Article

Intravitreal Trimethoprim and Sulfamethoxazole Toxicity to the Retina of Albino Rabbits

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Purpose: To evaluate retinal toxicity of intravitreal trimethoprim-sulfamethoxazole (TMP-SMX) in an albino rabbit model.

Methods: Albino rabbits ($N = 10$) were treated in the right eye with the maximum intravitreal dose of TMP-SMX mixture (1600 μg/8000 μg /0.1 mL), while 0.1 mL saline was injected into the vitreous of the left eye. Clinical examination and electrophysiological (electroretinogram [ERG] and visual evoked potentials [VEPs]) testing were conducted before injection, 3 days, 1, 2, and 4 weeks postinjection. Retinal structure and expression of glial fibrillary acidic protein (GFAP) were assessed from histology and immunocytochemistry respectively at the end of the follow-up period.

Results: Clinical examination was normal throughout the follow-up period. ERG responses from the experimental eyes were similar to those recorded from the control eyes, but the sum of oscillatory potentials decreased in the experimental eyes at 2 weeks postinjection. The VEP responses, elicited by stimulation of the experimental eyes, were abnormal having reduced amplitude and prolonged implicit time. Histological damage in the experimental eyes was expressed by thickness reduction of whole, outer, and inner nuclear layers. GFAP was expressed in retinal Müller cells of all experimental eyes, but none of control eyes.

Conclusions: A single intravitreal injection of TMP-SMX mixture (1600 μg/8000 μg, respectively) causes functional and structural damage to the inner retina and retinal output. Signs of retinal stress were also evident by GFAP expression in retinal Müller cells of all experimental eyes. Therefore, the use of TMP-SMX via intravitreal administration should be done with caution.

Translational Relevance: These findings highlight the risk of retinal toxicity after intravitreal injection of trimethoprim-sulfamethoxazole and emphasize that this treatment should be carefully considered.

Introduction

Ocular toxoplasmosis is the most common infectious cause of posterior uveitis worldwide and in some areas, such as South America and Asia, it is one of the leading causes of visual impairment.[1,2] Treatment options consist of various antibiotics, and it should be considered for lesions involving or threatening the macula, optic nerve, or a major retinal vessel and for peripheral lesions associated with severe vitritis.[3]

Trimethoprim-sulfamethoxazole (TMP-SMX) is a combined antibiotic consisting of one part trimethoprim to five parts sulfamethoxazole. Trimethoprim and sulfamethoxazole have a synergistic effect as they inhibit successive steps in the tetrahydrofolic acid (THF) synthesis pathway inside microbial organisms such as toxoplasmosis.[4] THF is the biologically active form of folate, a major coenzyme in the metabolism of amino acids and nucleic acids.

Trimethoprim is a competitive inhibitor of dihydrofolate reductase (DHFR), as such it inhibits de novo synthesis of THF. Sulfamethoxazole is a

sulfonamide, a p-aminobenzoic acid (PABA) analog that competes with PABA in the biosynthesis of dihydrofolic acid, an intermediate step in the formation of THF.[4]

Systemic use of TMP-SMX was shown to be effective against toxoplasma retinochoroiditis (TRC) either alone or in combination with azithromycin or corticosteroids.[5–9] In addition, it was found as an effective treatment for diffuse toxoplasmic retinochoroiditis in a patient with acquired immunodeficiency syndrome[10] and it is used for the prevention of toxoplasmic retinochoroiditis recurrences.[11–13] Systemic use of TMP-SMX may cause side effects, including fever, gastrointestinal pain, and weight loss. Severe adverse effects including Stevens-Johnson syndrome, toxic epidermal necrolysis, pancreatitis, serum sickness, hyperkalemia, and thrombocytopenia,[5] may restrict its use.

Two recent reports[14,15] showed that intravitreal administration of TMP-SMX with dexamethasone, given as multiple injections as needed, resulted in reduction of intraocular inflammation and improvement of visual acuity in most of the patients. These reports suggest that intravitreal TMP-SMX with dexamethasone may offer an alternative route for treatment of ocular toxoplasmosis that may be used in patients who cannot tolerate, have contraindications to, or do not respond to oral medications. The study by Choudhury et al.,[14] which included four patients who all underwent full-field ERG at 6-week follow-up visit, reported that the ERG of the treated eye was comparable to that of the normal fellow eye in all patients. However, another study, utilizing TMP-SMX with dexamethasone injection, did not test for retinal damage using electrophysiological recordings.[15]

Only one study reported on intravitreal toxicity of TMP-SMX in an animal model.[16] This study examined dark-adapted ERG and histology changes 10 days post intravitreal injection of TMP-SMX in 16 albino rabbits. Several concentrations of TMP-SMX were used and none were found to cause a dark-adapted ERG abnormality or histologic retinal changes. Fourteen eyes that underwent vitrectomy with TMP-SMX in the vitrectomy solution exhibited minimal alterations in the ERG b-waves, and in eyes treated with the highest concentration (50 μg/mL), some homogenization of the photoreceptors outer segments was found as well.[16]

The potential of using TMP-SMX for intravitreal treatment of ocular toxoplasmosis is promising, but the possibility that TMP-SMX may be toxic to the retina following intravitreal injection should be tested rigorously. The aim of our study was to conduct a thorough electrophysiological and morphological study over a sufficiently long period of follow-up in order to test the safety use of TMP-SMX for intravitreal injection.

We assessed the effects of intravitreal administration of commercial mixture of TMP-SMX (1600 μg/8000 μg, respectively) to albino rabbit retina and retinal output using electrophysiological and morphological techniques.

Materials and Methods

Animals

Twelve adult New Zealand white rabbits weighing 1.5 to 3 kg started this study. However, two rabbits died during the follow-up (4 weeks) period, and therefore only data from 10 rabbits were used to examine the effects of TMP-SMX upon the albino rabbit retina. The rabbits were housed in the vivarium of the Technion Faculty of Medicine under 12/12-hour light/dark cycle, and allowed free access to food and water. General anesthesia was induced, as described before[17] with a mixture containing ketamine hydrochloride, acepromazine maleate solution, and xylazine solution. Topical anesthesia (benoxinate hydrogen chloride 0.4%) was administered to the eyes to reduce animal's discomfort. The pupils were fully dilated with cyclopentolate hydrochloride 1%.

