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Noninvasive Electroretinography Assessment of Intravitreal Sustained-Release Methotrexate Microimplants in Rabbit Eyes

Soumyarwit Manna, James J. Augsburger, Zelia M. Correa, Marwan F. Al-Rjoub, Marepalli B. Rao, and Rupak K. Banerjee

Abstract

Purpose: The purpose of this study is to noninvasively evaluate the safety and toxicity of a chitosan (CS) and polylactic acid (PLA)-based sustained-release methotrexate (MTX) intravitreal microimplant in normal rabbit eyes using electroretinography (ERG).

Methods: PLA-coated CS-based microimplants containing 400 μg of MTX and placebo microimplants (without drug) were surgically implanted in the vitreous of the right and the left eyes, respectively, in each of the 8 New Zealand rabbits using minimally invasive technique. At each predetermined time points (days 5, 12, 19, and 33), ERG was conducted on 2 rabbits to evaluate the safety of the microimplants administered in each eye. ERG was carried out using 2 protocols, scotopic and photopic, on each eye prior to surgery (PS) and prior to euthanasia (PE) conditions. The safety of the microimplants was assessed using statistical analysis of the ERG data (B/A ratio analysis, oscillatory potential analysis, and Naka–Rushton analysis) and subsequently quantifying and comparing functional integrity of the retina between the PS and PE conditions of each eye.

Results: Statistical analysis of the ERG data showed no change in retinal functional integrity because of the PLA-coated CS-based MTX microimplant and the placebo microimplant. ERG analysis also revealed absence of any evident bioelectrical dysfunction caused by the microimplants.

Conclusion: ERGs were performed to determine whether the microimplants containing MTX and the placebo microimplants were associated with any profound retinal bioelectrical dysfunction that might be attributable to toxicity not apparent on histological studies of such eyes. The results shown in this report indicate that there were no such evident adverse effects of the microimplants or contained drug.

Keywords: electroretinogram, chitosan, methotrexate, polylactic acid, intravitreal, sustained drug delivery

Introduction

Methotrexate to treat vitreoretinal diseases

The antimetabolite chemotherapeutic drug, methotrexate (MTX), has a proven reputation for the management of selected vitreoretinal (VR) diseases such as chronic intraocular inflammation (uveitis) and primary central nervous system lymphoma involving the eye. The therapeutic efficacy and potential systemic complications of MTX are well recognized. A single intravitreal injection of MTX at the 200–400 μg/0.1 mL dose is well tolerated and usually uncomplicated. However, there is an increasing risk of probable side effects and complications such as rhegmatogenous retinal detachment, exogenous endophthalmitis, and elevated intraocular pressure when multiple intravitreal injections of MTX are used over a period of several months for effective treatment of VR diseases. MTX is hydrophilic in nature. When administered by intravitreal injection, MTX undergoes rapid clearance from the eye (intravitreal half-life of 14.3 h). Prior pharmacokinetic studies have revealed that each intravitreal injection of MTX causes therapeutic intravitreal concentrations of the MTX (0.1–1 μM) for only about 48–72 h. Since MTX is cleared rapidly, repetitive intravitreal injections of MTX are required for sustained therapeutic efficacy. A sustained-release intravitreal drug delivery device is expected to provide a therapeutic dose of MTX...
over a prolonged duration of time without causing collateral toxicity to the normal tissues. Therefore, such a device may be preferred over repetitive intravitreal injections of MTX if the safety of such an intravitreal device can be assured. During the past several years, our group developed a biodegradable slow-release device\(^4\) for intravitreal delivery of sustained therapeutic doses of MTX and performed pharmacokinetic studies of intravitreal dose levels over time and histopathological studies of rabbit eyes, in which one of these microimplants had been inserted.\(^5\)

**Assessment of toxicity from MTX delivery system**

The assessment of toxicity caused by the MTX administration from the sustained MTX delivery device is crucial for its safe use. The safety of various intravitreal drug delivery devices has been evaluated using conventional histopathology studies of the retina and intraocular tissues. However, a noninvasive technique for toxicity assessment, such as electoretinography (ERG), is always preferred compared with an invasive technique, such as histopathology analysis. Prior studies that involved assessment of the safety of the intravitreal implants using ERG were involved with sustained administration of lipophilic drugs, such as dexamethasone, triamcinolone, and 2-methoxyestradiol.\(^6\)–\(^9\) There has been no report of evaluation of the safety of sustained-release hydrophilic drugs, such as MTX, over a long duration using ERG.

For an intravitreal MTX microimplant, Palakurthi et al.\(^2\) of our group computed the effective drug distribution within the vitreous of the eye for sustained release of MTX to be within the therapeutic window of 0.1–1 \(\mu\)M or 0.2–2.0 \(\mu\)g/day for a period of 1 month or more. Accordingly in a subsequent study, our group developed a chitosan (CS) and polylactic acid (PLA)-based MTX intravitreal microimplant device that was able to sustain MTX release between 0.2 and 2 \(\mu\)g/day in vitro for more than 50 days.\(^4\) The fabrication of the microimplant, along with the MTX-release characteristics from the microimplant, was described in detail in that report.\(^4\) In addition, the potential benefits, limitations, and risks of MTX administration from an intravitreal MTX microimplant in comparison with those of systemic MTX administration or repeated intravitreal injections of MTX were discussed in detail in that report.\(^4\)

In our recent preclinical trials in rabbit eyes involving the same PLA-coated CS-MTX microimplant containing \(\sim 400 \mu\)g of MTX (similar dosage of MTX present in an intravitreal MTX injection), a sustained release of MTX (0.1–1 \(\mu\)M) was observed for more than 30 days.\(^5\) Furthermore, histopathological analysis of the study eyes showed no evidence of histological retinal toxicity of the microimplants.\(^5\)

**Noninvasive assessment of toxicity using ERG**

Although our published histopathological study\(^5\) of eyes containing one of our microimplants showed no evident anatomic toxicity in the retina, this does not prove absence of functional toxicity of the microimplants. To assess the possibility of retinal dysfunction induced by our microimplants, we performed ERG on the study eyes and analyzed the bioelectrical responses of the retina to light flashes.

