Sustained Connexin43 Mimetic Peptide Release From Loaded Nanoparticles Reduces Retinal and Choroidal Photodamage

Nasir Mat Nor,1–3 Cindy X. Guo,1,3 Ilva D. Rupenthal,3–5 Ying-Shan Chen,3–5 Colin R. Green,3,4 and Monica L. Acosta1,5

1School of Optometry and Vision Science, University of Auckland, Auckland, New Zealand
2Faculty of Medicine, University of Sultan Zainal Abidin, Kuala Terengganu, Malaysia
3New Zealand National Eye Centre, University of Auckland, Auckland, New Zealand
4Department of Ophthalmology, University of Auckland, Auckland, New Zealand
5Buchanan Ocular Therapeutics Unit, University of Auckland, Auckland, New Zealand

Purposes. To evaluate the long-term effect on inflammation and inflammasome activation of intravitreally delivered connexin43 mimetic peptide (Cx43MP) in saline or incorporated within nanoparticles (NPs) for the treatment of the light-damaged rat eye.

Methods. Light-induced damage to the retina was created by exposure of adult albino Sprague-Dawley rats to intense light for 24 hours. A single dose of Cx43MP, Cx43MP-NPs, or saline was injected intravitreally at 2 hours after onset of light damage. Fluorescein isothiocyanate (FITC)-labelled Cx43MP-NPs were intravitreally injected to confirm delivery into the retina. Electroretinogram (ERG) recordings were performed at 24 hours, 1 week, and 2 weeks post cessation of light damage. The retinal and choroidal layers were analyzed in vivo using optical coherence tomography (OCT) and immunohistochemistry was performed on harvested tissues using glial fibrillary acidic protein (GFAP), leukocyte common antigen (CD45), and Cx43 antibodies.

Results. FITC was visualized 30 minutes after injection in the ganglion cell layer and in the choroid. Cx43MP and Cx43MP-NP treatments improved a-wave and b-wave function of the ERG compared with saline-injected eyes at 1 week and 2 weeks post treatment, and prevented photoreceptor loss by 2 weeks post treatment. Inflammation was also reduced and this was in parallel with downregulation of Cx43 expression.

Conclusions. The slow release of Cx43MP incorporated into NPs is more effective at treating retinal injury than a single dose of native Cx43MP in solution by reducing inflammation and maintaining both retinal structure and function. This NP preparation has clinical relevance as it reduces possible ocular complications associated with repeated intravitreal injections.

Keywords: choroid, retina, light damage, connexins, retinal degeneration

Inflammation plays a significant role in the development of the pathogenesis of age-related macular degeneration (AMD).1–3 In AMD, local inflammation is associated with an immune cell-mediated process that leads to a chronic inflammatory environment.4 According to recent reports, the inflammatory reaction is associated with an increase in connexin43 (Cx43) expression, primarily in the choroid, RPE, and retina.5 Cx43 forms gap junctions that play a major role in the bidirectional movement of ions and metabolites between cells, contributing to the blood-retinal barrier in endothelial cells.6–10 However, Cx43 also forms hemichannels in the plasma membrane, including that of endothelial cells and astrocytes in response to injury, and these “pathological pores” play a major role in the inflammatory reaction and its perpetuation.11–17 This has been demonstrated in experiments in which connexin channel-blocking mimetic peptides have been used to prevent connexin hemichannel opening and reduce the inflammatory response.5,18–25 However, the short half-life of native Cx43 mimetic peptides26 in solution is potentially an obstacle for long-term therapeutic effects.

One Cx43 mimetic peptide (Cx43MP), also known as peptide5, is a short amino acid sequence of the extracellular loop of rat Cx43.27 This peptide can be delivered directly into the eye via intravitreal (IVT), subtenon or intravenous injections.28–30 The main advantage of IVT injection is that it provides a localized concentration of drug that diffuses directly to posterior segment tissues with minimal systemic side effects.31–33 IVT injection is the preferred and standard drug delivery method to treat posterior eye segment diseases, enabling the direct delivery of molecules with high molecular mass.34 However, in the treatment of certain conditions, repeated IVT injections are required to maintain drug availability and thus treatment efficacy, which may lead to a number of ocular complications such as subconjunctival hemorrhages, vitreous hemorrhage, endophthalmitis, retinal detachment, or cataract formation.28,29
Advances have been made in formulations that protect the active ingredient from enzymatic degradation and allow slow drug release over time.\textsuperscript{21,34} Nanoparticles (NPs) are one of the most widely used groups of drug delivery systems. A large number of studies have described the potential for polymeric NPs to be used in the treatment of retinal diseases because of their high tolerance, biocompatibility, biodegradability, and lack of intrinsic immunogenicity.\textsuperscript{21,35,36} We have previously shown that a double injection of unmodified Cx43MP is protective against damage in the intense light–exposed rat retina.\textsuperscript{5} In this study we investigated the effect of sustained Cx43MP delivery from NPs and monitored the effect on inflammation, as well as connexin expression that occurs in the light-damaged rat retina.