Before each electrophysiological (ERG and visual evoked potential [VEP]) testing session, the rabbits underwent complete clinical inspection by indirect ophthalmoscope for signs of ocular inflammation, cataract formation, and retinal damage. ERG and VEP recordings were performed before intravitreal injection, and again at 3 days, 1, 2, and 4 weeks after intravitreal TMP-SMX injection. At termination of the follow-up period, after final ERG and VEP recordings, the rabbits were euthanized by intravenous injection of sodium pentobarbital (80 mg/kg body weight), both eyes were enucleated, and the retinas were prepared for histological and immunocytochemical evaluation.

All experimental procedures adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and to institutional guidelines.

Drug Administration

The commercial Septrin for infusion (trimethoprim 80 mg and sulfamethoxazole 400 mg) solution

(Perrigo Israel Agencies Ltd., Bnei Brak, Israel) in a concentration of 1600 μg/8000 μg per 0.1 mL, the maximum concentration available from the commercial preparation, was used for intravitreal injection. The excipients in the commercial drug solution include 1.7 mmol sodium, 13.2 vol % ethanol (alcohol) per 5 mL and sodium metabisulphite, Propylene Glycol (E1520) and Tromethamine (Datapharm; https://www.medicines.org.uk/emc/product/4669/smpc). Intravitreal injections were performed as previously described.[17,18] A volume of 0.1 mL TMP-SMX solution was injected into the right eye, the experimental eye, and a similar volume (0.1 mL) of saline (BSS; Alcon, Fort Worth, TX) was injected into the left eye, the control eye.

Electroretinogram (ERG)

Flash ERG responses were recorded from the experimental and control eyes as described before.[17] Corneal electrodes (Medical Workshop, Groningen, The Netherlands) were used to record the ERG signals relative to a reference and ground electrodes, made of stainless steel surgical needles that were inserted into the ears. The Flash ERG responses were recorded with UTAS 3000 electrophysiology system (LKC Technologies, Gaithersburg, MD) using Ganzfeld white light source with a maximum strength of 760 cd-s/m^2.

The ERG responses were recorded in darkness, after at least 30 minutes in the dark, and then in the light-adapted state (background illumination of 30 cd/m^2). In order to improve signal/noise ratio, several responses of the same strength were averaged, depending upon the strength of the stimulus and the state of adaptation, using the International Society for Clinical Electrophysiology of Vision (ISCEV) guidelines for ERG recording.[19]

ERG analysis was based on amplitude measurements of the a-wave, the b-wave, and the sum of the oscillatory potentials, and of peak time measurement for the b-wave. The a-wave amplitude was measured from baseline to the trough of the a-wave, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. The sum of the oscillatory potentials[20] was derived by the built-in procedure of the LKC electrophysiology recording system that filtered (70–1000 Hz) and amplified the original averaged ERG signal. The b-wave peak time was measured from stimulus onset to the peak of the b-wave. The amplitudes of the dark-adapted ERG b-waves were plotted as a function of log stimulus strength, and fitted to a hyperbolic function.[21]

$$V/Vmax = I/(I + \sigma) \qquad (1)$$

Where V is the amplitude elicited by a stimulus strength I, and Vmax is the maximal response amplitude. The semi-saturation constant, σ, is the light stimulus strength needed to elicit a response of half maximal amplitude.

Functional damage of the rod system in the experimental eye was assessed from the maximal response amplitude (Vmax) and semi-saturation constant (σ) of the dark-adapted b-wave. Since the dark-adapted rabbits' a-wave did not reach a plateau with our recording protocol, we used the amplitude of the response to bright (7.5 cd-s/m^2) flash stimuli as the maximal a-wave amplitude. Since there can be large variability in the ERG responses of a rabbit between consecutive ERG recording sessions, we used the Vmax ratios and the sum of oscillatory potentials ratio (experimental eye/control eye) and logσ difference (experimental eye – control eye) to assess the toxic effects of intravitreal injected TMP-SMX.[22] The functional integrity of the cone system was evaluated from the b-wave amplitude ratios (experimental eye/control eye) that were calculated as the average of the amplitudes that were measured in the responses to 2.5 and 7.5 cd-s/m^2. The b-wave peak time differences (experimental eye – control eye) were measured in the ERG responses elicited by bright (7.5 cd-s/m^2) white light stimuli.

Visual Evoked Potentials

Flash VEPs were recorded, as described before[17] using a stainless-steel needle as the active electrode that was inserted from the base of the skull under the skin along the midline between the two ears. The reference and ground electrodes, made of curves surgical needles, were inserted in the ears. The VEP signals were amplified, filtered, and averaged by the UTAS-3000 electrophysiological system (LKC Technologies). Twenty-five responses elicited by identical stimuli of 2.5 cd-s/m^2, delivered at a rate of 1.1 Hz, were averaged. VEPs were recorded first in response to binocular stimulation in order to assure optimal VEP recording, and then in response to monocular stimulation. To assure monocular recording of VEP, the nonrecorded eye was tightly covered with half of a black rubber ball of 5 cm diameter. The flash VEP in rabbits is composed of a negative wave appearing at implicit (trough) time of 25 to 40 ms followed by a positive wave at 40 to 60 ms after stimulus onset.

VEP analysis was based on trough time measurement from stimulus onset to the trough of the

negative wave, and on amplitude measurement from the trough of the negative wave to the peak of the following positive wave. The functional integrity of the visual pathways from the photoreceptors to the retinal ganglion cells, and through the optic pathways to the visual cortex was evaluated from the VEP amplitude ratios (experimental eye/control eye) and from the VEP trough time differences (experimental eye – control eye).

Histology

As described before,[17,23] following fixation in a solution of 4% paraformaldehyde (in phosphate-buffered saline [PBS] buffer; 0.1 M; pH 7.4) a circumferential incision was made to remove the anterior segment of the eye (cornea and lens). After removing the vitreous, the posterior eyecup was bisected at the level of the optic disc. One half of the eyecup was rinsed and dehydrated in ethanol (twice in 70%, twice in 96%; 1.5 hours each). Then, soaked in a solution of resin and catalyst without the hardener overnight. Embedding was done in resin (JB-4, Bio-Rad, Watford, UK). The tissue was cut into 2-μm sections (Reichert-Jung, Nussloch, Germany), and mounted onto slides. For light microscopy, the sections were stained with Richardson's solution.