**Methods**

**Design of ERG study**

All procedures related to the rabbit surgery in this research were in accordance with the Institutional Animal Care and Use Committee protocol (IACUC No. 12-09-13-01, University of Cincinnati, dated: November 21, 2012) and followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In this feasibility study, 8 New Zealand white rabbits were used, each weighing 2–3 kg. Each rabbit was administered a sterilized PLA-coated CS-MTX microimplant in the right eye and a sterilized placebo microimplant (microimplant without MTX) in the left eye. Parallel to the limbs in the superotemporal quadrant, a full-thickness eye wall incision was made. An MTX-loaded microimplant was passed through incision of the right eye and a placebo microimplant was inserted into the left eye. Postinsertion, a mattress suture of 7-0 polyglactin 910 (suture material: Ethicon, Cincinnati, OH) was used to close the conjunctival–scleral wound. The PLA-coated CS-MTX microimplant contained 400 \(\mu\)g of MTX,\(^4,5\) which is similar to the dosage of MTX in an intravitreal injection. These microimplants were \(\sim 4.2 \text{ mm}\) in length and had a cross-sectional diameter of \(\sim 0.9 \text{ mm}\). The fabrication process of the microimplants has been reported in our prior in vitro study.\(^4\) Two rabbits were assessed for retinal toxicity using ERG on each of the predetermined time points: days 5, 12, 19, and 33. ERG recordings were obtained on each eye at the following 2 time points: (1) prior to the surgery (PS) involving the implantation of the microimplants and (2) prior to the euthanasia (PE) involving euthanasia of 2 rabbits at the predetermined time points. The rabbits had to be euthanized at the end of the study for pharmacokinetics and histopathological evaluation.\(^5\)

**ERG setup and recording**

The ERG study was conducted using a portable ERG machine (HMsERG system; Ocuscience LLC, Henderson, Nevada). Animal preparation involved anesthetizing the animals and then electrode connections were established between the rabbit and the HMsERG system. Isoflurane (1%–2.5%) was used for anesthetizing the animals during the ERG procedure. Animals were occasionally kept on a heated water bath to maintain their body temperature at 37°C. A droplet of 2.5% Hypromellose ophthalmic demulcent solution (Goniovisc™, Hub Pharma, Rancho Cucamonga, CA) was applied on the concave side of the ERG-Jet contact lens electrode (Fabrilin SA, La Chaux-de-Fonds, Switzerland), and then the contact lens electrode was placed on the cornea. A stainless steel needle electrode was inserted subcutaneously at the base of the ear, which was the reference electrode. Furthermore, another stainless steel needle electrode was inserted subcutaneously on top of the forehead (midline), which served as the ground electrode. The electrodes were secured with surgical tape after the connections were made.

ERG analysis at each condition, that is, PS and PE, involved recordings of 2 protocols on each eye: scotopic protocol primarily representing the activity of the rods and the photopic protocol primarily representing the activity of the cones in the eye. The HMsERG machine has preset programs, which provide the required light stimulus for the
scotopic (dark adaptation) and the photopic (light adaptation) protocols.

The eyes required 30 min of dark adaptation (completely dark room, without any light) before the scotopic protocol recording. The preset scotopic protocol in the HMsERG provided 6 stimulus intensities in an ascending order at an interval of 60 s between 2 subsequent intensities. The intensities of the light stimulus during the scotopic protocol were presented in the following sequence: 100, 300, 1,000, 3,000, 10,000, and 25,000 mcd s/m^2.

In the case of photopic protocol recording, the eyes were exposed to 10 min of light adaptation at 30,000 mcd s/m^2 by the HMsERG unit. The preset photopic protocol in the HMsERG provided 8 stimulus intensities in an ascending order at an interval of 0.5 s between 2 subsequent intensities. The intensities of the light stimulus during the photopic protocol were presented in the following sequence: 10, 30, 100, 300, 1,000, 3,000, 10,000, and 25,000 mcd s/m^2. An ophthalmic ointment of bacitracin zinc and polymyxin B sulfate was applied topically to each eye after the ERG procedure.

Statistical analysis of ERG data

For both scotopic and photopic protocols, the amplitude and the implicit time of the A-wave and the B-wave for each intensity were recorded for both the PS and PE conditions. As reported in prior studies, the ratio of the B-wave amplitude to the A-wave amplitude, also known as the B/A ratio, is often used as an indicator of the retinal functional integrity.\(^{10,11}\) The B/A ratio for each intensity was also recorded for both the PS and PE conditions. The mean values of all parameters for each intensity (A-wave amplitude, A-wave implicit time, B-wave amplitude, B-wave implicit time, and B/A ratio) were obtained for each time point (PE condition: days 5, 12, and 33; n = 2 for each time point) for both scotopic and photopic protocols. These mean values were compared with the mean values of the respective parameter for the corresponding intensity of the PS condition (n = 8, day 0).

Relative B/A ratio [(B/A)\(_{rel}\)] analysis. For each of the protocols, at every time point and for each of the intensities, the relative B/A ratio was computed for each rabbit. The relative B/A ratio (B/A)\(_{rel}\) is defined as

\[
(B/A)_{rel} = \frac{(B/A) \text{ ratio prior to euthanasia}}{(B/A) \text{ ratio prior to surgery}).
\]

(Eq. 1)

Subsequently, the effect of the protocols, the intensities, the days (observation time points), and their interaction on the (B/A)\(_{rel}\) was studied using a 3-way analysis of variance (ANOVA) model (Eq. 2). The 3-way ANOVA model is described as

\[
(B/A)_{rel} = \mu + I_i + D_j + (ID)_{ij} + (IP)_{ik} + (DP)_{jk} + e_{ijk},
\]

(Eq. 2)

where \(i = 3,000, 10,000, \) and 25,000 mcd s/m^2; \(j = \text{days 5, 9, 12, and 33}; k = \text{scotopic and photopic protocols}; l = 1, 2 \) rabbits (replication number); \(\mu\) = overall effect; \(I_i\) = effect of the \(i\)th level of intensity; \(D_j\) = effect of the \(j\)th level of day; \(P_k\) = effect of the \(k\)th level of protocol; \((ID)_{ij}\) = interaction between the \(i\)th level of intensity and \(j\)th level of days; \((IP)_{ik}\) = interaction between the \(i\)th level of intensity and \(k\)th level of protocol; \((DP)_{jk}\) = interaction between the \(j\)th level of days and \(k\)th level of protocol; and \(e_{ijk}\) = random error \(\sim N(0, \sigma)\). The responses to the intensities \(\leq 1,000\) mcd s/m^2 were ignored for both the protocols as the light stimulus of low intensities did not yield significant measurable A-wave responses. Thus, in total we had \(n = 48\) (3 intensities \(\times 4\) time points \(\times 2\) rabbits \(\times 2\) protocols) data points.