**Materials and Methods**

**Light Damage Procedure**

Six- to eight-week-old Sprague-Dawley (SD) rats (200–250 g; male or female) were used in this experiment. All experimental procedures were approved by the University of Auckland Animal Ethics Committee (approval No. 001462) and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. SD rats were obtained from the Vernon Jansen Unit, the University of Auckland. Light damage was induced using a light source with a luminance of 2700 lux from fluorescent light lamps with no heat emission (Philips Master TLD 18W/965; 380–760 nm; Koninklijke Philips Electronics N.V., Shanghai, China) when placed directly above the rat cage. Animals were allowed to roam free in the cages during light exposure and had ad libitum access to food and water. Light damage experiments were performed consistently starting at 9:00 AM. After the 24 hours of intense light exposure, animals were returned to normal light/dark cycle conditions of 12 hours of light (174 lux) and 12 hours of darkness (<62 lux). Figure 1 shows the experimental setup and the outline of the intervention protocols.

**Animal Anesthesia**

For IVT injections and ERG recordings animals were anesthetized by intraperitoneal (i.p.) injection using a combination of ketamine (75 mg/kg; Parnell Technologies, Auckland, New Zealand) and Domitor (0.5 mg/kg; Pfizer, Auckland, New Zealand). Following manipulations, anesthesia was reversed by intraperitoneally injecting atipamezole (1 mg/kg Antisedan, Pfizer) and animals were returned back to their cages. Warm water bottles were used to maintain the animals’ body temperature during recovery.

**Mimetic Peptide Preparation and Intravitreal Injections**

Poly-(d, l-lactic-co-glycolic acid) (PLGA, 50 lactic:50 glycolic acid; Sigma-Aldrich, Auckland, New Zealand) NPs were prepared according to the procedure described by Chen et al.\textsuperscript{21,37} using the double emulsion solvent evaporation method.\textsuperscript{26-37} Briefly, the 5 mM Cx43MP (MWt 1396 g/mol; Auspep, VIC, Australia) aqueous solution was emulsified in dichloromethane containing 30 mg/mL PLGA using a probe sonicator (amplitude 50 W; duty cycle 0.6 s/min; Hielscher, Teltow, Germany) to agitate particles and form the primary water-in-oil (w/o) emulsion. The primary w/o emulsion then underwent a second emulsification in 3% w/v polyvinylpyrrolidone (PVA) (Sigma-Aldrich, New Zealand) aqueous solution to form a water-in-oil-in-water (w/o/w) emulsion, which was then poured into a 0.1% wt/vol aqueous PVA solution. This procedure was performed to stabilize the double emulsion during the evaporation process and was followed by ultracentrifugation using a ProteomeLab XL-A/XL-1, Type 70 Ti rotor (Beckman Coulter, Auckland, New Zealand) at 11,000g and 4°C. Formulated Cx43MP-NPs were then lyophilized (VirTis) (SP Scientific, Gardiner, USA) for 24 hours.

Both the Cx43MP-NPs and the Cx43MP formulations were diluted in saline to a final peptide concentration of 280 μM. This peptide concentration was chosen based on previous intervention studies using the light-damaged rat model.\textsuperscript{11} Previous studies have shown that PLGA injected intravitreally does not result in any electrophysiological or histologic toxicity in the retina.\textsuperscript{38,39} Thus, saline was used as injection control, given the extensive previous evaluation and feasibility regarding PLGA NPs as suitable vehicles for ophthalmic administration.\textsuperscript{40-44} Treatment consisted of 4 μL Cx43MP, Cx43MP-NPs, or saline injected into the vitreous 2 hours after the onset of intense light exposure. A Hamilton syringe attached to a 30-gauge × 0.5-inch needle (BD PrecisionGlide; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used for the injection. In order to avoid damage to the lens, the injections were made into the temporal side of the eye after rotating the eyeball to the nasal side by holding the bulbar conjunctiva. Maxitrol (Alcon, Fort Worth, TX, USA) was also applied to the cornea after the IVT injection to avoid any ocular infection at the site of injection.