Quantitative assessment of retinal structure was obtained from thickness measurements of the outer nuclear layer (ONL), inner nuclear layer (INL), and of the entire retina. Measurements were done using the CellSens Dimension Software of the Olympus DP70 camera. Five retinal sections of each eye were measured and averaged to give a single value for thickness measurements of ONL, INL, and entire retinal from each rabbit.

Immunocytochemistry

Glial fibrillary acidic protein (GFAP) is normally expressed in retinal astrocytes and not in retinal Müller cells; however, following a variety of retinal trauma and injuries, GFAP expression in Müller cells increases. Therefore, GFAP expression in retinal Müller cells is used as a sensitive cellular marker for retinal trauma.[24–26]

As described before,[23] the fixated second half of the posterior eyecup was washed and cryoprotected, in 15% sucrose for 1 hour, in 20% sucrose for another hour, and in 30% sucrose overnight, embedded in optical coherence tomography solution, and cut into 16-μm thick sections along the vertical meridian on a cryostat (Leica CM1900, Leica Microsystems Nussloch GmbH, Nussloch, Germany). Cryostat sections were soaked in PBS 0.1 M, pH 7.4, and then incubated in normal nonimmune serum (3% serum + 0.1% TritonX-100 + PBS 0.1 M). Then, the sections were soaked separately overnight at 4°C in a moist chamber with primary antibody to GFAP (Chemicon International, Inc., Temecula, CA), at 1:400 dilution in PBS 0.1 M + 3% serum + 0.1% TritonX-100. For immunofluorescence visualization, the slides were rinsed, and then incubated for 1 hour in donkey antimouse Alexa Fluor 594–labeled antibody (Molecular Probes, Eugene, OR) at 1:200 dilution. The slides were also stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000) to allow visualization of cells' nuclei in order to identify the retina layered structure.

Statistical Analysis

Data were analyzed using SPSS software package version 22 (SPSS, Inc., Chicago, IL) and WINPEPI programs version 11.4 (http://www.brixtonhealth.com/pepi4windows.html). The ERGs and VEPs parameters were tested for statistical significance using analysis of variance (ANOVA) with repeated measures. Histological measurements were tested for statistical significance using paired t-test. All tests were 2-tailed, and the threshold for statistical significance was defined as a P-value <0.05.

Results

Clinical Observations

All 20 eyes (control eyes and experimental eyes) were checked at each testing time point with indirect ophthalmoscope. All clinical findings were normal with no evidence for intraocular inflammation.

Electrophysiology

Figure 1 shows dark-adapted ERG responses from one rabbit that was tested at all time points. For clarity of the figure, only ERG data that were recorded in three testing time points (baseline, 2 weeks, and 4 weeks) are represented.

Representative ERG responses that were elicited by bright (I = 2.5 cd-sm^2) white light stimuli (first row upper part of Fig. 1) show no significant difference between the experimental eye and control eye (upper and lower traces, respectively, in each pair of traces). It should be noted that large variations are seen in the ERG responses between different recording sessions,
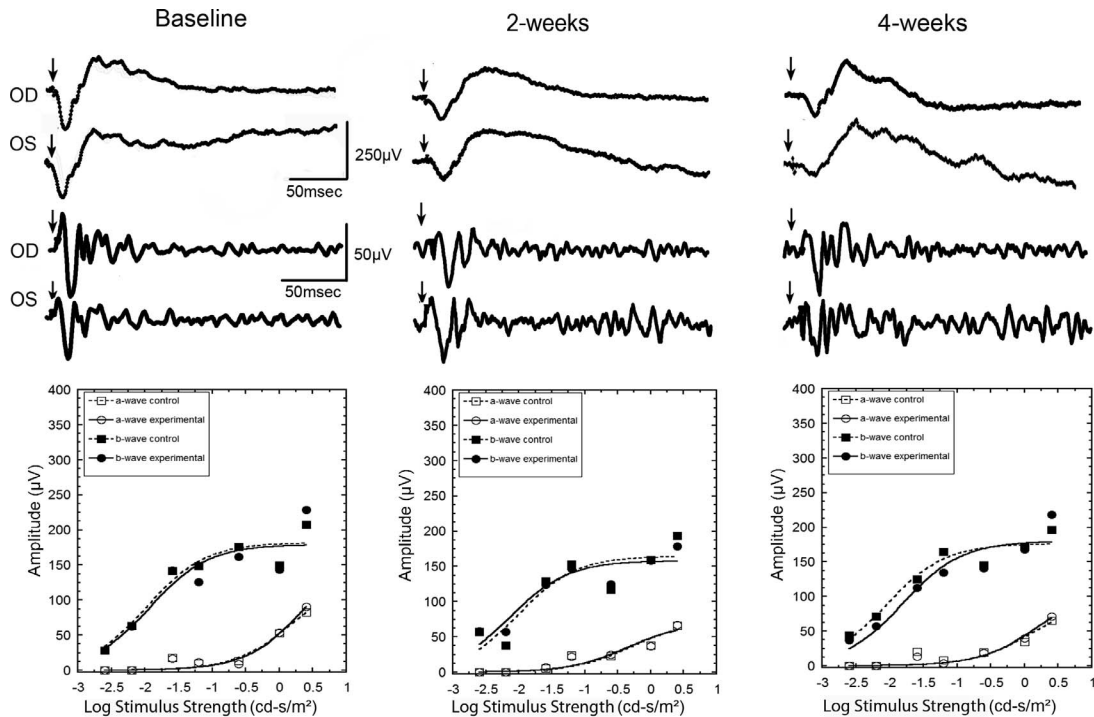
Figure 1. Representative ERG recording (*first row*), oscillatory potentials (*second row*), and response-stimulus strength relationship curves (*third row*) from one rabbit during the follow-up period of 4 weeks. The experimental eyes and control eyes (upper and lower traces, respectively) were elicited by white light stimuli of 2.5 cd-s/m². The ERG responses of the two eyes are similar in each recording session, but differ between sessions. Light stimuli of different strength were used to elicit ERG responses in order to construct the response-stimulus strength relationships (*third row*). These relationships were fitted to the hyperbolic equation (Eq. 1) in order to derive the maximal amplitude (Vmax) and the semi-saturation constant (σ). The onset of light stimulus is indicated by an arrowhead above the ERG traces.