\(P\) value > 0.05 is indicative of the statistically insignificant effect of the intensities, days, protocols, and their interaction on the (B/A)\(_{rel}\). The interactions between days and protocol \((DP)_{jk}\) \(P\) value = 0.02) and also between intensity and days \((ID)_{ij}\) \(P\) value = 0.02) were found to be statistically significant, where the linear model fit was moderate with \(r = 0.74\); \(P\) value = 0.0002.

Since the scotopic and the photopic protocols are treated independently, the effect of protocol on intensity and days (observation time points) can be eliminated. Therefore, we examined the significance of days and intensities along with their interaction within each protocol using a 2-way ANOVA model (Eq. 3). The 2-way ANOVA model is described as

\[
(B/A)_{rel} = \mu + I_i + D_j + (ID)_{ij} + e_{ijk},
\]

(Eq. 3)

where \(i = 3,000, 10,000, \) and 25,000 mcd s/m^2; \(j = \text{days 5, 9, 12, and 33}; k = 1, 2; \mu = \text{overall effect}; I_i = \text{effect of the \(i\)th level of intensity}; D_j = \text{effect of the \(j\)th level of days}; (ID)_{ij} = \text{interaction between the \(i\)th level of intensity and \(j\)th level of days}; e_{ijk} = \text{random error \(\sim N(0, \sigma)\)}; k = \text{replication number}.\) Thus, in total, we had \(n = 24\) (3 intensities \(\times 4\) time points \(\times 2\) rabbits) data points.

\(P\) value > 0.05 is indicative of the statistically insignificant effect of the intensities, days, and their interaction on the (B/A)\(_{rel}\) for the scotopic and the photopic protocols. If the \(P\) value for the interaction parameter is observed to be greater than 0.05, then the mentioned 2-way ANOVA model is conducted without the interaction parameter \((ID)_{ij}\) to study the influence of the main effects—intensity and days—on the (B/A)\(_{rel}\) for the scotopic and the photopic protocols (Eq. 4).

\[
(B/A)_{rel} = \mu + I_i + D_j + e_{ijk},
\]

(Eq. 4)

Subsequently, the mean (B/A)\(_{rel}\) is computed over all intensities for each day (days 5, 12, 19, and 33) and compared between each combination of protocol (scotopic and photopic) and eye (right eyes receiving the MTX microimplant and the left eyes receiving the placebo microimplant). Also, the mean (B/A)\(_{rel}\) is computed over all days for each intensity (3,000, 10,000, and 25,000 mcd s/m^2) and compared between each combination of protocol and eye.

Furthermore, for each protocol, the group mean (B/A)\(_{rel}\) computed over all intensities for each day (days 5, 12, 19, and 33) was compared between each eye (right eyes receiving the MTX microimplant and the left eyes receiving the placebo microimplant) using 2-tailed Student’s \(t\)-test (\(P < 0.05\), \(n = 6\), 3 (B/A)\(_{rel}\) obtained for each of the intensities 3,000, 10,000, and 25,000 mcd s/m^2, for each eye from 2 rabbits at each time point). Similarly, for each protocol, the mean (B/A)\(_{rel}\) computed over all days for each intensity (3,000, 10,000, and 25,000 mcd s/m^2) was compared between the right eye and the left eye using 2-tailed Student’s \(t\)-test (\(P < 0.05\), \(n = 8\), 4 (B/A)\(_{rel}\) obtained for each of the time
Oscillatory potentials. Oscillatory potentials (OPs) were obtained by using a band-pass filter between 34 and 300 Hz. For each eye receiving the MTX microimplant and the placebo microimplant, the OPs were retrieved for intensities 3,000, 10,000, and 25,000 mcd s/m² at each time point (days 5, 12, 19, and 33) and for each protocol (scotopic and photopic). As per the ISCEV (International Society for Clinical Electrophysiology of Vision), OP amplitude is considered the difference between the positive peak following the negative peak, and OP implicit time is the time where the OP amplitude peak is observed. The amplitude and the implicit time of the first 5 OPs after stimulation were recorded for each rabbit. Therefore, at each time point, \( n = 10 \) (5 OPs \( \times \) 2 rabbits) for each intensity for each eye at each protocol. For each of the intensities, the mean OP amplitude and OP implicit time were further compared between PS and PE conditions using 2-tailed Student’s \( t \)-test for each eye and protocol at each time point. \( P \) value >0.05 for mean comparisons of OP amplitude and OP implicit time between PS and PE conditions is indicative of them being statistically insignificant.

The retinal function was further verified by fitting the B-wave amplitude data to the Naka–Rushton equation, which is discussed in detail in the Appendix section.

Results

Summary of the ERG data

The summary of the ERG data obtained for PS and PE for all eyes at each time point is reported for both the scotopic (dark adapted) and the photopic (light adapted) protocols. Subsequently, B/A ratios have been used to compare between the PS and PE conditions, for each eye at each time point for both the scotopic and the photopic protocols.

The representative ERG plots shown in Fig. 1 provide a comparison between the scotopic ERG data obtained from PS and PE conditions on the 33rd day time point for the same eye treated with the MTX microimplant. Similar ERG plots were obtained for all eyes for both protocols at the predetermined time points and then compared between the PS and PE conditions. Tables 1 and 2 summarize the ERG findings for the scotopic and photopic protocols in our study animals. The values shown in Tables 1 and 2 represent the results obtained for flash intensity of 25,000 mcd s/m². The baseline values for each parameter were obtained from all 8 rabbits used in this study for PS condition (\( n = 8 \), day 0). However, the values for each parameter for PE condition were obtained from only 2 rabbits at each time point (\( n = 2 \), days 5, 12, 19, and 33). The baseline values (PS condition) are reported as mean ± standard deviation (\( n = 8 \)), whereas the PE values reported for each time point are mean values only (\( n = 2 \)).

In both the scotopic and the photopic protocols, the range of values obtained for each parameter during the PE conditions (days 5, 12, 19, and 33) is similar to the baseline values obtained in the PS condition (day 0). There was some variability from one testing encounter to another but we noted no trend of progressive reduction of ERG amplitudes and/or prolongations of ERG response times in our study animals. This indicates no change to the retinal function as a consequence of the sustained MTX release from the microimplant.