**Electroretinogram**

The procedure was performed as described previously.\textsuperscript{11,45} SD rats were dark-adapted overnight for 12 to 14 hours before the ERG recording. The ERG baseline was recorded before light damage and again at 24 hours, 1 week, and 2 weeks after the cessation of the light damage procedure. A dim red light generated by a light-emitting diode (λmax = 650 nm) was used during manipulations of dark-adapted animals. The corneas were maintained hydrated with 1% carbamoylmethylcellulose sodium (Celluvisc; Allergan, Irvine, CA, USA) throughout the whole ERG recording. Right- and left-eye ERGs were recorded at the same time. The active electrodes were gold ring electrodes (Roland Consult Stasche & Finger GmbH, Brandenburg, Germany) and were placed in contact with the center of the cornea. The inactive electrode was hooked around the front teeth and in contact with the wet tongue. Body temperature was kept approximately at 37°C to avoid temperature-driven ERG amplitude fluctuations.

Full-field ERG responses were elicited by a twin-flash (0.8 ms stimulus interval) generated from a photographic flash unit (Nikon SB900 Flash, Tokyo, Japan), via a Ganzfeld sphere. In the Ganzfeld method, an integrating sphere approximately 650 cm in diameter, painted white internally, was used to reflect the light onto the dilated pupil. The flash intensity range was from −2.9 to 2.1 log candela-seconds per meter squared (cd.s/m²) and was attenuated using neutral density filters (Kodak Watten; Eastman Kodak, Rochester, NY, USA) in order to obtain light intensities of −3.9, −2.9, −1.9, 0.1, 1.1, 1.6, 1.8, and 2.1 log cd.s/m². The flash intensity was calibrated using an IL1700 research radiometer (UV Process Supply, Inc., Chicago, IL, USA). This study utilized a twin-flash paradigm for the isolation of rod and cone pathways. Paired flashes of identical luminous energy were triggered from the flash unit. The rod and cone mixed responses were recorded after the initial flash, and the response from the second flash was recorded representing the function from cones only. The rod PHI response was derived through digital subtraction of the cone response.
from the initial mixed response. Recordings were performed in a Faraday cage to reduce electrical noise. The results of ERG signals were amplified 1000 times by a Dual Bio Amp (AD Instruments, NSW, Australia), and waveforms were recorded by using the Scope software (AD Instruments, Dunedin, New Zealand) and analyzed using published algorithms of the amplitudes of a-wave and b-wave.5,45

Optical Coherence Tomography

The optical coherence tomography (OCT) imaging technique was used to obtain information on in vivo retinal layers and choroid structure. Spectral-domain optical coherence tomography (SD-OCT, Micron IV; Phoenix Research Laboratories, Pleasanton, CA, USA) was employed. This procedure was executed immediately after ERG recordings under anesthesia and pupil dilation.5 Rats were placed on a 37°C heating pad to maintain their body temperature and to prevent the development of cold cataracts. Dilated eyes were covered with Poly Gel (3 mg/g Carbomer; Alcon, NS, Australia) and the retina was visualized by contacting the OCT lens to the gel. StreamPix 6 software, version 7.2.4.2 (Phoenix Research Laboratories, Dunedin, New Zealand) and analyzed using published algorithms of the amplitudes of a-wave and b-wave.5,45

