injection may be an adjuvant treatment that may immediately deliver drug to the posterior segment of the eye. Our results show that MPA is detectable in the vitreous up to 29 days after a single intravitreal injection. The rate of elimination from the vitreous depends on drug characteristics and partition characteristics of the surrounding tissues. MPA is a lipophilic drug and its half-life is expected to be greater than that of hydrophilic drugs. Our pharmacokinetic data cannot be directly compared to other studies as this is the first report of MPA vitreous half-life determination. However, triamcinolone acetonide (TA) is also a lipophilic drug slightly soluble in water and its behavior in the vitreous can be considered a reference for this study. TA vitreous half-life in rabbit eyes varies from 1.6 to 8.2 days,[35–37] confirming that MPA and TA, both lipophilic drugs, have vitreous half-lives in the range of few days.

ERG analysis shows significant differences in b-wave amplitude versus flash intensity curves in the dark-adapted state, suggesting that MPA causes dose- and time-related retinal toxicity. The increase on the intensity needed to reach 50% of V_{max} (parameter k) 30 days after intravitreal injection of 0.5 μg MPA and higher represents a decrease in retinal sensitivity, suggesting that injection of 0.5 μg MPA and higher causes time-related damage to either the photoreceptors or to the synaptic transmission between these and bipolar cells. Regarding the 100 μg dose, this effect is detectable after 15 days of MPA intravitreal injection and extends through day 30. However, postreceptoral changes seem less likely as the b- to a-wave amplitude ratios are not altered. If postreceptoral transmission pathways were affected by MPA, one might expect that b-wave would be reduced and a-wave would remain unchanged.

The increase in parameter n on days 7 and 30 after injection of 100 μg MPA represents a change in the gain of synaptic transmission between photoreceptors and bipolar cells, which ultimately drive the b-wave. Although the n value increase might corroborate the photoreceptor damage, the difference is not present on day 15. Therefore, it is not possible to assert that this finding represents experimental evidence of photoreceptor damage.

Flicker stimulation of the light-adapted retina isolates cone-driven ERG responses since the rod photoreceptors are saturated by the light adaptation and cannot respond even at low temporal frequencies. Some authors have shown that flicker responses may be more sensitive than light- and dark-adapted a- and b-wave parameters in detecting early toxic effects.[38–40] Our graphs show that MPA tends to cause reduction in the first harmonic of the mean response amplitude at stimulus frequencies of 12, 18, and 24 Hz, at days 7 and 15. The light-adapted ERG response to flicker contains contributions from cones and from the inner retina at the temporal frequencies used here.[41,42] The observed apparent reduction together with the other ERG findings may reflect a diffuse damage of the cones of eyes treated with the higher doses of MPA. However, rabbits have rod-rich retina and, therefore, toxicity studies of cone system should be viewed with caution.[43]

In contrast to the electrophysiological results, intravitreal injection of up to 100 μg of MPA did not cause morphological changes in the rabbit retina under light microscopy. Several studies using other drugs showed lack of association between functional and morphological findings.[27,44–46] Although we have not found morphological evidence of retinal toxicity after injection of a wide range of MPA doses, it may not be discarded for some reasons. In this study we analyzed sections of the inferior midperipheral posterior segment. The whole retina was not examined by light microscopy and focal damage may not have been detected. It is also possible that the retinal changes may have occurred at the ultramicroscopic level, as shown by Inan et al. for bevacizumab.[47]

In this case, toxic findings could only be evidenced by electron transmission microscopy, which was not performed in the present study. The injection of higher doses of MPA might increase the odds of detecting morphological retinal changes. However, it is not possible to obtain a suitable suspension of MPA in concentrations higher than 1.0 mg/mL in the appropriate suspending vehicle used as the re-suspension of the drug particles is significantly compromised and the dose uniformity (accuracy and precision of unit dose) cannot be reached.

Different mechanisms may be involved in the retinal toxicity of MPA, such as oxidative stress. Our group has shown that 100 μg/mL MPA causes significant decrease in *in vitro* cell viability and increase in caspase 3/7 activity in retinal pigment epithelium (RPE) and Müller cells compared with untreated cells, suggesting increased apoptosis.[48] The dispersion agent polysorbate 80 used in the vehicle of the intravitreal formulation of MPA may also play a role in the retina functional impairment. After systemic injection, polysorbate 80 has been shown to induce hypersensitivity reactions and plasma viscosity increase.[49] However, polysorbate is part of the formulation of other drugs widely used in intravitreal injections, such as bevacizumab (Avastin®; Genentech, Inc., San Francisco, CA) and preservative-free TA (Triesence®; Alcon Laboratories, Inc., Fort Worth, TX), and no significant safety concerns have been raised in terms of retina functional damage.[50–52] Differences in pH and osmolarity are also known as potential causes of retinal toxicity. However, the pH of the MPA preparation used in this study was 6.8 ± 0.2, and its osmolarity was 300 ± 8 mOsm, both in the physiological range. In addition, the lack of definite safe ranges for these parameters makes it difficult to predict whether a preparation is toxic or not.[53]

The design of this study does not allow drawing any conclusion on why MPA does not cause photoreceptor functional impairment on ERG early after intravitreal injection. It has been previously shown that MPA causes *in vitro* early RPE cell viability decrease and apoptosis increase.[48] We can speculate that an ultramicroscopic RPE damage may be the primary event on the MPA retinal toxicity cascade and, due to the interdependency between RPE and photoreceptors, these cells may start to present functional changes weeks after intravitreal injection of MPA that are not detectable by ERG and light microscopy. Alternatively, MPA might cause focal early damage to photoreceptors that is not detected by ERG as it only detects the mass response of the retina to a light stimulus. However, the areas of focal damage may increase with time until they are so spread that ERG can detect functional changes.

This study has several limitations. First, the relatively small number of animals included in each group (4 animals) may impair the drawing of assertive conclusions. As we could not rely on previous studies, we tried to increase the odds of detecting toxic retinal changes by using different approaches and analyzing several variables. For this reason, the number of subjects per group was relatively small. Second, if MPA causes focal damage to the retina, it would not have been detected by our morphological analysis, as only the inferior midperipheral retina was used on the light microscopy analysis. Third, we used albino rabbits in our experiments, whose eyes do not contain melanin, a molecule highly expressed in the human retina. However, the use of albino rabbits may be more predictive of acute retinal toxicity than the use of pigmented animals, as some drugs partially bind to melanin and it partially protects the retina from drug toxicity.[54] For this reason, a long-term administration of drugs with high melanin affinity may be necessary to induce ocular toxicity.[55,56] Finally, this is an experimental study and limitations of the rabbit model include differences in the retina vascularization when compared with the human eye, as well as differences in eye volume. For these reasons, results may not fully represent the findings in inflamed human eyes.

In conclusion, our data suggest that 0.05 μg MPA is safe for intravitreal injection. However, 0.5 μg MPA and higher may cause functional retinal changes that are detected 30 days after the intravitreal injection. The highest dose tested (100 μg) causes early functional retinal changes that may be irreversible, as the retina does not seem to functionally recover by day 30. This study contains evidences that may have both clinical and experimental significances of the use of intravitreal MPA as an adjuvant approach for the treatment of specific cases of non-infectious uveitis. It is unknown whether 0.05 μg MPA has therapeutic effects in an animal model of autoimmune uveitis or in humans, but our findings may be used as a reference for future *in vivo* studies.

Acknowledgments

Author Disclosure Statement

All authors do not have any competing financial interests.

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