Relative B/A ratio \([\text{(B/A)}_{\text{rel}}]\) analysis

The \( P \) values obtained from the 2-way ANOVA (24 data points) for each eye and each protocol are reported in Table 3. In the scotopic protocol, the effect of the intensities, days, and their interaction on the \( \text{(B/A)}_{\text{rel}} \) \( (P \) values: 0.60, 0.40, 0.99, respectively) was statistically insignificant \( (P>0.05) \), as observed in the right eye receiving the MTX microimplant. Moreover, in the scotopic protocol involving the right eye, the effect of the intensities and the days \( (P \) values: 0.48, 0.24, respectively) on the \( \text{(B/A)}_{\text{rel}} \) excluding their interaction was also statistically insignificant \( (P>0.05) \). Similarly, the effect of the intensities and days on the \( \text{(B/A)}_{\text{rel}} \) in the left eye receiving the placebo microimplant was statistically insignificant \( (P>0.05) \), both including and excluding their interaction. Furthermore, in the photopic protocol conducted in the right eye, the effect of the intensities and the days on the \( \text{(B/A)}_{\text{rel}} \) was statistically insignificant \( (P>0.05) \), both including their interaction and excluding their interaction. However, in the photopic protocol conducted in the left eye receiving the placebo microimplant, the effect of the intensities and the days on the \( \text{(B/A)}_{\text{rel}} \) was statistically significant \( (P<0.05) \), including their interaction. The discussion section includes additional explanation for significant \( P \) values. Subsequently, the effect of the intensities and the days on the \( \text{(B/A)}_{\text{rel}} \) was not analyzed using ANOVA excluding their interaction.
Table 1. Representative ERG Data Obtained from the Scotopic Protocol

<table>
<thead>
<tr>
<th>Day</th>
<th>No. of rabbits tested</th>
<th>No. of eyes tested for each type of microimplant</th>
<th>Mean A-wave amplitude (μV)</th>
<th>Mean A-wave implicit time (ms)</th>
<th>Mean B-wave amplitude (μV)</th>
<th>Mean B-wave implicit time (ms)</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (preoperative)</td>
<td>8</td>
<td>8</td>
<td>68.1±13.4</td>
<td>10.4±2.7</td>
<td>214.3±58.2</td>
<td>177.4±26.3</td>
<td>3.1±0.4</td>
</tr>
<tr>
<td>Day 5 after microimplant</td>
<td>2</td>
<td>2</td>
<td>93.4</td>
<td>9.2</td>
<td>241.0</td>
<td>196.3</td>
<td>2.7</td>
</tr>
<tr>
<td>implantation</td>
<td></td>
<td></td>
<td>82.4</td>
<td>8.6</td>
<td></td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>Day 12 after microimplant</td>
<td>2</td>
<td>2</td>
<td>64.6</td>
<td>8.2</td>
<td>195.0</td>
<td>211.6</td>
<td>3.0</td>
</tr>
<tr>
<td>implantation</td>
<td></td>
<td></td>
<td>76.1</td>
<td>9.0</td>
<td></td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>Day 19 after microimplant</td>
<td>2</td>
<td>2</td>
<td>81.6</td>
<td>9.0</td>
<td>279.3</td>
<td>230.5</td>
<td>3.4</td>
</tr>
<tr>
<td>implantation</td>
<td></td>
<td></td>
<td>53.8</td>
<td>9.4</td>
<td></td>
<td></td>
<td>5.7</td>
</tr>
<tr>
<td>Day 33 after microimplant</td>
<td>2</td>
<td>2</td>
<td>81.9</td>
<td>13.1</td>
<td>245.5</td>
<td>311.5</td>
<td>3.2</td>
</tr>
<tr>
<td>implantation</td>
<td></td>
<td></td>
<td>92.8</td>
<td>11.8</td>
<td></td>
<td></td>
<td>3.4</td>
</tr>
</tbody>
</table>

For stimulus intensity 25,000 mcd s/m²; right eye: MTX microimplant; left eye: placebo microimplant. Data reported as mean± standard deviation for baseline as n=8; and all other values are reported as mean as n=2 for days 5, 12, 19, and 33. ERG, electroretinography; MTX, methotrexate.

Table 2. Representative ERG Data Obtained from the Photopic Protocol

<table>
<thead>
<tr>
<th>Day</th>
<th>No. of rabbits tested</th>
<th>No. of eyes tested for each type of microimplant</th>
<th>Mean A-wave amplitude (μV)</th>
<th>Mean A-wave implicit time (ms)</th>
<th>Mean B-wave amplitude (μV)</th>
<th>Mean B-wave implicit time (ms)</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (preoperative)</td>
<td>8</td>
<td>8</td>
<td>18.4±2.8</td>
<td>13.4±0.9</td>
<td>54.0±12.1</td>
<td>31.2±3.2</td>
<td>2.9±0.4</td>
</tr>
<tr>
<td>Day 5 after microimplant</td>
<td>2</td>
<td>2</td>
<td>21.1</td>
<td>14.4</td>
<td>64.0</td>
<td>30.5</td>
<td>3.0</td>
</tr>
<tr>
<td>implantation</td>
<td></td>
<td></td>
<td>17.3</td>
<td>14.1</td>
<td></td>
<td></td>
<td>3.7</td>
</tr>
<tr>
<td>Day 12 after microimplant</td>
<td>2</td>
<td>2</td>
<td>15.4</td>
<td>12.8</td>
<td>42.9</td>
<td>31.3</td>
<td>2.9</td>
</tr>
<tr>
<td>implantation</td>
<td></td>
<td></td>
<td>21.8</td>
<td>13.5</td>
<td></td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>Day 19 after microimplant</td>
<td>2</td>
<td>2</td>
<td>19.1</td>
<td>15.6</td>
<td>49.3</td>
<td>38.8</td>
<td>2.6</td>
</tr>
<tr>
<td>implantation</td>
<td></td>
<td></td>
<td>20.4</td>
<td>14.5</td>
<td></td>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>Day 33 after microimplant</td>
<td>2</td>
<td>2</td>
<td>21.3</td>
<td>14.4</td>
<td>74.9</td>
<td>35.4</td>
<td>3.7</td>
</tr>
<tr>
<td>implantation</td>
<td></td>
<td></td>
<td>28.6</td>
<td>14.5</td>
<td></td>
<td></td>
<td>3.8</td>
</tr>
</tbody>
</table>

Data reported as mean± standard deviation for baseline as n=8; all other values are reported as mean as n=2 for days 5, 12, 19, and 33. For stimulus intensity 25,000 mcd s/m². Right eye: MTX microimplant; left eye: placebo microimplant.
Based on the ANOVA, it can be concluded statistically that there is no effect on the mean \((B/A)_{rel}\) caused by the intensities and the days (observation time points) for the scotopic protocol in both the eyes. Also, it can be inferred statistically that there is no effect on the mean \((B/A)_{rel}\) caused by the intensities and the days (observation time points) for the photopic protocol in the right eye. However, in the photopic protocol conducted in the left eye, there is an effect of the intensities, days, and their interaction on the mean \((B/A)_{rel}\) \((P<0.05)\).