Immunohistochemical Labelling of Tissue Sections

Immunohistochemical labelling was conducted using the indirect immunofluorescence technique. Briefly, frozen tissue sections were air-dried at room temperature for 10 to 15 minutes and washed with 0.1 M PBS. The tissue sections were blocked with a solution containing 6% normal goat serum or donkey serum (Invitrogen, Grand Island, NY, USA), 1% bovine serum albumin (BSA; Sigma-Aldrich, New Zealand) and 0.5% Triton X-100 in 0.1 M PBS for 1 hour at room temperature. The tissues were immunolabelled with primary antibodies, including rabbit anti-Cx43 mouse anti-leukocyte common antigen (CD45) and mouse anti-gliarial fibrillary acidic protein (GFAP) antibodies (Table 1), and were incubated overnight at room temperature. After the incubation period, sections were washed four times for 15 minutes each in 0.1 M PBS in order to remove excess primary antibody. The secondary antibody, goat anti-rabbit or goat anti-mouse, conjugated with Alexa 488 or Alexa 594 (Molecular Probes, Thermo Fisher Scientific, Auckland, New Zealand) was diluted 1:500 and incubated for 2 to 3 hours in the dark at room temperature. The slides were washed thoroughly with 0.1 M PBS to remove excess secondary antibody. Slides were mounted in Citifluor and sealed with nail polish.

Statistical Analysis

Graphing and statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). All data are presented as mean ± the standard error of the mean (SEM). Functional and morphologic data (n = 6 per group) were compared using analysis of variance with an α value of 0.05. A 2-way ANOVA followed by a Bonferroni post-test was used in the ERG response analysis to compare the effects of stimulus intensity. A 1-way ANOVA followed by Tukey’s test was used for the ERG response at the intensity of 2.1 log cd.s/
RESULTS

Localization of FITC-Cx43MP NPs in Choroid and Retina

To determine whether NPs delivered the peptide directly to retina and choroid, FITC-conjugated Cx43MP-NPs were injected into the vitreous. The whole-mount fluorescence signal and cross sections (Figs. 2A1, 2A2) analyzed 30 minutes post injection showed FITC in CD45⁺ round cells in the choroid (Figs. 2B1–4). The labelling also showed FITC in the ganglion cell layer (GCL), OPL, and photoreceptors in cross sections (Fig. 2A3) and in whole mounts it was observed between but not colocalized with GFAP (Figs. 2C1–4). The fluorescent label was seen around blood vessels, mainly in the choroid, but also in the RPE (Fig. 2B) and in the retinal nerve fiber layer (Fig. 2C). This demonstrates that the tagged extracellular acting peptide was able to penetrate at least the inner retinal layers and that IVT injection allows consistent delivery of the peptide to the RPE and choroid.

Effect of Sustained Cx43MP Delivery on Electroretinograms

Representative mixed ERG waveforms of each animal group at 2 weeks post light damage are shown in Figure 3A. The ERG allows assessment of changes in the function of the outer retina (a-wave) and inner retina (b-wave) and deciphering of the rod and cone pathway function using a range of light intensities. Compared with saline-injected animals, retinal function in the light-damaged rats did not improve at any of the time points studied (up to 2 weeks). Twenty-four hours after injection of the drug in the light-damaged rats, there were no significant differences between the Cx43MP-, Cx43MP-NP–, and the saline-injected groups in the a-wave (Figs. 3B, 3C). Significant improvements in mixed a-wave amplitude (rod and cone photoreceptors) in both Cx43MP- and Cx43MP-NP-treated animals at intensities of 1.1 to 2.1 log cd.s/m² were first detected at 1 week post treatment (P < 0.001, Figs. 3B, 3C). At 2 weeks post treatment, both Cx43MP and Cx43MP-NP groups showed a significant increase in rod and cone response (mixed a-wave amplitude) (Figs. 3B, 3C). Rats treated with Cx43MP had almost a 200-μV (on average) improvement in ERG a-wave compared with saline-injected rats at intensities of 1.1 to 2.1 log cd.s/m² (P < 0.001; Fig. 3B), but the improvement was bigger (more than 400 μV) in Cx43MP-NP–treated eyes compared with saline at intensities of 0.1 to 2.1 log cd.s/m² (P < 0.001; Fig. 3C). Table 2 shows the results of statistical comparison made between ERG results in Cx43MP- and Cx43MP-NP–treated animals. It shows that the improvement in b-wave of the ERG at 1 week post treatment reflects activation of the ON-bipolar cell pathway (rod and cone driven). While this rescue persisted at 2 weeks post treatment, a-wave improved only at 2 weeks, suggesting that functional recovery of photoreceptors (both cones and rods) was considerable only 2 weeks post treatment.

