Direct Effect of Sodium Iodate on Neurosensory Retina

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PURPOSE. To systematically characterize the effects of NaIO₃ on retinal morphology and function.

METHODS. NaIO₃ at 10, 20, or 30 mg/kg was administered by retro-orbital injection into adult C57BL/6J mice. Phenotypic and functional changes of the retina were assessed at 1, 3, 5, and 8 days postinjection by fundus imaging, optical coherence tomography (OCT), ERG, and histology. Direct NaIO₃ cytotoxicity on ARPE-19 and 661W cells was quantified using lactate dehydrogenase (LDH) apoptosis assay. Effect of NaIO₃ on RPE and photoreceptor gene expression was assessed in vitro and in vivo by quantitative PCR.

RESULTS. While little to no change was observed in the 10 mg/kg NaIO₃-injected group, significant retinal anomalies, such as RPE atrophy and retinal thinning, were observed in both 20 and 30 mg/kg NaIO₃-injected groups. Gene expression analysis showed rapid downregulation of RPE-specific genes, increase in heme oxygenase 1 expression, and induction of the ratio of *Bax* to *Bcl-2*. Electroretinographic response loss and photoreceptor gene repression preceded gross morphological changes. High NaIO₃ toxicity on 661W cells was observed in vitro along with reactive oxygen species (ROS) induction. NaIO₃ treatment also disrupted oxidative stress, phototransduction, and apoptosis gene expression in 661W cells. Exposure of ARPE-19 cells to NaIO₃ increased expression of neurotrophins and protected photoreceptors from direct NaIO₃ cytotoxicity.

CONCLUSIONS. Systematic characterization of changes associated with NaIO₃ injection revealed a large variability in the severity of toxicity induced. Treatment with >20 mg/kg NaIO₃ induced visual dysfunction associated with rapid suppression of phototransduction genes and induced oxidative stress in photoreceptors. These results suggest that NaIO₃ can directly alter photoreceptor function and survival.

Keywords: sodium iodate, retinal degeneration, cytotoxicity, photoreceptor, oxidative stress

Retinal degeneration, a characteristic of neurodegenerative diseases such as age-related macular degeneration (AMD) or retinitis pigmentosa, can lead to severe visual impairment and eventually blindness. Millions of people worldwide suffer varying degrees of irreversible vision loss because of these untreatable, degenerative eye disorders. To understand the molecular mechanisms of disease induction and progression, and to develop therapeutic strategies for vision preservation in these patients, extensive research has been done using a large number of both inherited and induced animal models.^{1,2} However, the mechanisms of formation/development of these diseases remain poorly understood. Systematic delivery of sodium iodate (NaIO₃), a stable oxidizing agent, has been proven to be an effective way to induce retinal degeneration associated with regional loss of retinal pigment epithelium (RPE) recapitulating some of the morphological features of geographic atrophy.^{3–5} NaIO₃ retinal toxicity has been demonstrated in many different mammalian species, including sheep,⁶ rabbit,^{7,8} rat,^{9–11} and mouse.^{12–14} In the retina, NaIO₃ is thought to target primarily the RPE cells,^{6,15} inducing their necrosis, followed by choriocapillaris atrophy¹⁶ and panretinal degeneration.^{10,15} Despite the fact that the NaIO₃-dependent model of retinal degeneration has been previously investigated^{9,17–19} and used for the evaluation of neuroprotective treatments,^{11,20} the acute effect of NaIO₃ toxicity

remains poorly characterized. Most studies used relatively high doses of NaIO₃ (from 50 to 100 mg/kg) and reported rapid RPE damage characterized by defragmentation and loss of RPE cell nuclei at ~2 to 12 hours,^{15,21} followed by disorganization of the rod outer segment discs at ~6 hours¹⁰—results consistent with the concept that RPE cells are the primary target of NaIO₃ toxicity. However, this mechanism was recently challenged by a study showing that high doses of NaIO₃ are able to directly induce intraretinal neuron injury.²² Because the model of NaIO₃ ocular toxicity is widely used to assess the efficacy of cell replacement therapy through transplantation,^{13,23,24} it is important to clarify whether and/or how NaIO₃ affects different retinal cells, whether or not this effect is direct or indirect, and, specifically, how NaIO₃ administration alters the gene expression profiles of photoreceptor (PR) and RPE cells. To date no report has characterized the early retinal function changes together with a precise, sequential, morphological observation in order to more precisely define this particular model of retinal degeneration. In the present study, we conducted a systematic characterization of the early effects of low NaIO₃ concentration (<30 mg/kg) on the morphology, function, and gene expression of the murine retina, and demonstrated evidence of direct NaIO₃ toxicity on PR cells both in vivo and in vitro.

TABLE 1. List of Genes Studied

Symbol	Name	Function
<i>Hprt1</i>	Hypoxanthine phosphoribosyl-transferase 1	Housekeeping
<i>B2m</i>	Beta-2-microglobulin	Housekeeping
<i>Rpe65</i>	Retinal pigment epithelium-specific protein 65kDa	Visual cycle
<i>Opn1mw</i>	Opsin 1 (cone pigments), medium wave sensitive	Color vision
<i>Pde6b</i>	Phosphodiesterase 6B, cGMP-specific, rod, beta	Phototransduction
<i>Rho</i>	Rhodopsin	Phototransduction
<i>Prph2</i>	Peripherin 2	Phototransduction
<i>Arr3</i>	Arrestin 3	Phototransduction
<i>Gnat2</i>	Guanine nucleotide binding protein, alpha transducing activity polypeptide 2	Phototransduction
<i>Cnga1</i>	Cyclic nucleotide gated channel alpha 1	Phototransduction
<i>Cnga3</i>	Cyclic nucleotide gated channel alpha 3	Phototransduction
<i>Cngb3</i>	Cyclic nucleotide gated channel beta 3	Phototransduction
<i>Rora</i>	RAR-related orphan receptor A	Transcription factor for photoreceptor development and functions
<i>Rorb</i>	RAR-related orphan receptor B	Transcription factor for photoreceptor development and functions
<i>Bax</i>	Bcl2-associated X protein	Proapoptotic
<i>Bcl2</i>	B-cell CLL/lymphoma 2	Antiapoptotic
<i>Gpx1</i>	Glutathione peroxidase 1	Antioxidant
<i>Gpx4</i>	Glutathione peroxidase 4	Antioxidant
<i>Sod2</i>	Superoxide dismutase 2, mitochondrial	Antioxidant
<i>Hmox1</i>	Heme oxygenase 1	Heme catabolism (inducible)
<i>Hmox2</i>	Heme oxygenase 2	Heme catabolism (constitutive)
<i>Txn2</i>	Thioredoxin 2	Antioxidant
<i>Pspn</i>	Persephin	Neurotrophic factor
<i>Ctf1</i>	Cardiotrophin 1	Neurotrophic factor
<i>Bdnf</i>	Brain-derived neurotrophic factor	Neurotrophic factor
<i>Ngf</i>	Nerve growth factor	Neurotrophic factor
<i>Vegf</i>	Vascular endothelial growth factor	Neurotrophic factor
<i>Pedf</i>	Pigment epithelium-derived factor	Neurotrophic factor
<i>NT4</i>	Neurotrophin-4	Neurotrophic factor
<i>Gdnf</i>	Glial cell-derived neurotrophic factor	Neurotrophic factor
<i>Tgfb1</i>	Transforming growth factor beta 1	Neurotrophic factor
<i>Igf1</i>	Insulin-like growth factor 1	Neurotrophic factor
<i>Lif</i>	Leukemia inhibitory factor	Neurotrophic factor