but the responses of the two eyes are similar indicating that the parameters affecting the amplitudes and pattern of the ERG responses affect the two eyes similarly as discussed before.[18] The oscillatory potentials that were isolated from the ERG responses are shown in the second row. Light stimuli of different strengths were used to elicit ERG of different amplitudes that were used to construct the response-stimulus strength relationships (third row of Fig. 1). The response-stimulus strength relationships were fitted to the hyperbolic function (Eq. 1) in order to derive the maximal response amplitudes (Vmax) and the semi-saturation constants (σ) for the dark-adapted b-wave in all the rabbits throughout the ERG follow-up period. Fitting the hyperbolic function (Eq. 1) to the response-stimulus strength relationships of the dark-adapted a-waves could not be used to derive a reliable value for the a-wave Vmax; therefore, we used the amplitudes of the dark-adapted a-waves that were elicited by bright (I = 7.5 cd-s/m²) white light stimuli as maximal (Vmax) amplitudes of the dark-adapted a-waves. Therefore,

the semi-saturation constant could not be derived for the dark-adapted a-wave. These ERG parameters, for the rabbit whose ERG responses are shown in Figure 1, are listed in Table 1 for all the ERG recording sessions. The ERG data (Fig. 1) and the derived ERG parameters (Table 1) indicate no apparent differences between the experimental and control eyes of that rabbit throughout the follow-up period.

The ERG responses that were recorded from the experimental eye were similar to those recorded following stimulation of the control eye in nine of the studied rabbits. Only in one rabbit the ERG responses of the experimental eye were considerably smaller than those of the control eye. Despite this outlier data point, ERG analysis included all 10 rabbits. Figure 2 shows the mean (± SD) of the Vmax ratios and the logσ differences for the dark-adapted ERG b-wave (B) and only for the Vmax ratio for the dark-adapted ERG a-wave (A) for the 10 rabbits during the entire 4 weeks of follow-up. The Vmax ratios values for the dark-adapted a-wave and b-wave fluctuated around 1, and values for the logσ

Table 1. ERG Parameters; Vmax and Logσ and Sum of Oscillatory Potentials for Dark-Adapted ERG Responses of the Rabbit Whose ERG Responses Are Shown in Figure 1

4 Weeks	2 Weeks	1 Week	3 Days	Baseline	
127.2	138.7	124.1	119.2	183	Vmax a-wave experimental eye (µV)
125.1	143.7	133.9	73.1	207.3	Vmax a-wave control eye (µV)
1.01	0.96	0.9	1.6	0.9	a-wave Vmax ratio
194.9	231.8	173.3	159.6	245.2	Vmax b-wave experimental eye (µV)
211.9	219.2	174.7	173.6	258.9	Vmax b-wave control eye (µV)
0.91	1.06	0.99	0.91	0.95	Vmax b-wave ratio
−1.8	−1.9	−1.65	−2.02	−1.97	Log σ b-wave experimental eye (cd-s/m^2)
−1.6	−1.8	−1.69	−2.01	−1.9	Log σ b-wave control eye (cd-s/m^2)
−0.2	−0.1	0.03	−0.01	−0.07	Log σ b-wave difference
55.1	75	77	74.4	87.4	Sum OPs[a] amplitude experimental eye (µV)
65	88.7	75.8	66.5	75.8	Sum OPs[a] amplitude control eye (µV)
0.85	0.8	1.02	1.12	1.15	OPs[a] amplitude ratio

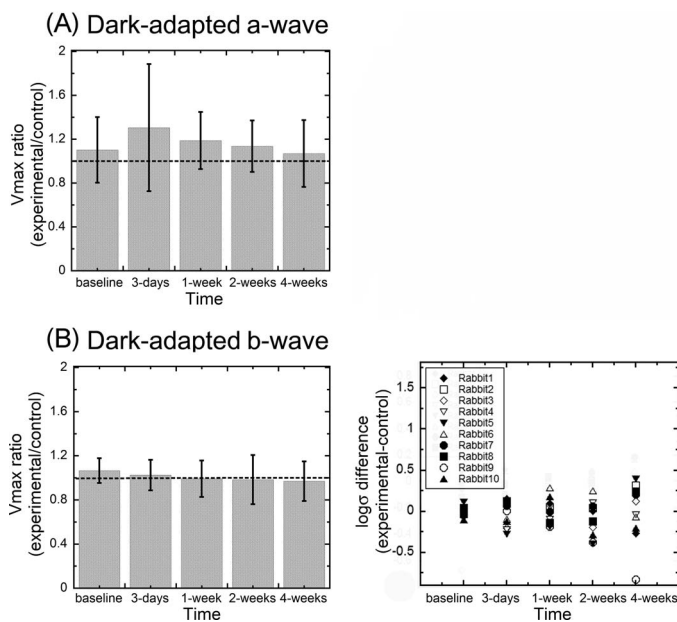[a] OPs, oscillatory potentials.

Figure 2. Vmax ratio (experimental eye/control eye) and logσ difference (experimental eye – control eye) were derived for the 10 rabbits at each ERG recording session. (A) Vmax for the dark-adapted a-wave was defined as the amplitude measured with bright (7.5 cd-s/m^2) light stimuli in the dark-adapted state. Mean (± SD) of the Vmax ratio (experimental eye/control eye) for the dark-adapted a-waves fluctuate around 1. (B) Mean (± SD) of the Vmax ratio (experimental/control) (*left*), and scatter-gram of logσ differences (experimental – control) (*right*) for the dark-adapted b-waves (*left and right*, respectively). Vmax ratios are around 1 and logσ differences are around 0, indicating no damage to the rod system.

differences (scatter-grams at Fig. 2, lower right) fluctuated around 0 with random fluctuation, suggesting no damaging effect of intravitreal TMP-SMX on the dark-adapted function of the distal retina. Statistical analysis for Vmax of b-waves and a-waves in dark-adapted state showed no interaction between time and treatment ($P = 0.241$ and $P = 0.806$, respectively). There was also no interaction between time and treatment for logσ difference of the dark-adapted b-wave ($P = 0.878$).