Subsequently, the comparison of the mean \((B/A)_{rel}\) computed over all intensities for each day (days 5, 12, 19, and 33) is presented in Fig. 2A for each combination of protocol and eye. All data presented in Fig. 2 are shown as mean \(\pm 2\times\) standard error (SE).

The group mean values of the \((B/A)_{rel}\), as obtained for both the scotopic and photopic protocols, have been compared between each eye at each time point and each intensity (Table 4A, B, respectively). The values are reported as mean \(\pm 2\times\) SE. \(P\) value >0.05 is indicative of the statistically insignificant mean comparisons of the \((B/A)_{rel}\) between the right and the left eyes.

**Mean \((B/A)_{rel}\) values comparison for each day.** In the case of scotopic protocol, there is an insignificant difference \((P>0.05)\) in the mean \((B/A)_{rel}\) of the right eye \((0.9\pm0.1)\) and that of left eye \((1.0\pm0.6)\), as obtained on the 5th day of the study. Similarly, the difference between the mean \((B/A)_{rel}\) of the right eye \((1.1\pm0.3)\) and that of the left eye \((0.9\pm0.2)\), as obtained on the 12th day of the study, is insignificant \((P>0.05)\). In addition, on the 19th day of the study, the mean \((B/A)_{rel}\) of the right eye \((1.3\pm0.4)\) is not significantly different \((P>0.05)\) from that of the left eye \((1.3\pm0.5)\). Lastly, on the 33rd day of the study, the mean \((B/A)_{rel}\) of the right eye \((1.3\pm0.4)\) also does not differ significantly \((P>0.05)\) from the mean \((B/A)_{rel}\) of the left eye \((1.2\pm1.2)\). Similarly, in the case of photopic protocol, there was no significant difference \((P>0.05)\) between the mean \((B/A)_{rel}\) values obtained between the right eyes and the left eyes at all time points.

**Mean \((B/A)_{rel}\) values comparison for each intensity.** In the case of scotopic protocol, there is an insignificant difference \((P>0.05)\) in the mean \((B/A)_{rel}\) of the right eye \((1.2\pm0.3)\) and that of the left eye \((1.6\pm0.8)\), as obtained for 3,000 mcd s/m². The difference between the mean \((B/A)_{rel}\) of the right eye \((1.2\pm0.4)\) and that of the left eye \((0.7\pm0.3)\), as obtained for 10,000 mcd s/m², is also insignificant \((P>0.05)\). The mean \((B/A)_{rel}\) of the right eye \((1.0\pm0.2)\) is not significantly different \((P>0.05)\) from that of the left eye \((1.0\pm0.4)\), as obtained for 25,000 mcd s/m². Similarly, in the case of photopic protocol, there was no significant difference \((P>0.05)\) between the mean \((B/A)_{rel}\) values obtained between the right eyes and the left eyes at all intensities. Lastly, the comparison of the group mean values of the \((B/A)_{rel}\), as obtained for both the scotopic and photopic protocols, between each eye at each time point is presented in Fig. 3A, B, respectively, and that for each intensity is presented in Fig. 3C, D, respectively.

There was no significant difference between the mean \((B/A)_{rel}\) values in any of the eyes at any time point and any intensity for either of the protocols. This indicates no significant change in the functional integrity of the retina.
because of the presence of the PLA-coated CS-MTX or the placebo microimplants.

**OP analysis of the scotopic protocol.** The mean OP amplitudes and the mean OP implicit times observed at each of the intensities for each time point and each eye for the scotopic protocol are reported in Table 5. All values are reported as mean ± SE. As observed for the stimulus intensity of 25,000 mcd s/m², there is an insignificant difference (P > 0.05) between the mean OP amplitude obtained in PS and PE conditions for day 5 (PS: 18.9 ± 4.6 μV, PE: 19.3 ± 6.2 μV), day 12 (PS: 12.9 ± 3.3 μV, PE: 18.2 ± 4.9 μV), day 19 (PS: 16 ± 4.3 μV, PE: 16.7 ± 4.6 μV), and day 33 (PS: 9.1 ± 2.9 μV, PE: 20.8 ± 6.1 μV) in the right eyes receiving the MTX microimplant.

### Table 4. (A) Summary of (B/A)_{rel} Obtained on Each Day (n=6); (B) Summary of (B/A)_{rel} Obtained on Each Intensity (n=8)

<table>
<thead>
<tr>
<th>A (Days)</th>
<th>Right eye (MTX)</th>
<th>Left eye (placebo)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.6</td>
<td>0.74</td>
</tr>
<tr>
<td>12</td>
<td>1.1 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>0.50</td>
</tr>
<tr>
<td>19</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.5</td>
<td>0.93</td>
</tr>
<tr>
<td>33</td>
<td>1.3 ± 0.4</td>
<td>1.2 ± 1.2</td>
<td>0.93</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B (Intensity: mcd s/m²)</th>
<th>Right eye (MTX)</th>
<th>Left eye (placebo)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,000</td>
<td>1.2 ± 0.3</td>
<td>1.6 ± 0.8</td>
<td>0.36</td>
</tr>
<tr>
<td>10,000</td>
<td>1.2 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>0.06</td>
</tr>
<tr>
<td>25,000</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.4</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Data reported as mean ± 2 × SE.

SE, standard error.

FIG. 3. Comparison of the group mean values of the (B/A)_{rel}. (A) Between each eye at each time point for scotopic protocol, (B) between each eye at each time point for photopic protocol, (C) between each eye at each intensity for scotopic protocol, and (D) between each eye at each intensity for photopic protocol.
Table 5. Summary of OP Analysis for the Scotopic Protocol (n=10 for Each Intensity)