MATERIALS AND METHODS

Animals

Six- to eight-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbour, ME) were used in this study. The mice were housed in a standard laboratory environment and maintained on a 12-hour light-dark cycle at 21°C. All experimental procedures involving animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local institutional animal care and use committee.

NaIO₃ Injection

A sterile 1% NaIO₃ solution was freshly prepared from solid NaIO₃ (S4007; Sigma St. Louis, MO) diluted in 0.9% sterile sodium chloride. Mice were anesthetized with isoflurane/oxygen and injected via the orbital venous plexus with three different doses of NaIO₃: 10, 20, and 30 mg/kg body weight. Control mice were injected with similar volumes of physiological saline.

Electroretinography (ERG)

Following 6 hours of dark adaptation, mice were anesthetized and prepared under red light. Eyes were dilated with 1% tropicamide solution (Bausch and Lomb, Tampa, FL). Ground

and reference electrodes were placed subdermally near the tail and in the forehead, respectively. Scotopic and photopic ERGs were recorded from gold loop electrodes placed on the cornea using the Espion E³ machine (Diagnosys, Boxborough, MA). Scotopic responses were elicited using a graded series of flashes from -4 to $2 \log \text{cd}^*\text{s/m}^2$, with increasing interflash intervals for each step. Before photopic ERG recordings, mice were light adapted using a series of bright white flashes. Photopic responses were elicited using a graded series of flashes from -1 to $3 \log \text{cd}^*\text{s/m}^2$ presented on a constant $30 \text{cd}^*\text{s/m}^2$ amber background, with increasing interflash intervals for each step. All ERG data were collected at the same time of day. The a-wave amplitude was measured from the baseline to the trough of the first negative wave; the b-wave amplitude was measured from the trough of the a-wave to the peak of the first positive wave or, if the a-wave was absent, from baseline to the peak of the first positive wave. The scotopic a-wave implicit time and the ratio of b- to a-wave were calculated at $500 \text{cd}^*\text{s/m}^2$.

Spectral Domain Optical Coherence Tomography (SD-OCT) and Fundus Photography

Ultrahigh-resolution SD-OCT images centered on the optic nerve head (ONH) were acquired on live anesthetized animals using an SD-OCT system (Biophtigen, Morrisville, NC). Fundus images were obtained using the Micron III retinal imaging system (Phoenix Research Laboratories, Pleasanton, CA).

TABLE 2. Primer Sequences for qPCR Analysis

Symbol	Forward Primer (5'–3')	Reverse Primer (5'–3')
<i>Hprt1</i>	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAAACTT
<i>B2m</i>	GTGGCCCTTAGCTGTGCTCG	ACCTGAATGCTGGATAGCCTC
<i>Opn1mw</i>	TGCAAGCCCTTTGGCAATG	GCCGTCCATATAGCAGCCC
<i>Pde6b</i>	TGAAGATGAAGATGTTTTACG	CTCTGCGTGTCTCAGATTG
<i>Rbo</i>	GCCACACTTGGAGGTGAAATC	AAGCGGAAGTTGCTCATCG
<i>Prph2</i>	CGGGACTGGTTCGAGATTC	ATCCACGTTGCTCTTGATGC
<i>Arr3-2</i>	CCATTGATGGAGTCGTCTCTT	CTTTGCGAAATGTCAGACCA
<i>Gnat2</i>	CTGTCAAGCTGCTGTTGCTC	CTAGGCACTCTTCGGGTGAG
<i>Cnga1</i>	ACTCGTACAAAAGGCGAGGAC	CTTTGTTGCTGCTGTTGTTGAC
<i>Cnga3</i>	CCTTCGATAAGCAGGCATTG	GGTGTTCACCTTTGCCATCT
<i>Cngb3</i>	GACTGGGAAAAGAAAGGCCAG	AGGTTGTGTAGGTGGGCATC
<i>Rora</i>	AGGCAGAGCTATGCGAGC	TTCTTGACGATTTGTCTCCA
<i>Rorb</i>	ATGCCAGCTGATGGAGTTCT	TAGTCTCCCGGATAACAATG
<i>Bax</i>	GATCAGCTCGGGCACTTTAG	TTGCTGATGGCAACTTCAAC
<i>Bcl2</i>	CCGGGAGAACAGGGTATGATAA	CCCACTCGTAGCCCCCTCG
<i>Gpx1</i>	GTCTGGGACCTCGTGGACT	CAGTCCGGACGTACTTGAGG
<i>Gpx4</i>	CCGTCTGAGCCGCTTACTTA	GCTGAGAATTCGTGCATGG
<i>Sod2</i>	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
<i>Hmox1</i>	GAGCCTGAATCGAGCAGAAC	CCTTCAAGGCCTCAGACAAA
<i>Hmox2</i>	AGCACATGACCGAGCAGAAAA	GCTCCGTGGGGAAATATAAGGG
<i>Txn2</i>	CACACAGACCTTGCCATTGA	ATCCCCACAACTTGTCAC
<i>PSPN</i>	GTCTGAACAGGTGGCAAAGG	ACAGGGTCAGGCTCCACAG
<i>CTF1</i>	CGGAGGGAGGGAAGTCTG	AGGCTGTGTGTCTGACGGAT
<i>VEGFA</i>	GGGCAGAATCATCAGAAAGTG	ATTGGATGGCAGTAGCTGCG
<i>PEDF</i>	TATCACCTTAACCAGCCTTTCATC	GGGTCCAGAACTTGTCAATG
<i>NT4</i>	CCTCCGCCAGTACTTCTTTGA	CCGGGCCACCTTCCCTC
<i>GDNF</i>	GACTCAAATATGCCAGAGGTTATCC	GGTGGCTTGAATAAAATCCATGAC

Rpe65, BDNF, and NGF primers were ordered from SABiosciences (Valencia, CA).