No significant difference in mixed b-wave amplitude (cone and rod pathways) was detected at 24 hours post treatment (Figs. 3D, 3E). Further analysis on mixed b-wave amplitude in the ERG showed a significant increase only for intensity 1.1 log cd.s/m² for the Cx43MP group compared with saline (P < 0.01; Fig. 3D), suggesting that most changes were in the rod and cone function. A significant improvement in the Cx43MP-NP-treated group 1 week post treatment was seen for intensities 1.1 log cd.s/m² (P < 0.01; Fig. 3D), suggesting that most changes were in the rod and cone function. A significant improvement in the Cx43MP-NP-treated group 1 week post treatment was seen for intensities 1.1 log cd.s/m² (P < 0.01; Fig. 3D). An overt improvement on inner retinal function demonstrated by an increased mixed b-wave amplitude throughout all stimulus intensities was found at 2 weeks post treatment in both Cx43MP- and Cx43MP-NP–treated animals compared with saline-injected rats (P < 0.001; Figs. 3D, 3E). NP-treated eyes showed an additional increase of approximately 200 μV in the absolute amplitude of the b-wave compared to native Cx43MP-treated eyes (compare Figs. 3D, 3E).

The ERG PIII component can be reconstructed from bright-flash ERG responses using the phototransduction model, and PII can be estimated by subtracting the estimated PIII. Isolated rod PII (rod photoreceptor function only) and rod PII.
FIGURE 2. The location of FITC-Cx43MP-NPs injected in the vitreous in light-damaged rats. Eyes were enucleated 30 minutes post injection. In a whole mount, FITC was seen in the choroid (A1), and cross sections of the eye show FITC label in the choroid (A2) and in the GCL, OPL, and photoreceptors (A3). (B1) Whole-mount FITC label colocalizing with CD45 (B2), and DAPI (B3) shows FITC colocation with CD45⁺ cells. Whole-mount FITC label (C1) did not extensively colocalize with GFAP (C2) or DAPI (C3) in the GCL layer. Scale bar: 50 µm.

FIGURE 3. Representative ERG waveforms for light intensities ranging from −3.9 to 2.1 log cd.s/m² in saline-injected, Cx43MP-, or Cx43MP-NP–treated light-damaged rats (A). The effects of Cx43MP (B) and Cx43MP-NPs (C) on mixed a-wave amplitude; mixed b-wave amplitude effect of Cx43MP (D) and Cx43MP-NPs (E). Data for saline-injected light-damaged rats at 24 hours and 1 week are similar (nonsignificantly different) to those at 2 weeks. Statistical analysis was conducted using ANOVA, followed by Tukey’s multiple post hoc test. Significant values in comparison with saline treatment are indicated with asterisks: *P < 0.05; **P < 0.01; ***P < 0.001.
Sustained Delivery of Connexin43 Halts Retinal Damage

**Table 2.** Statistical Comparison Between Single-Injection Cx43MP and Cx43MP-NP ERG Results

<table>
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<th>Time Post Intervention</th>
<th>Cx43MP vs. Saline</th>
<th>Cx43MP-NPs vs. Saline</th>
<th>Cx43MP vs. Cx43MP-NPs</th>
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<td>Mixed a-wave amplitude</td>
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<tr>
<td>24 h</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>$P = 0.0014$</td>
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<td>$P &lt; 0.001$</td>
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<td>0.1 to 2.1 log cd.s/m²</td>
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<tr>
<td>Mixed b-wave amplitude</td>
<td></td>
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</tr>
<tr>
<td>24 h</td>
<td>NS</td>
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<tr>
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<td>Rod PIII amplitude</td>
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NS, nonsignificant.

Effect of Sustained Cx43 Delivery on Retinal and Choroid Structure

Analysis of retinal and choroidal thickness was obtained from in vivo OCT scans at 2 weeks post treatment (Fig. 5). Both Cx43MP- and Cx43MP-NP-treated eyes showed a significant improvement in choroid and retinal thickness 2 weeks post light damage (Figs. 5A, 5B). OCT imaging showed that the choroid was significantly thinner in the light-damaged salin-injected eyes 2 weeks post treatment compared to imaging prior to damage ($P < 0.01$; Fig. 5C). The retina was also thinner in the saline-injected group compared to before damage (Fig. 5A), with the loss of retinal thickness mainly due to significant thinning of the outer nuclear layer (ONL; $P < 0.001$; Fig. 5D). Both Cx43MP-NP and Cx43MP preserved retinal structure (Figs. 5B, 5D). Cx43MP-NP-treated eyes retained full choroidal thickness (Fig. 5C); eyes treated with a single dose of native Cx43MP, however, did not show preservation of choroidal thickness, with the thickness being similar to that in the saline group and significantly thinner than preinjury ($P < 0.01$; Fig. 5C).