In order to examine the toxic effect of TMP-SMX on the dark-adapted ERG of the proximal retina, we used the sum of oscillatory potentials that reflect the electrical activity in the neural networks involving bipolar cells, amacrine cells, and ganglion cells.[20,27] Analysis of the oscillatory potentials, similar to that shown in Figure 1 and Table 1, was done to all 10 rabbits for ERG recording sessions. Figure 3 shows the mean (± SD) of the sum of oscillatory potentials ratios (experimental eye/control eye), that were elicited by bright (2.5 cd-s/m^2) white light stimuli. The mean (± SD) sum of oscillatory potential ratios (experimental eye/control eye) fluctuate around 1 for three ERG recording sessions (baseline, 3 days, 1 week) as shown in Figure 3, but were reduced (∼0.8) in the ERG responses that were recorded at 2 weeks and 4 weeks of follow-up. Statistical analysis showed interaction between time and treatment ($P = 0.037$) with decrease in the average of sum oscillatory potential ratio (experimental eye/control eye) between baseline and 2 weeks but not between baseline and 4 weeks because of the high variability between the
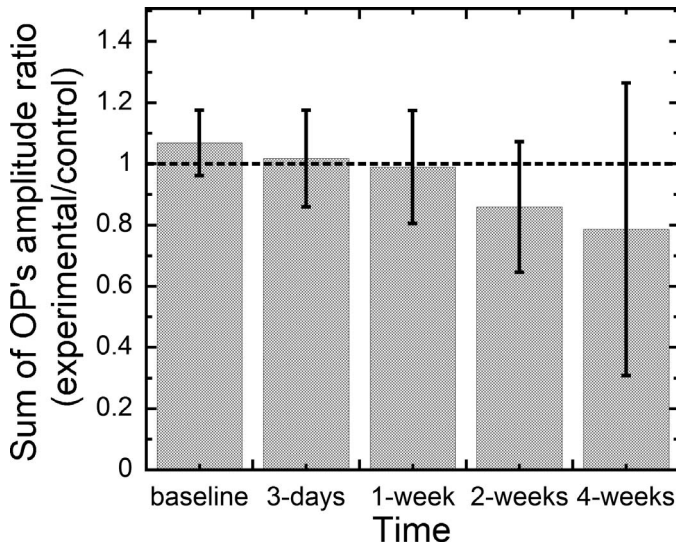
Figure 3. Sum of oscillatory potentials were measured from ERG responses that were elicited by bright (I = 2.5 cd-s/m^2) white light stimuli. Mean ($\pm$ SD) of the sums of the oscillatory potentials (experimental eye/control eye) are shown for all recording sessions throughout the follow-up period. Ratios of the sum of the oscillatory potentials, measured at 2 weeks and 4 weeks, are reduced in comparison to baseline.

measurements, suggesting toxic effect of TMP-SMX upon inner retina function.

As discussed above (Figs. 1, 2), there were no statistically significant differences between the experimental eyes and the control eyes for the Vmax ratios (experimental eye/control eye) and logσ differences (experimental eye – control eye) of dark-adapted b-waves, while analysis of the oscillatory potentials at 2 weeks revealed signs of mild retinal damage in the inner retina. Therefore, further examination of the relative relationship between the b-wave and a-wave was conducted in order to test signal transmission between photoreceptors and ON-center bipolar cells and the functional integrity of these cells.[28] A scatter-grams of b-wave as a function of a-wave were constructed for the experimental eyes and control eyes of all 10 rabbits in each recording session as shown in Figure 4. Regression lines for the b-wave to
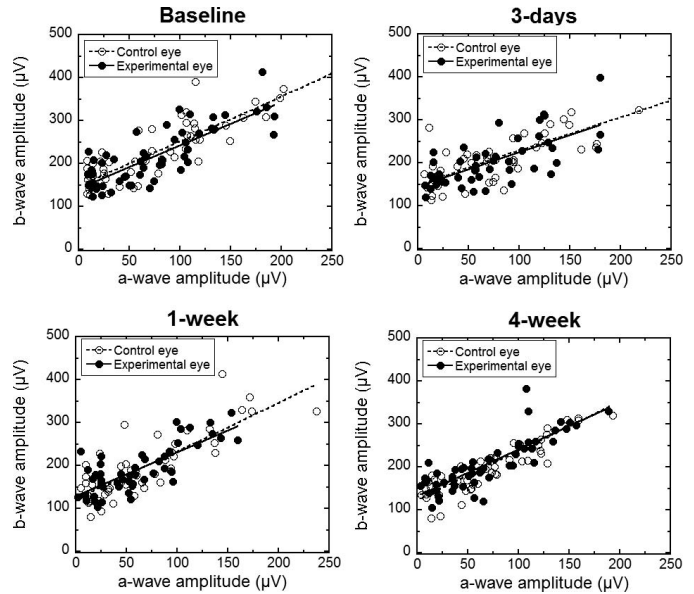
Figure 4. Scatter-grams of b-wave amplitudes as function of a-wave amplitudes in dark-adapted state for all 10 rabbits are shown for four recording sessions during the follow-up period: baseline, 3 days, 1 week, and 4 weeks after intravitreal injection of TMP-SMX. Data from the experimental eyes and control eyes are compared (*filled* and *open circles*, respectively). Regression lines for the experimental eyes (*continuous line*) are similar to those of the control eyes (*dotted line*) indicating no damage to the photoreceptors, ON-center bipolar cells, and signal transmission between them.

a-wave relationships of the experimental eyes (filled circles) and control eyes (open circles) are very similar, and the slope ratios fluctuate around 1 as listed in Table 2. No statistically significant differences were found between the slopes of the regression lines during the follow-up period (Table 2).