FIGURE 1. Color fundus photographs of C57BL/6J mice treated with increasing doses of NaIO₃. Top, middle, bottom: 10, 20, 30 mg/kg NaIO₃ groups, respectively. Left to right: fundus at days 1, 3, 5, and 8, respectively. Estimated scale bar based on mouse optic nerve diameter: 220 μ m.

FIGURE 2. OCT of C57BL/6J mice treated with increasing doses of NaIO₃. *Top, middle, bottom:* 10, 20, 30 mg/kg NaIO₃ groups, respectively. *Left to right:* days 1, 3, 5, and 8, respectively. *Arrows* point to hyperreflective spots in both the vitreous (green) and the retina (yellow). *Red double-headed arrows* mark total retinal thickness. *Scale bar:* 100 μ m.

Histology and Immunofluorescence

For histology, whole eyes were enucleated, fixed in 10% formalin, and prepared for paraffin embedding; then 3- μ m-thick slides were sectioned. The thickness of the retinal layers and nuclei/row numbers were measured on hematoxylin- and eosin-stained slides. For each section, photographs were taken at a distance of 1000 to 1500 μ m from the optic nerve (midperiphery). Three measurements were made per photograph and averaged. For immunohistochemistry of the posterior eye cups, the eyes were fixed in 4% paraformaldehyde overnight, and the neural retina was dissected out. The remaining RPE cell-containing eye cups were flattened by making long radial cuts and incubated in a blocking buffer containing 1% BSA, 3% goat serum in 1 $\times$ PBS (vol/vol) and 0.1% Triton X-100 for 2 hours at room temperature (RT). Retinal pigment epithelium-choroid flat mounts were incubated for 2 hours at RT with rabbit anti-RPE65 primary antibody (1:300; a gift from Michael Redmond, National Eye Institute, Bethesda, MD). The secondary antibody Alexa Fluor 488-conjugated anti-rabbit antibody (A11034, 1:500; Invitrogen, Carlsbad, CA) was added with Alexa 586-phalloidin (1:100; Invitrogen) to stain F-actin. After mounting, images were taken with an Axioscope Mot 2 microscope (Carl Zeiss Meditec, Inc., Dublin, CA).

661W and ARPE-19 Cell Cultures In Vitro

Murine 661W PR-derived cells were plated at 15,000 cells/cm² in 12-well plates and cultured in regular Dulbecco's modified Eagle's medium (DMEM) medium containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) for 36 hours before the experiments were performed. The human RPE cell line ARPE-19 was expanded in DMEM plus 10% FBS in plastic culture dishes. Once cells reached confluence, medium

was replaced with RPE differentiation medium (DMEM plus 1% FBS) for another 5 days before the experiments were performed. For conditioned medium collection, ARPE-19 cell cultures grown to confluence in 48-well plates, and LDH was measured after 250, 500, 750, 1000, or 3000 μ g/mL NaIO₃ for 24 hours in serum-free, phenol-free medium. After collection, the supernatants were centrifuged at 250g for 10 minutes to remove suspended cells. The supernatants were then assayed at a 1:4 dilution for a final volume of 100 μ L/well. Absorbance was measured at 490 nm using a microplate reader (Synergy2; BioTek, Winooski, VT). For each experiment, each condition was replicated six times; and background levels, determined using media not exposed to cells, were subtracted from absorbance values obtained for each condition. Lactate dehydrogenase values were normalized to the mean maximal value (defined as 100%) in parallel cultures exposed to 2% Triton X-100 for 3 hours—conditions that resulted in the death of all cells.

Lactate Dehydrogenase Assay

Cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in cell culture supernatants by a colorimetric cell death assay (Roche Molecular Diagnostics, Pleasanton, CA). The LDH assays were performed using 661W or ARPE-19 cell cultures grown to confluence in 48-well plates, and LDH was measured after 250, 500, 750, 1000, or 3000 μ g/mL NaIO₃ for 24 hours in serum-free, phenol-free medium. After collection, the supernatants were centrifuged at 250g for 10 minutes to remove suspended cells. The supernatants were then assayed at a 1:4 dilution for a final volume of 100 μ L/well. Absorbance was measured at 490 nm using a microplate reader (Synergy2; BioTek, Winooski, VT). For each experiment, each condition was replicated six times; and background levels, determined using media not exposed to cells, were subtracted from absorbance values obtained for each condition. Lactate dehydrogenase values were normalized to the mean maximal value (defined as 100%) in parallel cultures exposed to 2% Triton X-100 for 3 hours—conditions that resulted in the death of all cells.

Measurement of Reactive Oxygen Species (ROS) Production

The levels of intracellular ROS were measured using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) following the manufacturer's instructions (C6827; Invitrogen). In brief, 661W cells

FIGURE 3. Hematoxylin and eosin staining of paraffin-embedded retinal cross sections of NaIO₃-treated C57BL/6J mice at different time points. *Top, middle, bottom:* 10, 20, 30 mg/kg NaIO₃-injected group, respectively. *Left to right:* days 1, 3, 5, and 8, respectively. Starting from day 3, swelling and bundling of the RPE cells (*arrowheads*) associated with macrophage migration and photoreceptor disorganization (*arrows*) were observed. Significant loss of RPE cells and ONL thinning were detected at days 5 and 8 (*double-headed arrows*). Scale bar: 100 μ m.

were incubated with 10 μ M CM-H₂DCFDA for 30 minutes at 37°C, then washed in serum-free, phenol red-free medium, before the addition of 250 μ g/mL NaIO₃. Twenty-four hours later, DCF fluorescence was measured and corrected for cell number.