<table>
<thead>
<tr>
<th>Microimplant type</th>
<th>Days</th>
<th>Intensity (mcd s/m²)</th>
<th>OP amplitude (µV)(mean±SE)</th>
<th>OP implicit time (ms)(mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PS</td>
<td>PE</td>
<td>P</td>
</tr>
<tr>
<td>MTX (right eyes)</td>
<td>5</td>
<td>3,000</td>
<td>12.4±4.7</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>14.7±3.3</td>
<td>18.3±5.4</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>18.9±4.6</td>
<td>19.3±6.2</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3,000</td>
<td>13.4±4.1</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>12.7±3.1</td>
<td>13.7±4.3</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>12.9±3.3</td>
<td>18.2±4.9</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>3,000</td>
<td>15.8±5</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>15.7±5</td>
<td>14.7±4.6</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>16.4±3.3</td>
<td>16.7±4.6</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>3,000</td>
<td>13.8±4.7</td>
<td>18.3±7.1</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>10.6±3.4</td>
<td>22.1±7.2</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>9.1±2.9</td>
<td>20.8±6.1</td>
<td>0.11</td>
</tr>
<tr>
<td>Placebo (left eyes)</td>
<td>5</td>
<td>3,000</td>
<td>8.9±4.2</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>15.7±6</td>
<td>12±3.4</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>16.9±5.4</td>
<td>20.4±5.4</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3,000</td>
<td>10.1±3.8</td>
<td>19.5±6.8</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>10.6±3.1</td>
<td>18.1±5.1</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>11.7±3.6</td>
<td>18.8±4.9</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>3,000</td>
<td>13.9±4.3</td>
<td>12.1±3.4</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>15.5±4.6</td>
<td>12.1±3.7</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>16.6±5.2</td>
<td>13.2±3.2</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>3,000</td>
<td>14.0±3.2</td>
<td>17.5±6.2</td>
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<td></td>
<td>10,000</td>
<td>9.9±2.3</td>
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</tr>
<tr>
<td></td>
<td>25,000</td>
<td>10.8±2.6</td>
<td>19.1±6.6</td>
<td>0.26</td>
</tr>
</tbody>
</table>

OP, oscillatory potential; PE, prior to euthanasia; PS, prior to surgery.

In addition, the difference of mean OP implicit times corresponding to the same stimulus intensity observed in PS and PE conditions in the right eyes for day 5 (PS: 8.7±3.6 ms, PE: 8.3±3.6 ms), day 12 (PS: 11.2±3.8 ms, PE: 11.7±3.7 ms), day 19 (PS: 11.2±3.9 ms, PE: 13.2±3.9 ms), and day 33 (PS: 9.3±3.7 ms, PE: 11.8±3.8 ms) also showed no significant difference (P>0.05). Similarly, there is no significant difference (P>0.05) observed in the mean OP amplitude and the mean OP implicit time obtained for PS and PE conditions corresponding to the stimulus intensity of 25,000 mcd s/m² and other intensities (3,000, 10,000 mcd s/m²) in the right eye, as well as in the left eyes receiving the placebo microimplant for all the time points.

OP analysis of the photopic protocol. Table 6 shows the mean OP amplitudes and the mean OP implicit times observed at each of the intensities for each time point and each eye for the photopic protocol. All values are reported as mean±SE. Similarly to the scotopic protocol, a stimulus intensity of 25,000 mcd s/m² shows no significant difference (P>0.05) between the mean OP amplitude obtained in PS and PE conditions for day 5 (PS: 5.1±1.5 µV, PE: 5.9±1.6 µV), day 12 (PS: 5.1±1.3 µV, PE: 5.2±1.4 µV), day 19 (PS: 4.3±1.2 µV, PE: 3.9±1 µV), and day 33 (PS: 7.4±1.7 µV, PE: 6.4±1.8 µV) in the right eyes receiving the MTX microimplant.

Furthermore, an insignificant difference (P>0.05) was observed for the mean OP implicit time corresponding to the same stimulus intensity obtained in PS and PE conditions in the right eyes for day 5 (PS: 14.5±4.3 ms, PE: 12.6±4.5 ms), day 12 (PS: 13.5±4.8 ms, PE: 12.8±4.7 ms), day 19 (PS: 11.9±4.6 ms, PE: 15.9±4.5 ms), and day 33 (PS: 16.8±4.6 ms, PE: 16.7±4.5 ms). Lastly for all the time points, an insignificant difference is observed between the mean OP amplitudes and also between the mean OP implicit times obtained for PS and PE conditions corresponding to the stimulus intensity of 25,000 mcd s/m² and other intensities (3,000, 10,000 mcd s/m²) in the right eye, as well as those in the left eyes.

P>0.05 is consistently observed for all the mean values comparison of OP amplitude and OP implicit time across all intensities and time points in each eye and for each protocol. This indicates that there is no alteration in the inner retinal function caused by the MTX microimplant and the placebo microimplant. This analysis further justifies the safety of the microimplants. On a similar note, the B-wave amplitude data, when fitted to the Naka–Rushton equation, also revealed no change in the retinal function, as presented in the Appendix section.

Discussion

The ERG evaluation conducted in this study showed no evident retinal bioelectrical dysfunction attributable to our microimplants or the MTX released from the loaded implants. These findings are consistent with the findings of our previously published histopathological study on the same rabbits. Based on these findings, we expect the PLA-coated CS-MTX microimplants we developed to be well tolerated by human eyes in future studies.

Previous ERG studies involving MTX administrations

There have been limited studies in the past involving ERG analysis after MTX administration. Velez et al. reported that an
intravitreal MTX injection (400 μg of MTX provided therapeutic concentration (>0.5 μM) of MTX for a period of 2–3 days in a preclinical setting involving rabbit eyes.3 ERG analysis conducted in this study showed no statistically significant difference in the mean of the B- and A-wave amplitudes between the eyes receiving MTX injection and control eyes (placebo) after 162 days (P=0.11, n=10 rabbits/20 eyes).3 Also, the difference in the ratio of the B- and the A-wave amplitudes was not statistically significant between the treated eyes and the control eyes (P>0.20).

The PLA-coated CS-MTX microimplant that was used in this study contained the same drug loading as administered

<table>
<thead>
<tr>
<th>Microimplant type</th>
<th>Days</th>
<th>Intensity (mcd s/m²)</th>
<th>OP amplitude (μV)(mean±SE)</th>
<th>OP implicit time (ms)(mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PS</td>
<td>PE</td>
<td>P</td>
</tr>
<tr>
<td>MTX (right eyes)</td>
<td>5</td>
<td>3,000</td>
<td>3.7±1.2</td>
<td>4.8±1.8</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>5.1±1.5</td>
<td>5.9±1.6</td>
<td>7.0±1.7</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3,000</td>
<td>3.4±1.1</td>
<td>2.7±0.9</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>3.5±1.0</td>
<td>4.5±1.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>5.1±1.3</td>
<td>5.2±1.4</td>
<td>0.94</td>
</tr>
<tr>
<td>19</td>
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<td>4.7±1.6</td>
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<td>3.9±1.0</td>
<td>0.78</td>
</tr>
<tr>
<td>33</td>
<td>3,000</td>
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<tr>
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<td>5.5±1.3</td>
<td>5.7±1.8</td>
<td>0.93</td>
</tr>
<tr>
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<td>25,000</td>
<td>7.4±1.7</td>
<td>6.4±1.8</td>
<td>0.69</td>
</tr>
<tr>
<td>Placebo (left eyes)</td>
<td>5</td>
<td>3,000</td>
<td>3.1±1.3</td>
<td>2.9±0.8</td>
</tr>
<tr>
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<td>6.7±1.6</td>
<td>0.28</td>
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<tr>
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<td>4.4±1.9</td>
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<td>3.7±0.8</td>
<td>0.46</td>
</tr>
<tr>
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<td>3.8±0.8</td>
<td>0.4</td>
</tr>
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<td>7.4±2.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>3.8±1</td>
<td>8.4±2.6</td>
<td>0.12</td>
</tr>
</tbody>
</table>