Under background illumination (30 cd/m^2), the rod system is saturated, and the functional integrity of the cone system can be evaluated from the light-adapted ERG responses. The ERG under these conditions is characterized by fast kinetics (peak time of about 30 to 35 ms), and small b-wave amplitudes. Thus, in order to derive reliable data, we averaged ERG responses that were elicited by white light

Table 2. Mean of the Slopes of the Linear Regression Fits to the B-Wave to A-Wave Relationships for the Experimental Eye and Control Eye of All 10 Rabbits for Each ERG Recording Session

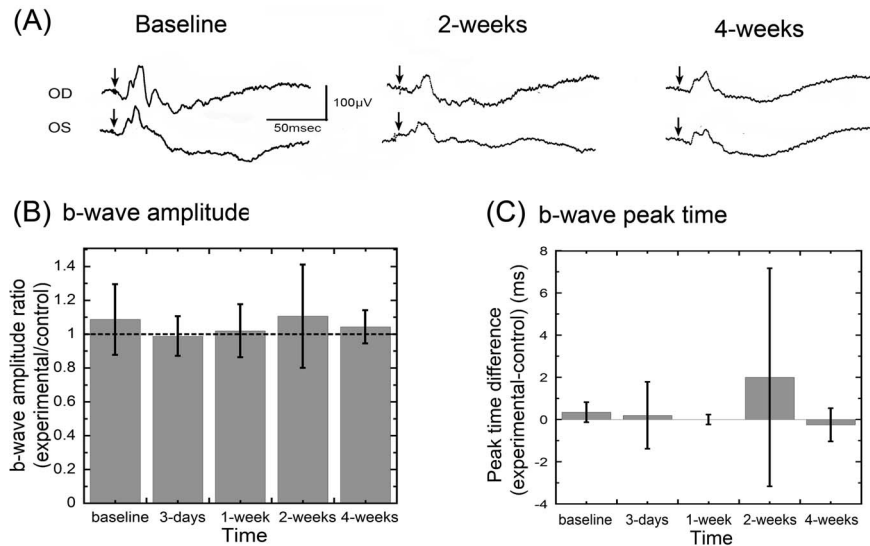| 4 Weeks	2 Weeks	1 Week	3 Days	Baseline	
1.15	1.1	1.1	0.8	1.04	Slope experimental eyes
1.1	0.96	1.01	0.8	1.0	Slope control eyes
1.06	1.2	1.1	1.0	1.04	Slopes ratio
0.66	0.3	0.5	0.96	0.7	*P* value

Figure 5. Light-adapted (30 cd/m^2) ERG responses and analysis after intravitreal injection of TMP-SMX. (A) Light-adapted ERG, elicited by bright (2.5 cd-s/m^2) light stimuli, from the experimental eye and control eye (*upper* and *lower traces*, respectively) for one rabbit at three ERG recording sessions. (B) Mean amplitude ratio (experimental eye/control eye) ($\pm$ SD) for all 10 rabbits during the follow-up period. (C) Mean peak time differences (experimental eye – control eye) ($\pm$ SD) for all 10 rabbits during the follow-up period.

stimuli of 2.5 and 7.5 cd-s/m^2 strength to derive the maximal b-wave amplitudes. Figure 5A shows ERG recording in the light-adapted state of one rabbit that was tested at all time points, but for clarity of the figure, only ERG responses that were recorded at three time points of the follow-up period are shown (baseline, 2 weeks, 4 weeks). Each pair of ERG responses compares the experimental eye (OD) to the control eye (OS) (upper and lower traces, respectively), elicited by bright (I = 2.5 cd-s/m^2) light stimulation. In order to test the toxic effect of intravitreal TMP-SMX on the cone system, we measured from the ERG responses the b-wave amplitude, and peak time for the experimental eyes and the control eyes, as listed in Table 3 for all follow-up periods.

Note that the ERG data gathered at the 1-week session were considerably smaller in amplitude compared to those recorded in all other recording sessions, at earlier or later times of follow-up. The amplitude ratio was 0.8, while in other ERG recording sessions, the amplitude ratio was around 1. These observations suggest that the amplitude reduction was affected similarly in both eyes, and most probably represents variability due to technical factors such as period of the light-adaptation, body temperature, and depth of anesthesia that affected both eyes similarly. Therefore, using amplitude ratio and peak time difference circumvent such differences.[18,22]

Light-adapted ERG responses were recorded in all 10 rabbits at all time points. Figure 5B shows the mean ($\pm$ SD) b-wave amplitude ratios (experimental eye/control eye) for the all 10 rabbits, which fluctuated around 1 during the follow-up period, and Figure 5C shows the mean ($\pm$ SD) b-wave peak

Table 3. Light-Adapted ERG B-Wave Amplitudes and Peak Times of the Rabbit Whose ERG Responses Are Presented in Figure 5

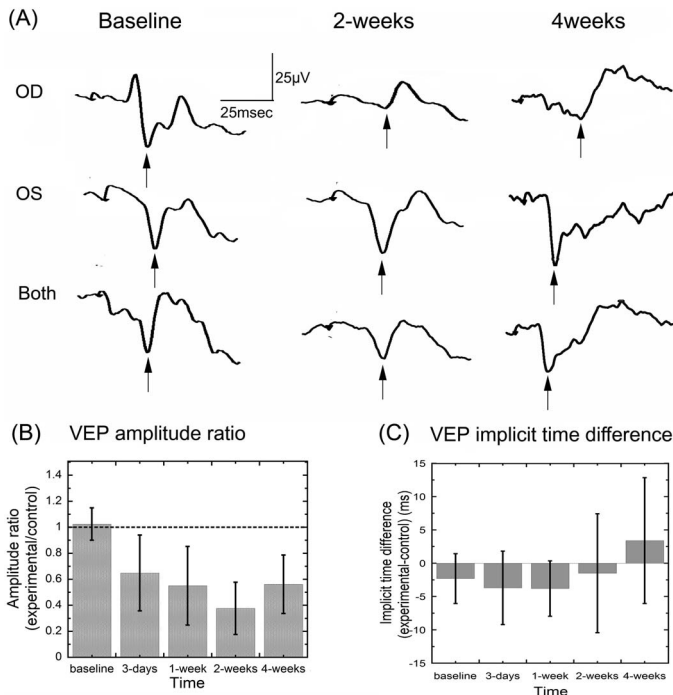| 4 Weeks	2 Weeks	1 Week	3 Days	Baseline	
139.4	102	36.4	74.7	97.3	Amplitude experimental eye (μV)
132.2	101.1	45.9	74.7	85	Amplitude control eye (μV)
1.05	1.0	0.8	1.0	1.1	Amplitude ratio
33	34.5	33.5	31	32.5	Peak time experimental eye (ms)
32	33	33	31	32.5	Peak time control eye (ms)
1.0	1.5	0.5	0.0	0.0	Peak time difference (ms)

Figure 6. Flash VEP responses and analysis after intravitreal injection of TMP-SMX. (A) Representative VEPs were elicited by photic stimuli of 2.5 cd-s/m². VEP responses were elicited by monocular stimulation of the experimental eyes and control eyes (*upper* and *middle rows*, respectively) and binocular stimulation (*lower row*) of one rabbit at baseline, 2 weeks, and 4 weeks postinjection. (B) Mean amplitude ratio (experimental eye/control eye) (± SD) for all 10 rabbits during the follow-up period. (C) Mean implicit time differences (experimental eye − control eye) (± SD) for all 10 rabbits during the follow-up period.