RNA Isolation and Quantitative PCR (qPCR)

Total RNA from RPE cells was isolated using the simultaneous RPE cell isolation and RNA stabilization (SRIRS) method.²⁵ Total RNA from the neuroretina was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). Residual DNA was removed by treatment with DNase I (Qiagen) during the extraction procedures. Ribonucleic acid (1 μ g) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON). Real-time qPCR was performed using the

SYBR green Master Mix and the Lightcycler 480 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. To ensure accurate gene quantification, the PCR data were normalized to the mean of multiple internal control genes (*Hprt1* and *B2m*),²⁶ and gene expression was calculated according to the $\Delta\Delta C_T$ method. Each sample was run in triplicate, and each experiment included three non-template control wells. The genes investigated by qPCR are listed in Table 1, and the respective primers used are listed in Table 2.

Statistical Methods

Statistical comparisons were made by Student's two-tailed *t*-test. Significance was set at $P < 0.05$, and the data are presented as the mean $\pm$ SEM unless otherwise specified (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

FIGURE 4. Immunofluorescence of RPE/choroid flat mounts 8 days post- NaIO_3 injection. F-actin was stained with phalloidin (red) and RPE65 (green). (A, B) Saline-injected control eyes displayed normal RPE morphology with evenly distributed RPE65 expression in the central (A) and peripheral retina (B). (C, D) 10 mg/kg NaIO_3 -treated RPE flat mounts were indistinguishable from saline control (E, F). In 20 mg/kg NaIO_3 -treated animals, RPE cells appeared highly disorganized in the central retina (E). Significantly enlarged RPE cells characterized by strongly reduced RPE65 expression were observed in the peripheral retina (F). OD, optic disc. Scale bar: 100 μm .

RESULTS

Morphological Changes in Retinal Tissue After NaIO_3 Administration

In order to evaluate the ocular effects of a low-dose NaIO_3 injection (i.e., 10 mg/kg), fundus photographs at days 1, 3, 5, and 8 were collected. No obvious changes were found for the 10 mg/kg group at any time point evaluated (Fig. 1, top) when compared to saline-injected controls (see Supplementary Fig. S1A). Higher doses (i.e., 20 and 30 mg/kg) were associated with progressive retinal degeneration characterized by significant pigmentary changes of the RPE layer at day 3 (Fig. 1, middle and bottom). The RPE pigmentary disruption and

atrophy quickly became more widespread in the following days. Retinal vasculature and optic nerve tissue appeared to remain normal at all examined time points and concentrations.

Spectral domain OCT evaluation confirmed a lack of obvious anatomical changes in mice treated with the lower doses of NaIO_3 (Fig. 2) when compared with control animals (see Supplementary Fig. S1B). However, higher concentrations of NaIO_3 injected were associated with loss of retinal lamination at day 3 and significant outer retina damage by day 5. Interestingly, hyperreflective opacities were observed in both the vitreous and neuroretina for both the 20 and 30 mg/kg groups as early as 1 day postinjection. Inside retinal tissue, these hyperreflective foci are likely to represent glia cell migration or the presence of phagocytic retinal cells. The

FIGURE 5. Altered outer retina thickness following NaIO_3 administration. Outer nuclear layer ([A], ONL), photoreceptor inner and outer segment layer thicknesses ([B], IS/OS), and ONL nuclei/row numbers ([C]) were quantified on histological sections. Asterisks indicate the statistically significant deviation of thickness from the control. $**P < 0.01$.

origin of the vitreal opacities is unknown but could represent migratory inflammatory cells. However, no sign of acute ocular inflammation was observed by fundus photography (Fig. 1). Loss of lamination was also accompanied by significant total retinal thinning, with prominent loss of the outer nuclear layer (ONL) and the outer segment (OS) layers by day 8 (Fig. 2).

Results from fundus and OCT studies were confirmed by histological examination of ocular specimens. Retinal pigment epithelium swelling and migration of pigmented cells into the

OS layer were observed for both the 20 and 30 mg/kg-injected groups as early as day 3 (Fig. 3). Cystic spaces in the inner nuclear layer were also observed; however, they were not present in either the control retinas (see Supplementary Fig. S1C) or the lower NaIO_3 -dose groups. By day 5, disorganization of PRs and significant thinning of the entire retina, especially the ONL, were observed along with large regions of complete RPE loss, which had expanded over time (Fig. 3).

To better characterize changes of the RPE monolayer, we used immunostaining for F-actin and RPE65 on flat-mounted posterior eye cups. There were no observable changes in the organization or RPE65 expression of the RPE layer of mice treated with 10 mg/kg NaIO_3 (Figs. 4A–D). Retinal pigment epithelium cells presented their normal cuboidal shape and even distribution, with robust expression of RPE65 in both the central and peripheral retina—similar to the appearance of RPE cells in normal saline-injected mice. Day 8 following the 20 mg/kg NaIO_3 injection was associated with dramatic RPE atrophy in large areas of the retina. In the peripheral region, many of the remaining cells appeared significantly enlarged and disorganized, and had lost RPE65 expression (Figs. 4E, 4F). Groups treated with 30 mg/kg NaIO_3 demonstrated similar results (data not shown).

Characterization of PR Degeneration and Functional Defects Following NaIO_3 Treatment

Quantification of ONL, inner and outer segment layer thicknesses (IS/OS), and ONL nuclei/row demonstrated significant and progressive PR degeneration by day 5 in mice treated with either 20 or 30 mg/kg NaIO_3 ; this led to over ~50% thinning of the ONL by day 8 (Fig. 5). Retinal function changes were examined by scotopic and photopic ERG 1 week before the NaIO_3 injections at baseline and at 1, 3, 5, and 8 days postinjection. As we observed through our anatomical examination, the 10 mg/kg injection group maintained a normal ERG response (Fig. 6). Surprisingly, however, an attenuated ERG response, characterized by a reduction of both scotopic and photopic b-wave amplitudes, was observed as early as 1 day post- NaIO_3 injection in both the 20 and 30 mg/kg groups, preceding any morphological indication of PR damage (Figs. 6B, 6C). These rapid ERG changes were also characterized by a reduction of the ratio of b to a amplitude (Fig. 6D) and a delayed a-wave implicit time (Fig. 6E). Moreover, visual function decreased over the experimental time course and, by day 8, both scotopic (a- and b-wave) and photopic (b-wave) responses were near zero (Fig. 6).