We also monitored the effect of saline injection or drug treatment on retinal and choroidal thickness changes over time (Fig. 6). In saline-injected animals, intense light exposure caused an immediate change in the ONL thickness, previously reported to be due to death of photoreceptor cells,39,50 that deteriorated further over the course of 2 weeks. Neither Cx43MP- nor Cx43MP-NP-treated animals showed a change in ONL thickness over the first week, although the Cx43MP rats did have a slight decrease in ONL thickness at the end of 2 weeks post injury (Fig. 6A). This decrease was nonsignificant compared to preinjury retinas. Light-exposed drug-treated retinal structure was significantly preserved compared to saline-injected light-exposed eyes (Fig. 6A). Conversely, light exposure caused a significant increase in choroidal thickness in saline-injected animals 24 hours after injury, followed by a reduction in thickness over the next 2 weeks (Fig. 6B). In comparison, Cx43MP-NP-treated rats had no significant fluctuation in choroidal thickness at any time point over the 2 weeks (Fig. 6B). However, Cx43MP-treated eyes had a decrease in choroidal thickness from 24 hours to 2 weeks compared with the Cx43MP-NP-treated rats (or choroidal thickness prior to injury; Fig. 6B).

Treatment Effect on Connexin43 Expression, Retinal Gliosis, and Inflammation

The effect of Cx43MP and Cx43MP-NPs on inflammation in the retina and choroid was investigated using immunohistochemical markers for GFAP (marker for astrocytes and Müller cell activation), CD45 (common lymphocyte antigen), and Cx43...
(hemichannel protein) in tissues collected 2 weeks post light damage and saline or drug treatment (Fig. 7).

Cx43MP-NP–treated rats had less Cx43 immunoreactivity in the choroid compared with saline-injected or Cx43MP-treated eyes (Figs. 7A–C). There were also fewer CD45-positive cells in the choroid in Cx43MP-NP–treated animals compared with saline-injected animals (Figs. 7D, 7F). Treatment with Cx43MP also resulted in fewer CD45-positive cells in the choroid (Fig. 7E), although there were more than in the Cx43MP-NP–treated eyes (Fig. 7F). GFAP was used to determine the effect of Cx43MP and Cx43MP-NPs on astrocyte and Müller cell activation (Figs. 7G–I). Cx43MP-NP–treated rats had normal (preinjury) levels of GFAP label in the GCL (Fig. 7I). However, saline-injected animals showed a marked increase in GFAP expression in both the GCL and within Müller cells, indicating that these had become activated (Fig. 7G). The extent of GFAP immunoreactivity was less in Cx43MP-treated eyes (Fig. 7H) compared to saline-injected retina (Fig. 7G) but was more extensive than in Cx43MP-NP–treated rats (Fig. 7I). Taken together, these results suggest that there is a strong association between functional recovery and morphologic improvement with Cx43MP-NP treatment, especially over saline-injected controls, but also over eyes treated with Cx43MP alone.

**DISCUSSION**

This study was conducted to determine the effect of sustained Cx43MP release from NPs on the light-damaged rat retina in comparison with a single dose of native Cx43MP or with saline-injected control eyes. Nanoparticles allowed quick tissue distribution and slow release of mimetic peptide resulting in improved retinal function, preserved retinal morphology, and reduced inflammation when followed through to 2 weeks after a single treatment. A single injection of Cx43MP alone was also beneficial, but improvements were more modest.