from ganglion cells through the optic pathways, and up to the visual cortex after intravitreal injection of TMP-SMX. Figure 6A shows VEP records that were evoked by binocular and monocular stimulations of the experimental eye (OD) and control eye (OS) in one rabbit that was tested in all time points (baseline, 3 days, 1 week, 2 weeks, and 4 weeks after injection) during the follow-up period. For clarity of the figure, only VEP data that were obtained at baseline, 2 weeks, and 4 weeks are shown. Records following monocular stimulations are compared (upper and middle traces, respectively), indicating that the amplitude of the VEP responses from stimulation of the experimental eye after intravitreal injection of TMP-SMX are reduced in comparison to those of the control eye that were recorded in the same session, and from that recorded from the experimental eyes at baseline. Two parameters were measured in each VEP response; the trough time of the first negative wave (arrow in Fig. 6A), and the amplitude between the trough of the first negative wave and the peak of the following positive wave. Data for the VEP parameters of the rabbit, whose responses represented at Figure 6A, are listed at Table 4 showing a reduction in VEP amplitudes of the experimental eyes, but not in the VEP amplitudes from control eyes. Additionally, prolongation of the trough time in the VEP from the experimental eye is evident at 4 weeks post-intravitreal injection. Similar VEP responses were recorded from all 10 rabbits following monocular photic stimulation of the experimental eye and control eye.

The amplitude ratios (experimental eye/control eye), and the VEP trough time differences (experimental eye − control eye) were derived. The mean (± SD) of VEP amplitude ratio was around 1 at baseline, and was significantly reduced after the intravitreal injection of TMP-SMX during the follow-up period (Fig. 6B). The mean (± SD) trough time differences were around 0 (Fig. 6C), except at the end of the

time differences (experimental eye − control eye), which are around 0. No statistical significance was found for the light-adapted b-wave amplitudes ($P = 0.512$), and for the peak time differences ($P = 0.253$), indicating no toxicity damage to the cone system after intravitreal injection of TMP-SMX.

VEP responses were recorded during the entire follow-up period in order to test the retinal output

Table 4. VEP Parameters: Amplitudes and Trough Times of the Rabbit Whose Responses Are Presented in Figure 6

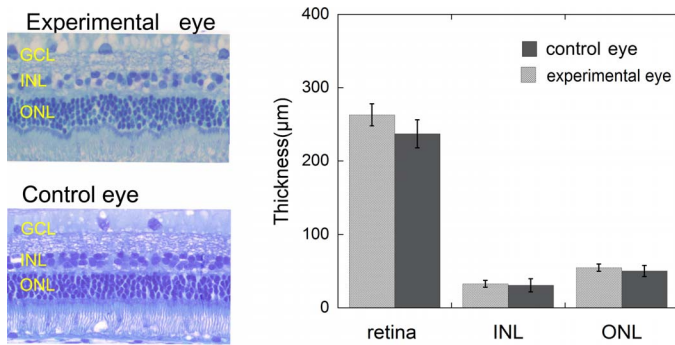| 4 Weeks	2 Weeks	1 Week	3 Days	Baseline	
6	5.1	16.2	10.5	18.5	Amplitude experimental eye (μV)
18	17.9	21.5	17.6	18.3	Amplitude control eye (μV)
0.3	0.3	0.75	0.6	1.01	Amplitude ratio
38	25	29	32	25	Trough time experimental eye (ms)
29	28	29	31	28	Trough time control eye (ms)
9	−3	0	1	−3	Trough time difference (ms)

Figure 7. Representative micrographs of retinal histology that were processed at termination of the follow-up period, 4 weeks postintravitreal injection of TMP-SMX, for the experimental eye and control eye (*left*) of one rabbit. The retinal structure is similar between the retinas. Mean (± SD) of retinal thicknesses, and thicknesses of INL and ONL of the experimental retinas and control retinas for all 10 rabbits are compared in the *right side* of the figure. GCL, ganglion cell layer.

follow-up period (4 weeks postinjection) when the implicit time of the experimental eyes were prolonged compared to the control eyes, indicating damaging effect of intravitreal TMP-SMX to the functional integrity of the visual pathways. Statistical significance was found for the VEP amplitude ratios ($P < 0.001$), and for the implicit time differences ($P = 0.022$).

Retinal Histology

Histological analysis was performed at the end of the follow-up period (4 weeks) for both the experimental eyes and control eyes, as described in the Methods section. Qualitative examination of the structural integrity of the retinas showed no differences between the retinas as shown at the representative histological micrographs in Figure 7 (left). Further evaluation was conducted in order to test quantitative changes in the retinas. Measurements of thicknesses of the entire retina, the INL, and the ONL were done in the retinas from the experimental eyes and control eyes. These parameters were averaged, and the mean (± SD) thickness measurements of the

experimental retinas were compared to the corresponding values from control retinas and found reduced, as shown in Figure 7 (right) and listed in Table 5. A Student-paired *t*-test, conducted on these parameters, showed statistical significance as listed in Table 5, indicating mild structural retinal damage.

Immunocytochemistry

Expression of GFAP in retinal Müller cells is a widely accepted cellular marker for retinal stress, even in cases in which the retinal structure is normal.[24–26]

Representative micrographs of experimental and control retinas from two rabbits are shown in Figure 8. GFAP expression in Müller cells is evident in the retinas from the experimental eyes (Fig. 8, left), but not in the retinas from the control retinas (Fig. 8, right). Similar results were found in the retinas of all 10 rabbits; all the experimental eyes showed GFAP expression in Müller cells, but not in the control eyes.

Discussion

This study assessed the functional and morphological changes following an intravitreal injection of 1600 μg/8000 μg per 0.1 mL TMP-SMX in albino rabbit eyes. The clinical examination and ERG assessment of outer retina function (b-wave and a-wave) were normal. However, the ERG oscillatory potential results, the histological findings, and the positive GFAP immunostaining of Müller cells suggested a mild degree of retinal trauma to the inner retina. This conclusion was strengthened by the VEP findings, showing significant amplitude reduction, and slight implicit time prolongation in the VEPs that were recorded by stimulation of the experimental eyes compared to the VEPs from the control eyes. These electrophysiological and morphological findings suggest that TMP-SMX may induce retinal toxicity.