\(^a\)The P value is 0.0521 (rounded off to 4 digits after decimal).
in an intravitreal MTX injection reported by Velez et al.\(^3\) (400 mg of MTX). The findings concerning the ERG analysis of retinal toxicity in this study are consistent with that of Velez et al.\(^3\)

Comparison of toxicity with histopathology results

The pathological examination has been conducted by an experienced ocular oncologist and pathologist, Dr. Zelia Correa of the Department of Ophthalmology at the University of Cincinnati. As observed in our prior concurrent \textit{in vivo} study involving the administration of the PLA-coated CS-MTX microimplant and the placebo microimplant in the same rabbits, histopathological findings showed no major structural abnormalities between the right and the left eyes for all time points.\(^5\)

Histology assessment of all eyes after hematoxylin and eosin (H&E) staining revealed normal retina post euthanasia. Pathological examination confirmed normal retina of the eyes receiving (a) MTX microimplant on the 19th day time point (H&E, 60×) (Fig. 4A) and on the 33rd day time point (H&E, 30×) (Fig. 4C) and on the 33rd day time point (H&E, 15×) (Fig. 4D), thus indicating no toxicity.

The ANOVA indicates that there is no effect of the intensities and days on the mean (B/A) rel in the right eye receiving the MTX microimplant, as observed in both the

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{A} & & & & & & & \\
\textbf{Scotopic protocol} & & & & & & & \\
\hline
\textbf{n Values} & \textbf{Right eyes (MTX)} & \textbf{Left eyes (placebo)} & \textbf{Right eyes (MTX)} & \textbf{Left eyes (placebo)} & \textbf{Right eyes (MTX)} & \textbf{Left eyes (placebo)} \\
\hline
\textbf{Days} & PS & PE & PS & PE & PS & PE \\
\hline
5 & 0.4 & 0.3 & 0.5 & 0.4 & 3.0 & 1.6 \\
12 & 0.3 & 0.2 & 0.2 & 0.5 & 1.7 & 2.3 \\
19 & -0.1 & 0.6 & 0.7 & 0.1 & -7.5\(^a\) & 2.3 \\
33 & 0.5 & 0.3 & 0.0 & 0.7 & 2.2 & 2.5 \\
\hline
\end{tabular}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Photopic protocol} & & & & & & \\
\hline
\textbf{n Values} & \textbf{Right eyes (MTX)} & \textbf{Left eyes (placebo)} & \textbf{Right eyes (MTX)} & \textbf{Left eyes (placebo)} & \textbf{Right eyes (MTX)} & \textbf{Left eyes (placebo)} \\
\hline
\textbf{Days} & PS & PE & PS & PE & PS & PE \\
\hline
5 & 1.0 & 0.9 & 1.1 & 0.7 & 2.7 & 3.0 \\
12 & 0.7 & 0.4 & 0.7 & 0.8 & 3.0 & 3.1 \\
19 & 0.8 & 0.9 & 1.0 & 0.7 & 3.0 & 2.8 \\
33 & 0.8 & 0.8 & 0.6 & 0.9 & 3.0 & 2.9 \\
\hline
\end{tabular}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{B} & & & & & & \\
\textbf{Naka–Rushton parameters obtained from scotopic protocol (mean±SE)} & & & & & & \\
\hline
\textbf{Microimplant type} & \textbf{n Values} & \textbf{–log K (log mcd s/m\(^2\))} & \textbf{P} & \textbf{PS} & \textbf{PE} & \textbf{P} & \textbf{PS} & \textbf{PE} & \textbf{P} \\
\hline
Right eyes (MTX microimplant) & 0.3±0.1 & 0.4±0.9 & 0.71 & 2.3±0.4\(^b\) & 2.2±0.2 & 0.82 & PS & PE & P \\
Left eyes (placebo microimplant) & 0.4±0.1 & 0.4±0.1 & 0.84 & 2.0±0.1\(^b\) & 1.7±0.6\(^b\) & 0.67 & PS & PE & P \\
\hline
\end{tabular}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{C} & & & & & & \\
\textbf{Naka–Rushton parameters obtained from photopic protocol (mean±SE)} & & & & & & \\
\hline
\textbf{Microimplant type} & \textbf{n Values} & \textbf{–log K (log mcd s/m\(^2\))} & \textbf{P} & \textbf{PS} & \textbf{PE} & \textbf{P} & \textbf{PS} & \textbf{PE} & \textbf{P} \\
\hline
Right eyes (MTX microimplant) & 0.8±0.7 & 0.8±0.1 & 0.66 & 2.9±0.9 & 2.9±0.1 & 0.88 & PS & PE & P \\
Left eyes (placebo microimplant) & 0.8±0.1 & 0.8±0.1 & 0.67 & 3.0±0.1 & 2.9±0.1 & 0.86 & PS & PE & P \\
\hline
\end{tabular}
\end{table}

\(\^a\)indicates that the corresponding value is not registered as it is an outlier.

\(\^b\)indicates \(n=3\).

SE, standard error.
scotopic and the photopic protocols. This indicates that the MTX microimplant is safe and does not alter the functional integrity of the retina. The \( P \) value observed in the left eyes (eyes receiving the placebo microimplant) for the scotopic protocol was statistically insignificant (\( >0.05 \)). However, the \( P \) value observed in the left eyes for the photopic protocol was statistically significant (\( <0.05 \)). This indicates changes in the functional integrity of the retina of the left eyes receiving the placebo microimplant. These changes could possibly be caused by (a) improper light and dark adaptation of the rabbit eyes while recording the ERG data and (b) the toxicity and inflammation induced by the constituents of the placebo microimplant, such as CS and dichloromethane (DCM). On the contrary, in the right eyes, which received the MTX microimplant, the inflammation and toxicity, which may have been caused by the microimplant constituents, are neutralized by the anti-inflammatory action of the drug, MTX. As a precaution, we have evaporated the DCM during the manufacturing process of the microimplant. However, there could be traces of DCM in the placebo microimplant causing minimal toxicity and inflammation in the left eyes, as reflected by the ANOVA. Nonetheless, the range of values observed for each ERG parameter, such as A-wave amplitude, A-wave implicit time, B-wave amplitude, B-wave implicit time, and the B/A ratio for the PE conditions, is similar to the values observed for the respective parameter in the PS condition. Overall, the ERG analysis supports the findings of our recent histopathology study and assures that there is no major retinal toxicity caused by the microimplants.