Induction of Oxidative Stress and Phototransduction Gene Dysregulation by NaIO_3

The acute effect of NaIO_3 on the expression profile of oxidative stress, cell death, and phototransduction-relative genes was measured using qPCR on isolated RPE and neuroretina (free of RPE) samples 1 and 3 days postinjection of 10 or 20 mg/kg NaIO_3 . Treatment with 20 mg/kg NaIO_3 was associated with a dramatic loss of *Rpe65* expression within 24 hours, followed by repression of both *Mitf* and *Otx2* expression at day 3 (Fig. 7A). This acute NaIO_3 effect on *RPE65* gene expression was also accompanied by the induction of *Hmox1* (iron-generated ROS antioxidant) expression and increase of ratio of *Bax* to *Bcl2* (indicative of increased apoptosis) at day 1 (Figs. 7C, 7D), a time point at which no major RPE morphological changes were observed (Figs. 1, 3). Two other markers of oxidative stress, *Txn2* and *Gpx4*, were also found at slightly decreased levels at day 3 in the RPE cells, while other ROS markers, such as *Sod2*, showed no significant changes (data not shown). Gene analysis of the neuroretina

FIGURE 6. Longitudinal analysis of visual function of mice treated with NaIO_3 . (A) Scotopic a-wave recorded at $500 \text{ cd}^*/\text{m}^2$. (B) Scotopic b-wave recorded at $0.1076 \text{ cd}^*/\text{m}^2$. (C) Saturating photopic b-wave recorded at $10^3 \text{ cd}^*/\text{m}^2$. Significant reduction of all ERG responses was observed at day 1 in both the 20 and 30 mg/kg NaIO_3 groups. Both a- and b-waves continued to decrease over time, and by day 8, all ERG responses were abolished. No functional change was detected for the 10 mg/kg NaIO_3 group at any time point. At day 1, a significant reduction of the ratio of b to a amplitude (D) and increase of the a-wave implicit time (E) in animals treated with 20 and 30 mg/kg NaIO_3 were also observed. The results are presented as mean $\pm$ SEM. $\dagger$, $\ddagger$, or $*$ $P < 0.05$, $\ddagger\dagger$, $\ddagger\dagger\dagger$, or $**P < 0.01$, $\ddagger\dagger\dagger\dagger$, $\ddagger\dagger\dagger\dagger\dagger$ or $***P < 0.001$.

revealed a rapid dysregulation of many phototransduction genes, such as *Rho*, *Opn1mw*, and *Pde6b*, following treatment with 20 mg/kg NaIO_3 (Fig. 7B). Similarly, as observed in the RPE cells, both *Hmox1* expression and the ratio of *Bax* to *Bcl2* were strongly induced in neuroretina treated with 20 mg/kg NaIO_3 . However, no changes in *Gpx4* and *Sod2* expression were detected (data not shown). Intriguingly, treatment with 10 mg/kg NaIO_3 was associated with induction of *Rpe65*, *Mitf*, and *Otx2* expression at day 3 (Fig. 7A). The same induction trend was observed in the neuroretina with the phototransduction genes *Rho*, *Pde6b* (Fig. 7B), and *Cnga1* (data not shown).

Characterization of a Direct Toxic Effect of NaIO_3 on PRs

The observation of a major alteration of the phototransduction pathway in the neuroretina of animals treated with $>20 \text{ mg/kg}$ NaIO_3 , together with the identification of ERG anomalies, suggests that NaIO_3 could exert a direct toxic effect on PRs. This is supported by our observations that these changes occurred at days 1 to 3 (Figs. 6, 7), preceding a measurable PR loss at day 5 (Fig. 5). To test this hypothesis, we utilized the murine cone-like 661W cell line and characterized the effect of experimentally relevant doses of NaIO_3 . In a serum-containing culture system, NaIO_3 treatment led to dose-dependent 661W cell death, as measured by LDH release, and was detectable only after high doses of NaIO_3 (above $750 \mu\text{g/mL}$). However, significant cytotoxicity was detected in the serum-free culture

system at doses as low as $250 \mu\text{g/mL}$ (Fig. 8A). This NaIO_3 -dependent cell death was also associated with a robust increase in ROS production (Fig. 8B). Similar to our observations in vivo, gene expression analysis revealed a rapid increase of the oxidative stress-related genes *Hmox1* and *Gpx1* along with a strong induction of the ratio of *Bax* to *Bcl2* (Fig. 8E). Furthermore, most of the cone phototransduction-related genes tested were significantly downregulated (Fig. 8D), which is also consistent with our in vivo data.

In contrast, results of similar experiments using the human RPE cell line ARPE-19 indicated that RPE cells were less vulnerable to NaIO_3 -induced cell death than were 661W cells. Moreover, only high concentrations of NaIO_3 ($>750 \mu\text{g/mL}$) induced a significant, but low, cytotoxicity, although an excessive concentration ($3000 \mu\text{g/mL}$) led to 80% cell death (Fig. 9A). To identify if the paracrine interaction between RPE cells and PRs could affect PR sensitivity to NaIO_3 in a context similar to in vivo conditions, we exposed 661W cells to conditioned media (CM) collected from ARPE-19 cells pre-cultured with or without $250 \mu\text{g/mL}$ NaIO_3 . Interestingly, conditioning the NaIO_3 -containing media on ARPE-19 cells significantly decreased (by 50%) NaIO_3 toxicity on 661W cells (Fig. 9B). Through gene expression analysis, we observed that only a few known neurotrophic factors released from RPE cells—cardiotrophin 1 (CTF1), persephin (PSPN), and brain-derived neurotrophic factor (BDNF)—were significantly increased after NaIO_3 treatment (Fig. 9C). Expression of other neurotrophic factors analyzed, which included *NGF*, *VEGF*,

FIGURE 7. Gene expression changes in mouse RPE and neuroretina following NaIO_3 treatment. RPE cells and neuroretina were isolated from mice treated with 10 or 20 mg/kg NaIO_3 for 1 or 3 days. (A) Expression of *Rpe65*, *Mitf*, and *Otx2* from mouse RPE cells. (B) Expression of phototransduction-related genes from mouse neuroretinas. (C, D) Expression of *Hmox1* (C) and the apoptosis marker ratio of *Bax* to *Bcl2* (D) in mouse RPE cells and neuroretinas, at days 1 and 3, respectively. The results are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. RE, relative expression.

PEDF, *N4*, *GDNF* (Fig. 9C), *TGF β 1*, *IGF1*, and *LIF* (data not shown), were not affected by NaIO_3 treatment.