Prior studies have shown that administration of Cx43MP decreased inflammation and oxidative stress in this light-damaged rat retina model, but two injections of the peptide were required (2 hours post onset and at the end of the 24-
proved effective, most likely owing to the prolonged peptide insult retina ischemia–reperfusion model these NPs also in a more acute (shorter hour light exposure period) in order to decrease the light-induced chorioretinal damage. In the present study, a single injection of Cx43MP-NPs improved retinal function over the long term. Drug bioavailability was attained using NPs as the delivery system. These are capable of transretinal passage after IVT injection and enable drug delivery into the RPE and choroid as shown in this study by the imaging of fluorescence-tagged peptides present in these layers within 30 minutes of administration. In this study, we cannot differentiate between transretinal passage and other passage mechanisms. It is possible that NPs were delivered to the choroid following endocytosis into Müller cells at the inner limiting membrane, followed by exocytosis at the external limiting membrane and into the interphotoreceptor matrix as has been previously proposed. In any case, administration of the Cx43MP-NPs 2 hours after initiation of intense light exposure was effective at reducing the initial wave of damage that is characteristic of this animal model. Progressive degradation of NPs then allowed slow release of Cx43MP to prevent photoreceptor loss and extensive retinal remodeling that are otherwise features of this disease model.

PLGA are biodegradable polymers extensively used in ocular studies and also in pharmaceutical drug delivery studies as drug carriers. Previous studies have shown that particles loaded with Cx43MP release the drug in a triphasic manner—an initial burst (49.5%), a long sustained period of slow release, and a final short burst when particles break down with Cx43MP release for up to 63 days. In a more acute (shorter insult) retina ischemia–reperfusion model these NPs also proved effective, most likely owing to the prolonged peptide release over time. Despite a slight delay in functional recovery compared with delivery of the drug in solution, the Cx43MP-NPs resulted in retinal structure and function protection. The long-term effect of slowly released Cx43 had an outcome similar to the effect of double injections of native Cx43MP in our previous study, suggesting a need to act on sequential critical damaging events that occur in this model. PLGA has Food and Drug Administration (FDA) approval for the delivery of some ocular drugs, which, based on our studies, combined with Cx43, should be applicable in humans.

In the long term, Cx43MP-NP treatment restored ERG function and immunolabeling with stress markers showing no alteration in glial reactivity. These results are consistent with data obtained in a previous, double-injection Cx43MP study. Both a-wave, reflecting photoreceptor function, and b-wave, representing mainly bipolar cell function in the inner retina, were spared. In this study, Cx43MP-NPs also improved the amplitude of rod and cone PIII and PII waveforms, which can be explained as neuroprotection of rod cells in the outer retina and rod bipolar cells and amacrine cells in the inner retina, respectively. A similar improvement in the rod pathway was associated with treatment of the light-damaged eye with mimic peptide in saline. Although the effect of Cx43MP on rod and cone pathways is still not clear, most of our experiments suggest that there is inflammation and Cx43 expression-mediated damage to the photoreceptors.

The choroid plays a key role in preventing retinal damage. We have found an increase in choroidal thickness soon after light damage that is associated with an increased infiltration of leukocytes. However, after 1 week, choroidal thickness decreased. This finding is consistent with findings of past studies indicating that the thinning of the choroid is associated with CD8+ cytotoxic T lymphocytes infiltrating toward the retina and choroid interface in mice and albino rats. This infiltration of T lymphocytes could exert deleterious effects on the choroid, RPE, and retina environments. In this study, reduced Cx43 levels were seen as in previous studies, indicating that there is a reduced inflammatory response (the peptide does not in itself reduce Cx43 expression). Furthermore, the hallmarks of retinal stress and upregulation of GFAP in astrocytes and Müller cells (signs of reactive gliosis) were diminished with Cx43MP-NP treatment. It appears that the mimetic peptide reduces the inflammatory process that leads to retinal damage in this model.
FIGURE 7. Cx43MP-NP–treated rat eyes showed less Cx43 immunoreactivity in the choroid (C) compared to native Cx43MP- (B) and saline-injected rats (A). CD45-immunolabelled cells were fewer in number in the choroid of Cx43MP-NP–treated rats (F) compared to native Cx43MP- (E) and saline-injected rats (D). GFAP immunoreactivity did not increase in the retina of Cx43MP-NP–treated rats (I) compared with native Cx43MP- (H) or saline-injected rats (G). Scale bar: 50 μm.

FIGURE 6. Quantification of retinal and choroidal thickness before light damage and up to 2 weeks post light damage in saline-, Cx43MP-, or Cx43MP-NP–treated eyes. The graphs show the changes in thickness of the ONL (A) and choroid (B) with n = 6 per group. Data are expressed as mean ± standard error of the mean. Significant values are indicated with asterisks: *P < 0.05; **P < 0.01; ***P < 0.001.
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