·Basic Research·

Effects of hydralazine on $NaIO_3$ –induced rat retinal pigment epithelium degeneration

Wei Jiang, Wan-Yu Zhang, George C Y Chiou

Institute of Ocular Pharmacology, College of Medicine, Texas A&M Health Science Center, College Station, TX 77843, USA

Correspondence to: George C Y Chiou. Institute of Ocular Pharmacology, College of Medicine, Texas A&M Health Science Center, College Station, TX 77843, USA. chiou@medicine.tamhsc.edu

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Abstract

• AIM: To study the effects of 10g/L hydralazine eye drops on 35mg/kg $NaIO_3$ -induced degeneration in rat eyes.

• METHODS: Various doses of $NaIO_3$ and/or saline alone were injected into Brown Norway rats from hypoglossal vein. After 3, 7, 14 or 28 days of injection, ERG a-, b-, c-wave, fast oscillation (FO) and light peak (LP) were measured along with retinal colored pictures and fluorescein angiography (FA) taken. Some rats were chosen to study the histology of retinas by light microscopy and autofluorescence of retina flatmounts. Different concentrations of $NaIO_3$ were given to RPE-19 cells, and cell proliferation rate was measured. For hydralazine study, 35mg/kg $NaIO_3$ was injected into Brown Norway rat from hypoglossal vein. $NaIO_3$ group was treated with saline alone after $NaIO_3$ injection, 10g/L hydralazine+ $NaIO_3$ group was treated with 10g/L hydralazine eyedrops after $NaIO_3$ injection whereas normal group was treated with saline alone without $NaIO_3$ injection. All eyedrops were instilled locally 3 times a day for 4 weeks and ERG c-wave was measured at the end of 2 and 4 weeks.

• RESULTS: After $NaIO_3$ administration, the amplitude of all ERG waves fell markedly in large dose groups at 30, 40 or 60mg/kg $NaIO_3$. Not many changes were observed in groups treated with <30mg/kg $NaIO_3$. Some retinal necrosis appeared from 3 days post-injection (PI) in 30mg/kg $NaIO_3$ group, which became more serious in larger dose groups or longer treatment time, but no apparent change was found in smaller dose groups. Similarly, on the retina flatmount, RPE monolayer showed necrosis from 3 days PI in the 30mg/kg $NaIO_3$ and larger dose groups. On histological examination, no significant change was seen in 30mg/kg $NaIO_3$ and lower concentration groups. In cell culture experiment, changes were found in RPE-19 cells proliferation rate with a concentration of $NaIO_3$ at 30mg/L or higher. In hydralazine experiments, 4 weeks after injection of $NaIO_3$, ERG c-wave fell markedly in $NaIO_3$ group to 31% of control group($P<0.01$). The ERG c-wave of hydralazine +$NaIO_3$ group fell only to 50% of control group ($P<0.05$). This was a 61% reversal of the c-wave of $NaIO_3$ treated group.

• CONCLUSION: Retinal pigment epithelium (RPE) degeneration induced by $NaIO_3$ was both dose and time dependent. Around 30 to 40mg/kg $NaIO_3$ would be the optimal to be used as a non-exudative age-related macular degeneration (AMD) rat model. Hydralazine may postpone the development of non-exudative AMD.

• KEYWORDS: retinal pigment epithelium; sodium iodate; age-related macular degeneration; hydralazine

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INTRODUCTION

Retinal pigment epithelium (RPE) monolayer plays a very important role in retinal function and many pathologic processes in eye diseases, especially the age-related macular degeneration (AMD). In non- exudative or 'dry' age-related macular degeneration, dysfunction of RPE is the first step followed by lipofuscin accumulation, presence of drusen, RPE atrophy and loss of photoreceptors. For searching a good model of dry-AMD, many people focused on sodium iodate, which could lead to RPE degeneration and atrophy selectively. Previous studies showed that after systemic injection of $NaIO_3$, ocular fundus showed following changes [1,2]. First, degeneration of RPE cells which could be seen from the histology and the suppression of ERG c-wave. It was then followed by the reduction of ERG a- and b- waves. The last part affected by $NaIO_3$ was the inner retina. However, the results were inconsistent depending on doses, time period and species used. When ICR albino mice were injected with 40mg/kg of sodium iodate intravenously, a- and b-wave amplitudes decreased first but could recover to the normal levels in 4 hours after injection [3]. If Balb/c mice were injected with 40mg/kg of $NaIO_3$ solution through the caudal vein, the suppressed b-wave amplitude began to recover at 14 days and totally recovered at 6 weeks after injection [4]. When New Zealand albino rabbits were injected with 0.5mg/kg $NaIO_3$, regeneration of RPE was noted at 6 to 7 days after $NaIO_3$ administration [5].

There are various explanations given to the different sensitivity of RPE cells to $NaIO_3$ actions. One theory indicated that $NaIO_3$ might act on melanin, a large component in RPE cells, which could be released from melanosome to convert glycine into glyoxylate [6]. Another study suggested that $NaIO_3$ could denature retinal proteins manifested by changes of SH level in retina [7]. Another suggestion indicated that retinotoxic effect of $NaIO_3$ was through inhibition of sulphydryl enzyme activity[8]. A group of articles considered the structural changes induced by sodium iodate, either via breakdown of retinal pigment epithelium diffusion barrier [9,10], or reduction of adhesion between RPE and photoreceptor cells[11,12]. As a strong oxidizing agent and selectively affecting RPE cells, $NaIO_3$ was used as a model to evaluate drug actions on AMD or similar retinopathy diseases [13-15].