DISCUSSION

In this study, we have reassessed the mechanisms of NaIO_3 -induced retinal damage model in pigmented C57BL/6J mice. To this end, we examined the effects of different doses and postinjection time points on several aspects of both retinal structure and function. Our data showed that even low doses of NaIO_3 (20 or 30 mg/kg) led to significant loss of retinal function at day 1, followed by local morphological changes in RPE cells and PRs at day 3, and significant ONL thinning at day 5. Thus, we were able to detect functional deficits in the absence of overt morphological changes.

Although it is difficult to compare results of the current study to previously published reports, due to differences in the

source of NaIO_3 , methods of administration, animals (type, strain, and age), and time points analyzed after injection, trends in changes can still be meaningfully compared. The retinal toxicity following administration of high doses of NaIO_3 (30–100 mg/kg) was characterized by whole retina thinning associated with degeneration of RPE, rods, and cones, leading to the complete ablation of the outer nuclear and RPE layers.²⁷ Under these conditions, cell toxicity was too rapid to allow unequivocal identification of the mechanisms leading to PR damage.

NaIO_3 is traditionally considered to be highly specific to RPE cells. However, this concept was challenged by a recent study demonstrating that NaIO_3 injection in the rat leads to rapid and direct intraretinal neuron injury.²² In our study, we observed acute PR dysfunction before notable changes to the RPE and ONL layers. Such function loss could be due to several factors. First, NaIO_3 treatment could cause synaptic damage, as a defect in synaptic transmission between the PRs and

FIGURE 8. Effect of NaIO₃ on 661W cells in vitro. (A) 661W cell death secondary to NaIO₃ treatment was quantified by assessing LDH activity in serum-containing and serum-free culture media. NaIO₃ toxicity appears strongly dependent on serum level, as serum-free media condition elicited a much higher LDH release. (B) 24-hour treatment of 661W cells with 250 µg/mL NaIO₃ in a serum-free condition was also associated with increased ROS production. (C, D) Gene expression analysis on 661W cells treated with 250 µg/mL NaIO₃ showed upregulation of oxidative stress (C) and downregulation of phototransduction-related genes (D). (E) Expression ratio of *Bax* to *Bcl2* was strongly induced by NaIO₃ treatment. The results are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to controls. RE, relative expression.

depolarizing bipolar cells can lead to a diminished ERG b-wave.²⁸ This idea is supported by a recent study using SD-OCT to characterize early changes associated with injection of high doses of NaIO₃ (40 mg/kg) in the rat and demonstrating a rapid

swelling of PR outer segments due to hydropic change,⁹ although no significant change of the inner plexiform layer (IPL), where the synapses are located, was observed.⁹ In our study, we were unable to accurately measure IPL thickness due

FIGURE 9. Effect of NaIO₃ on RPE cell neurotrophic function. (A) ARPE-19 cells are resistant to direct NaIO₃ cytotoxicity, as only concentrations over 750 µg/mL are associated with increased cell death. (B) Conditioned medium was transferred from either control ARPE-19 cells or 24-hour 250 µg/mL NaIO₃-treated ARPE-19 cells onto 661W cells for 24-hour culture, and comparison was made between 661W cells cultured with regular serum-free medium, medium with NaIO₃, and conditioned medium from either ARPE-19 cells or NaIO₃-treated ARPE-19 cells. CM, conditioned media collected from 24-hour ARPE-19 culture; CM+NaIO₃, conditioned media collected from 24-hour ARPE-19 culture treated with 250 µg/mL NaIO₃. (C) Expression of neurotrophic factors was significantly upregulated in ARPE-19 cells after 250 µg/mL NaIO₃ treatment at 24 hours. Asterisks indicate the statistically significant deviation of amplitude from the control. The results are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. RE, relative expression.

to the ONL disorganization and loss of uniformity at the IPL, but swelling in the inner retina was clearly observed in histological sections after NaIO₃ treatment (Fig. 3). However, our observations of a rapid reduction of the ratio of b to a amplitude following NaIO₃ injection is in agreement with a defective synaptic transmission to the second-order neurons. Secondly, NaIO₃ could directly alter PR function and survival. We tested this hypothesis by studying both RPE cells and PRs in vivo and in vitro, with a special emphasis on oxidative stress and phototransduction processes. We observed that rhodopsin, opsin, Cnga1, and Pde6b were strongly downregulated secondary to NaIO₃ treatment both in vivo and in vitro. As these genes are critically involved in the visual function of the PR, such dysregulation might be sufficient to cause the early ERG response loss with the lengthened a-wave implicit time observed in NaIO₃-treated mice.

We also observed an induction of the oxidative stress-related gene *Hmox1* in PRs by 7-fold in vivo (Fig. 7C) and 2-fold in vitro (Fig. 8C). This result was further supported by the accumulation of ROS in 661W cells after NaIO₃ exposure. Involvement of excessive generation of ROS and induction of oxidative stress has been considered to be a major factor in PR apoptosis.^{29,30} Indeed, we measured a significant, directly toxic effect of NaIO₃ on 661W cells at a concentration (250 µg/mL) similar to that used in vivo (20 mg/kg or 342 µg/mL in blood). Interestingly, serum content in the culture media strongly affected the sensitivity of PR to NaIO₃, as serum-free conditions were associated with much higher percentages of cell death. It has been previously shown that FBS-containing culture medium can lessen the oxidative injury caused by the transition metal ion in pheochromocytoma cells,³¹ likely due to the presence of many growth factors able to alter cell antioxidant responses. Together, our findings suggest that NaIO₃-induced oxidative stress in the neuroretina is able to dysregulate phototransduction gene expression and lead to subsequent PR cell death.