**Limitations of the study**

This study involved a limited number of rabbits \( (n = 2) \) per time point. As 2 rabbits were euthanized at every time point, the histopathology data could not be compared for the same retinal area of a particular rabbit between any 2 time points. Furthermore, in the B/A ratio analysis of this study, the ERG responses obtained for intensities \( \leq 1,000 \text{mcd s/m}^2 \) were ignored for both the protocols as the light stimulus of low intensities yields immeasurable A-wave amplitude, which subsequently inflates the B/A ratio values. A larger preclinical trial involving more animals and time points would be required to obtain more robust statistical comparison of the ERG results and histopathology analysis.

The ERG responses may be affected by the ERG machine setup in relation to rabbit eye. The ERG results are influenced by the contact lens electrode, which is required to be in contact with the cornea throughout the duration of the study. However, if there is any movement of the eye, it may be possible that the contact lens remains in partial contact instead of complete contact with the cornea, which is expected to somewhat alter the ERG reading.

The ERG responses are further governed by the dark and light adaptation, pupil diameter, and the level of anesthesia attained during the course of the ERG procedure. The consistency of the pupil diameter, dark and light adaptation, and level of anesthesia is a challenge to implement. The dark adaptation of the left eye of one of the rabbits of the 5th day time point was momentarily compromised during the ERG recording PE. This inadvertent light exposure caused improper adaptation and may have resulted in inaccurate ERG data recording PE for the 5th day time point. Furthermore, the pupil diameter can potentially vary depending on the drug administered for dilation. If the pupil diameter changes by an order of magnitude, then the effective stimulus intensity on the retina photoreceptor changes by a couple of log units.

**Concluding remarks**

This ERG study of rabbit eyes receiving a PLA-coated CS-MTX microimplant or a placebo microimplant showed no evident functional bioelectrical toxicity to the retina during the course of the study. These findings are consistent with the observations of our prior histopathological evaluation.

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**Author Disclosure Statement**

No competing financial interests exist.

**References**


**Appendix**

**Naka–Rushton Analysis**

The retinal function was also assessed by fitting the B-wave amplitude data to the Naka–Rushton equation.\(^8,11,13,14\)

The generic Naka–Rushton equation is defined as follows:

\[
\frac{V}{V_{\text{max}}} = \frac{I^n}{(I^n + K^n)},
\]

(Eq. 5)

where \(V\) is the B-wave amplitude, \(V_{\text{max}}\) is the maximum B-wave amplitude, \(I\) is the intensity of the light stimulus, \(K\) is the intensity of light necessary for the B-wave to reach its half of \(V_{\text{max}}\), and \(n\) is the slope when \(I\) is equal to \(K\). As reported in the analysis of Aylward,\(^13\) Equation (5) can be rewritten as

\[
\log\left[\frac{(V/V_{\text{max}})/(1-V/V_{\text{max}})}{nI} \right] = n \log I - n \log K.
\]

(Eq. 6)

The “\(n\)” values and the “–log \(K\)” values obtained by fitting the ERG data to the Naka–Rushton equation (Eq. 6) were used as indices to assess retinal sensitivity.\(^8,11\) The value of “\(n\)” and the value of “–log \(K\)” (log mcd s/m\(^2\)) have been reported to be \(1^{11}\) and \(3^{8}\), respectively, for a normal retina.

At each time point, \(V\) is the mean B-wave amplitude \((n=2)\), mean of the B-wave amplitudes from 2 rabbits) obtained for each intensity \(I\). Using Equation (6), the “\(n\)” and “–log \(K\)” are subsequently obtained as the slope and the \(y\) axis intercept from the linear regression fit of “\(\log\left[(V/V_{\text{max}})/(1-V/V_{\text{max}})\right]\) versus “\(\log I\)” for each time point.

Such fits are obtained independently for either eye (right eyes receiving the MTX microimplant and left eyes receiving the placebo microimplant) and for each protocol (scotopic and photopic). The “\(n\)” values and the “–log \(K\)” values at each time point for different combinations of eyes and protocols are presented in Table 7A. Thereafter, to assess the deviation of the state of the retina from the normal condition, the group mean values of “\(n\)” and “–log \(K\)” of all time points \((m=4)\) are compared between the PS and the PE conditions for both eyes and for both the scotopic (Table 7B) and the photopic (Table 7C) protocols.

For the scotopic protocol involving the right eye (MTX microimplant), there is an insignificant difference \((P>0.05)\) in the mean “\(n\)” values of the PS condition \((0.3\pm0.1)\) and PE condition \((0.4\pm0.9)\). Similarly, in the left eyes (placebo microimplant), the difference of the mean “\(n\)” values of the PS condition \((0.4\pm0.1)\) and of the PE condition \((0.4\pm0.1)\) is insignificant. Furthermore, in the right eye, there is an insignificant difference in the mean “–log \(K\)” values of the PS condition \((2.3\pm0.4)\) and the PE condition \((2.2\pm0.2)\). Lastly, in the left eye, the difference of the mean “–log \(K\)” values of the PS condition \((2.0\pm0.1)\) and of the PE condition \((1.7\pm0.6)\) is insignificant. This indicates no significant change in the retinal sensitivity because of the presence of the PLA-coated CS-MTX microimplant or the placebo microimplant. Similar to the scotopic protocol, all comparisons of the photopic protocol are found to be statistically insignificant (Table 7C).

**Prior Naka–Rushton Analysis**

There have been no prior reports of Naka–Rushton analysis in preclinical studies involving intravitreal administrations of MTX. However, research by Oliveira et al.\(^8\) on intravitreal administration of triamcinolone acetonide in normal rabbit eyes reported “–log \(K\)” values in the range of 2.82–3.37 for both treated and nontreated eyes. The range of “–log \(K\)” values obtained in the photopic protocol of the current study (2.92–2.97) is similar to that obtained in the study of Oliveira et al.\(^8\) Furthermore, the Naka–Rushton analysis showed that there is no significant difference in the means of the “\(n\)” values and the means of the “–log \(K\)” values in any of the eyes for both the scotopic and photopic protocols, which also implies no significant change in the retinal sensitivity because of the presence of the PLA-coated CS-MTX microimplant or the placebo microimplants.