TMP-SMX did not cause transient or permanent decrease in the functional integrity of the rod system as the maximal amplitudes of the dark-adapted a-

Table 5. Mean (± SD) of Retinal Thickness, INL Thickness, and ONL Thickness in the Retinas From the Experimental Eyes and From the Control Eyes of All 10 Rabbits

P Value	Control Eyes (μm)	Experimental Eyes (μm)	
0.001	295.5 ± 24	263 ± 15.1	Retina thickness ± SD[a]
<0.001	38.5 ± 9.4	32.7 ± 5	INL thickness ± SD[a]
<0.001	63 ± 11.1	54.8 ± 4.7	ONL thickness ± SD[a]

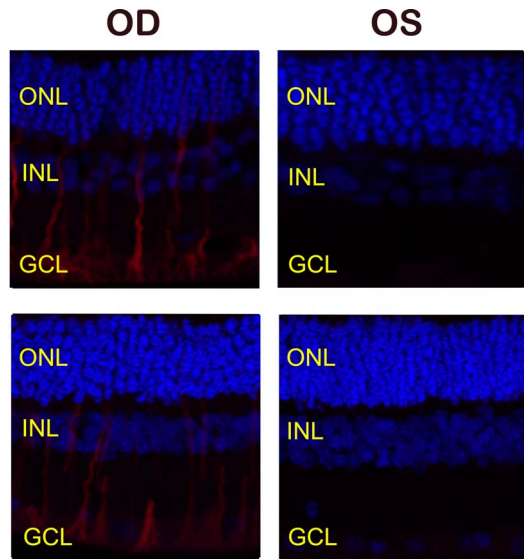[a] SD and thickness values are given in micrometers.

OD   OS

Figure 8. Immunocytochemistry for GFAP (*red*) and DAPI (*blue*) for the experimental eyes (OD, *left column*) and control eyes (OS, *right column*) of two rabbits (*rows*). Immunocytochemistry was performed on retinal cryosections that were prepared after termination of follow-up period. DAPI is used for nuclear staining and allows visualization of the nuclear layers in the retina; ONL, INL, and GCL with laser scanning confocal microscope. GFAP is a marker of retinal damage when expressed in Müller cells, as shown in the retinal sections from the experimental eye (*left column*) and not in the retinal sections from the control eyes (*right column*). Retinas from the experimental eyes of all studied rabbits were positive to GFAP staining in Müller cells, whereas none of the retinas from the control eyes showed GFAP staining in Müller cells. GCL, ganglion cell layer.

wave and b-wave, and the semi-saturation constant of the dark-adapted b-wave were similar in the experimental eyes and control eyes throughout the follow-up period (Fig. 2). TMP-SMX did not alter synaptic transmission in the outer plexiform layer (OPL) as indicated by the similar b-wave amplitude to a-wave amplitude relationships that were measured throughout the follow-up period (Fig 4; Table 2). Similarly, no transient or permanent damage to the functional integrity of the cone system was found as the b-wave amplitude and peak time of the ERG responses that were recorded under background illumination (30 cd/m^2) did not differ between the experimental eyes and control eyes (Fig. 5). However, we found that TMP-SMX caused mild damage to inner retina function because it reduced the sum amplitudes of the oscillatory potentials (Fig. 3),[20,27] which is used as an electrophysiological marker for the activity of the neural networks between bipolar cells, amacrine cells, and ganglion cells.

The effects of TMP-SMX on VEP responses (Fig. 6) demonstrated a significantly reduced VEP amplitude ratios during the follow-up period, and a prolonged trough time at 4 weeks postinjection. These results indicate reduced retinal output to the visual cortex and subnormal conduction via the optic nerve from the eyes that were treated with intravitreal TMP-SMX.

The functional damage to the inner retina was supported by retinal morphological changes that were demonstrated by the analysis of retinal histology. Quantitative assessment of retinal structure showed a significant thinning of the whole retina, the ONL, and the INL in retinas from the experimental eyes compared to retinas from the control eyes. In addition, positive GFAP immunostaining of Müller cells in the retina was found in all of the retinas from the experimental eyes, and none in the retinas from the control eyes (Fig. 8). GFAP upregulation in Müller cells is indicative for Müller cell activation, which usually reflects retinal damage.[24–26]

To date, only one study reported on testing intravitreal toxicity of TMP-SMX in albino rabbits either after a single intravitreal injection or following vitrectomy by adding the TMP-SMX to the vitrectomy solution.[16] This study examined dark-adapted ERG and histology changes 10 days postintravitreal injection of different concentrations (100, 200, 400, 800, and 1600 µg/0.1 mL) of TMP-SMX. TMP-SMX was found to be nontoxic, for all concentrations, as judged from ERG measurements and histological assessment. In 14 eyes that underwent vitrectomy with TMP-SMX in the vitrectomy solution small alterations in the ERG b-waves were reported. In eyes treated with the highest concentration (50 µg/mL), some homogenization of the photoreceptor outer segments was observed as well. Our findings are similar, showing no effect of a single intravitreal injection of TMP-SMX on the dark-adapted ERG responses, indicating no toxic effects on the distal retina, but we expanded on the electrophysiological and morphological testing, and prolonged the follow-up period to show toxic effects on the proximal retina.

One clinical study[14] assessed full-field ERG changes in four patients treated with intravitreal mixture of 1.28 mg/0.08 mL TMP-SMX with 400 µg/0.1 mL dexamethasone as weekly or biweekly treatment for TRC. ERG was performed at the 6-week follow-up visit and was unremarkable and comparable with the normal fellow eye in all patients. No other animal or clinical studies examined the plausible toxicity of intravitreal TMP-SMX.

In summary, our study shows that a single

intravitreal injection of TMP-SMX mixture (1600 µg/8000 µg per 0.1 mL) is toxic to the albino rabbit retina, mainly to the inner retina and retinal output. These findings do not necessarily suggest that the active ingredients (trimethoprim 80 mg and sulfamethoxazole 400 mg) are the toxic substance; the excipients can be the toxic elements. Nevertheless, our results advocate careful use of the available commercial drug Septrin that was used here for an intravitreal injection treatment.

Acknowledgments

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