In our study, the 10 mg/kg NaIO₃-injected group did not display any morphological or functional changes; however, gene expression analysis revealed that this low dose led to some modulation of RPE-specific (*Rpe65*, *Mitf*) and antioxidant (*Hmox1*) genes. In vivo, RPE cells are particularly exposed to oxidative stress due to their location between the choriocapillaris and PRs, high levels of light irradiation, and abundance of photosensitizers such as lipofuscin. Retinal pigment epithelium cells' endogenous antioxidant capability as a defense system allows them to withstand such oxidative conditions.³² Moreover, induction of antioxidant enzymes secondary to oxidative stress in RPE has been well documented.^{33,34} However, the effect of oxidative stress on RPE-specific genes, especially *RPE65*, is less well understood. Treatment of ARPE-19 with cytotoxic doses of the oxidant t-butyl hydroperoxide (tBH) has been shown to inhibit *RPE65* expression,³⁵ however, not to the extent observed in our study. Controversially, *RPE65* has been shown to be upregulated in RPE cells after short-term exposure to intense light, as a cellular oxidizer.³⁶ Thus, *RPE65* expression appears to be tightly controlled by the oxidative status of RPE cells. It is possible that noncytotoxic oxidative stress levels, such as those triggered by administration of 10 mg/kg NaIO₃, may be associated not only with induction of antioxidant enzymes but also with induction of other genes critical for RPE function such as *Rpe65* and *Mitf*. However, excess oxidative stress associated with activation of apoptotic pathways could lead to major disruption of RPE gene expression including loss of *Rpe65*. Indeed, decreased *Rpe65* expression secondary to intense photic injury was recently reported.³⁷

Our in vitro study clearly demonstrated that neuronal cells are more sensitive to NaIO₃ toxicity than are RPE cells. It is

well established not only that RPE cells are highly resistant to oxidative stress,³⁸ but also that one of their main functions is to support PR survival under homeostatic or pathologic oxidative stress conditions. Indeed, we observed that conditioning the NaIO₃-containing medium on ARPE-19 cells significantly reduced PR cell death, suggesting that RPE cells are able to process NaIO₃ and/or secrete neurotrophic factors in response. Further analysis confirmed that known neurotrophic factors, including CTF1, PSPN, and BDNF, were induced in RPE cells after NaIO₃ exposure. Retinal pigment epithelium cells are known for their ability to secrete a wide variety of growth factors³⁹ that are able to support surrounding cells by paracrine and autocrine signaling and hence promote PR survival and retinal homeostasis.^{40,41} In addition to their survival effect on PRs, many RPE-secreted neurotrophins, including BDNF and PSPN, can promote RPE cell survival due in part to their synthesis of neuroprotectin D1, which counteracts the cytotoxic effect of various proapoptotic stressors such as the accumulation of A2E.⁴² The neurotrophic function of CTF1 in the eye, unlike that of BDNF and PSPN, has not been evaluated. Although our data suggest that CTF1 is involved in RPE cells' protective role against direct NaIO₃ toxicity on PRs, further research is required to better characterize RPE-derived CTF1 functions.

Taken together, our results indicate that intravenous administration of low-dose NaIO₃ triggers a direct effect on PRs, leading to rapid retinal dysfunction before the appearance of measurable RPE atrophy. This direct and early PR damage likely plays a causative role in the pathological events associated with NaIO₃-induced retinal degeneration. The identification of this new mechanism of retinal NaIO₃ toxicity is also critical for the accurate interpretation of results from experiments testing approaches such as RPE replacement strategies using this model.

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References

- Samardzija M, Neuhauss SCF, Joly S, Kurz-Levin M, Grimm C. Animal models for retinal degeneration. In: *Animal Models for Retinal Diseases*. Totowa, NJ: Humana Press; 2009. *Neuro-methods*; vol 46:51–79.
- Zeiss CJ. Animals as models of age-related macular degeneration: an imperfect measure of the truth. *Vet Pathol*. 2010;47:396–413.
- Webster SH, Rice ME, Highman B, Von Oettingen WF. The toxicology of potassium and sodium iodates: acute toxicity in mice. *J Pharmacol Exp Ther*. 1957;120:171–178.
- Enzmann V, Row BW, Yamauchi Y, et al. Behavioral and anatomical abnormalities in a sodium iodate-induced model of retinal pigment epithelium degeneration. *Exp Eye Res*. 2006;82:441–448.
- Mizota A, Adachi-Usami E. Functional recovery of retina after sodium iodate injection in mice. *Vision Res*. 1997;37:1859–1865.
- Nilsson SE, Knave B, Persson HE. Changes in ultrastructure and function of the sheep pigment epithelium and retina induced by sodium iodate. I. The ultrastructure of the normal pigment epithelium of the sheep. *Acta Ophthalmol (Copenh)*. 1977;55:994–1006.

7. Shen Y, Zhuang P, Lin B-Q, Zhang W-Y, Cy Chiou G. Effect of tetramethylpyrazine on RPE degeneration, choroidal blood flow and oxidative stress of RPE cells. *Int J Ophthalmol*. 2010;3:205-210.
8. Obata R, Yanagi Y, Tamaki Y, Hozumi K, Mutoh M, Tanaka Y. Retinal degeneration is delayed by tissue factor pathway inhibitor-2 in RCS rats and a sodium-iodate-induced model in rabbits. *Eye*. 2004;19:464-468.
9. Hariri S. Noninvasive imaging of the early effect of sodium iodate toxicity in a rat model of outer retina degeneration with spectral domain optical coherence tomography. *J Biomed Opt*. 2013;18:026017.
10. Hariri S, Moayed AA, Choh V, Bizheva K. In vivo assessment of thickness and reflectivity in a rat outer retinal degeneration model with ultrahigh resolution optical coherence tomography. *Invest Ophthalmol Vis Sci*. 2012;53:1982-1989.
11. Chen W, Yu M, Wang Y, et al. Non-mitogenic human acidic fibroblast growth factor reduces retinal degeneration induced by sodium iodate. *J Ocul Pharmacol Ther*. 2009;25:315-320.
12. Xia H, Krebs MP, Kaushal S, Scott FW. Enhanced retinal pigment epithelium regeneration after injury in MRL/MpJ mice. *Exp Eye Res*. 2011;93:862-872.
13. Atmaca-Sonmez P, Li Y, Yamauchi Y, et al. Systemically transferred hematopoietic stem cells home to the subretinal space and express RPE-65 in a mouse model of retinal pigment epithelium damage. *Exp Eye Res*. 2006;83:1295-1302.
14. Machalińska A, Klos P, Baumert B, et al. Stem cells are mobilized from the bone marrow into the peripheral circulation in response to retinal pigment epithelium damage—a pathophysiological attempt to induce endogenous regeneration. *Curr Eye Res*. 2011;36:663-672.
15. Kiuchi K, Yoshizawa K, Shikata N, Moriguchi K, Tsubura A. Morphologic characteristics of retinal degeneration induced by sodium iodate in mice. *Curr Eye Res*. 2002;25:373-379.
16. Korte GE, Reppucci V, Henkind P. RPE destruction causes choriocapillary atrophy. *Invest Ophthalmol Vis Sci*. 1984;25:1135-1145.
17. Sorsby A, Harding R. Oxidizing agents as potentiators of the retinotoxic action of sodium fluoride, sodium iodate and sodium iodoacetate. *Nature*. 1966;210:997-998.
18. Yoon YH, Marmor MF. Retinal pigment epithelium adhesion to Bruch's membrane is weakened by hemicholinium-3 and sodium iodate. *Ophthalmic Res*. 1993;25:386-392.
19. Franco LM, Zulliger R, Wolf-Schnurrbusch UEK, et al. Decreased visual function after patchy loss of retinal pigment epithelium induced by low-dose sodium iodate. *Invest Ophthalmol Vis Sci*. 2009;50:4004-4010.
20. Ohtaka K, Machida S, Ohzeki T, et al. Protective effect of hepatocyte growth factor against degeneration of the retinal pigment epithelium and photoreceptor in sodium iodate-injected rats. *Curr Eye Res*. 2006;31:347-355.
21. Redfern WS, Storey S, Tse K, et al. Evaluation of a convenient method of assessing rodent visual function in safety pharmacology studies: effects of sodium iodate on visual acuity and retinal morphology in albino and pigmented rats and mice. *J Pharmacol Toxicol Methods*. 2011;63:102-114.
22. Tao Z, Dai J, He J, Li C, Li Y, Yin ZQ. The influence of NaIO₃-induced retinal degeneration on intra-retinal layer and the changes of expression profile/morphology of DA-ACs and mRGCs. *Mol Neurobiol*. 2012;47:241-260.
23. Amirpour N, Karamali F, Rabiee F, et al. Differentiation of human embryonic stem cell-derived retinal progenitors into retinal cells by sonic hedgehog and/or retinal pigmented epithelium and transplantation into the subretinal space of sodium iodate-injected rabbits. *Stem Cells Dev*. 2012;21:42-53.
24. Li Y, Atmaca-Sonmez P, Schanie CL, Ildstad ST, Kaplan HJ, Enzmann V. Endogenous bone marrow derived cells express retinal pigment epithelium cell markers and migrate to focal areas of RPE damage. *Invest Ophthalmol Vis Sci*. 2007;48:4321-4327.
25. Wang CX-Z, Zhang K, Aredo B, Lu H, Ufret-Vincenty RL. Novel method for the rapid isolation of RPE cells specifically for RNA extraction and analysis. *Exp Eye Res*. 2012;102:1-9.
26. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3:RESEARCH0034.
27. Machalińska A, Lubiński W, Klos P, et al. Sodium iodate selectively injures the posterior pole of the retina in a dose-dependent manner: morphological and electrophysiological study. *Neurochem Res*. 2010;35:1819-1827.
28. Pang JJ, Abd-El-Barr MM, Gao F, Bramblett DE, Paul DL, Wu SM. Relative contributions of rod and cone bipolar cell inputs to AII amacrine cell light responses in the mouse retina. *J Physiol*. 2006;580:397-410.
29. Sanvicens N, Gómez-Vicente V, Masip I, Messegueur A, Cotter TG. Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6. *J Biol Chem*. 2004;279:39268-39278.
30. Carmody RJ, McGowan AJ, Cotter TG. Reactive oxygen species as mediators of photoreceptor apoptosis in vitro. *Exp Cell Res*. 1999;248:520-530.
31. Song JH, Harris MS, Shin SH. Effects of fetal bovine serum on ferrous ion-induced oxidative stress in pheochromocytoma (PC12) cells. *Neurochem Res*. 2001;26:407-414.
32. Lee H, Chung H, Arnouk H, et al. Cleavage of the retinal pigment epithelium-specific protein RPE65 under oxidative stress. *Int J Biol Macromol*. 2010;47:104-108.
33. Woo JM, Shin D-Y, Lee SJ, et al. Curcumin protects retinal pigment epithelial cells against oxidative stress via induction of heme oxygenase-1 expression and reduction of reactive oxygen. *Mol Vis*. 2012;18:901-908.
34. Milbury PE, Graf B, Curran-Celentano JM, Blumberg JB. Bilberry (Vaccinium myrtillus) anthocyanins modulate heme oxygenase-1 and glutathione S-transferase-pi expression in ARPE-19 cells. *Invest Ophthalmol Vis Sci*. 2007;48:2343-2349.
35. Alizadeh M, Wada M, Gelfman CM, Handa JT, Hjelmeland LM. Downregulation of differentiation specific gene expression by oxidative stress in ARPE-19 cells. *Invest Ophthalmol Vis Sci*. 2001;42:2706-2713.
36. Jahng WJ. New biomarkers in the retina and RPE under oxidative stress. In: Adio A, ed. *Ocular Diseases*. Rijeka, Croatia: INTECH; 2012:1-36.
37. Hadziahmetovic M, Kumar U, Song Y, et al. Microarray analysis of murine retinal light damage reveals changes in iron regulatory, complement, and antioxidant genes in the neurosensory retina and isolated RPE. *Invest Ophthalmol Vis Sci*. 2012;53:5231-5241.
38. Kurz TT, Karlsson MM, Brunk UTU, Nilsson SES, Frennsson CC. ARPE-19 retinal pigment epithelial cells are highly resistant to oxidative stress and exercise strict control over their lysosomal redox-active iron. *Autophagy*. 2009;5:494-501.
39. Tanihara H, Inatani M, Honda Y. Growth factors and their receptors in the retina and pigment epithelium. *Prog Retin Eye Res*. 1997;16:271-301.
40. LaVail MMM, Yasumura DD, Matthes MTM, et al. Protection of mouse photoreceptors by survival factors in retinal degenerations. *Invest Ophthalmol Vis Sci*. 1998;39:592-602.
41. Politi LEL, Rotstein NPN, Carri NGN. Effect of GDNF on neuroblast proliferation and photoreceptor survival: additive protection with docosahexaenoic acid. *Invest Ophthalmol Vis Sci*. 2001;42:3008-3015.
42. Bazan NGN. Neurotrophins induce neuroprotective signaling in the retinal pigment epithelial cell by activating the synthesis of the anti-inflammatory and anti-apoptotic neuroprotectin D1. *Adv Exp Med Biol*. 2008;613:39-44.