ERG measurement is widely used in various animal experiments. It is well known that, the ERG comprised two major components, the a- and b-waves. The former reflects the function of rod photoreceptor outer segments [16] and the latter represents the activity of retinal bipolar cells [17]. Those two waves can be recorded by ac-coupled amplification. After b-wave, a series of slow potentials follow to which dc-coupled amplification is required [18]. C-wave is the second positive potential related to the transepithelial potential of the RPE. Fast oscillation (FO) has a negative trough that follows c-wave which relates to the basal membrane of the RPE. Following FO is a positive trough named light peak (LP) which also relates to the basal membrane of RPE.

It was tried to determine the morphological and functional changes in Brown Norway rats induced by various doses of $NaIO_3$. During the study of various ERG waves, an optimal dose of $NaIO_3$ was established in Brown Norway rats as a model to study the anti-AMD drugs. Hydralazine is a vasodilator clinically used to treat hypertension. Furthermore, it has previously been shown in microdialysis experiments to cause vasodilatation [19,20]. Local instillation of hydralazine eye drops has been found to alter intraocular pressure in animal eyes. We used hydralazine to treat rats after $NaIO_3$ injection and tried to observe effects of hydralazine eyedrops on reversal of $NaIO_3$ induced RPE degeneration.

MATERIALS AND METHODS

Materials 8-week-old male Brown-Norway (BN) rats were purchased from LARR (Texas A&M University, USA). All rats were housed in a standard animal room for a 12:12 hour cyclic lighting schedule. Animals were fed with normal food and water. All of the procedures conformed to the ARVO Resolution on the use of animals in ophthalmic and vision research. $NaIO_3$ (Sigma-Aldrich) was dissolved by saline at a mass concentration of 30g/L. Single injection of different doses of $NaIO_3$ (0, 7.5, 15, 20, 30, 40, 60mg/kg) was made through sublingual vein. Functional and histological changes examined at post injection (PI) 3 to 28 days or 2 months selectively. 10g/L hydralazine solution was prepared by Pam Louis Assoc. (San Antonio, TX).

Animal Procedure After single injection of $NaIO_3$, rats in different groups were measured with ERG, fundus pictures and fluorescein angiography at different time periods from 3 days to 2 months PI selectively. After functional examination, some rats from different groups were sacrificed and the eyes were removed and fixed in 25g/L glutaraldehyde for 2 hours and then in 20g/L formaldehyde overnight. One eye of each animal was used for histology and immunohistology studies, the other eye was prepared for autofluorescence measurement on flatmounts. For hydralazine studies, normal group was instilled with saline alone without $NaIO_3$ injection. $NaIO_3$ group was instilled with saline alone after 35mg/kg $NaIO_3$ injection, whereas 10g/L hydralazine+ $NaIO_3$ group was instilled with 10g/L hydralazine eye drops after 35mg/kg $NaIO_3$ injection. All eyedrops were instilled 3 times a day for 4 weeks. At the end, all rats in different groups were measured with ERG c-wave.

ERG Recordings BN rats were dark adapted overnight, and then anesthetized with ketamine 35mg/kg plus xylazine 5mg/kg intramuscularly. Half of the initial dose was given to each 1 hour thereafter. Pupils of all rats were dilated with one drop of 10g/L atropine, 10g/L tropicamide and 25g/L phenylephrine. Before recording, one drop of opticaine was used for surface anesthetization. All animals were kept warm during ERG measurement. Each rat was measured by dc-ERG recording firstly then by ac-ERG recording.

AC-ERG recording When ERG was recorded, Ag/AgCl electrode was placed gently in contact with the cornea as a reference electrode. A drop of 9g/L NaCl was used between them to establish stable signals. One stainless steel long electrode was inserted beneath the forehead's skin between the two eyes and another stainless steel short electrode was inserted to the leg as a ground electrode. A photostimulator (Grass PS22 Flash) was used to produce flashes of light five inches from the eye. EPIC-2000 was purchased from LKC Technologies, Inc. (Gaithersburg, MD). A single scotopic white flash (20ms duration) was used to elicit ERG a-and b-waves. The intensity of the stimuli was 628 cds/m^2 and bandpass filtered from 0.3 to 500Hz.

DC–ERG recording Methods developed by Dr. Peachey were followed [18]. Briefly, a 1mm diameter glass capillary tube with filament (Sutter Instruments, Novato, CA) that was filled with Hanks balanced salt solution (Invitrogen, Carlsbad, CA) was used to contact with a Ag/AgCl wire electrode with a attached connector. The capillary tube was connected with rat's corneal surface completely. Another similar electrode placed on the surface of the other eye served as a reference lead. Responses were amplified (dc-100Hz; gain=1 000×; DP-301, Warner Instruments, Hamden, CT) and digitized at 10Hz or 1 000Hz. Data were analyzed by iWORX LabScribe Data Recording Software (iWorx0CB Sciences, Dover, NH). Light stimuli was derived from an optical channel using a fiber-lite high intensity illuminator (Dolan-Jenner Industries, MA), with neutral density filters (Oriel, Stratford, CT) placed in the light path to adjust stimulus luminance. The stimulus luminance used in this experiment was 3.22logcd/m^2 and stimulated for 4 minutes. Luminance calibration was made by a Minolta (Ramsey, NJ) LS-110 photometer focused on the output side of the fiber optic bundle where the rat eye was located.

Fundus Pictures and Fluorescein Angiography Digital fundus camera (TRC-50EX; TOPCON, Tokyo, Japan) and Imagenet 2000 digital imaging systems (Topcon Medical Systems, Inc., Paramus, NJ) were used to capture retinal colored pictures and fluorescein angiography. When using fluorescein angiography, 10mg of fluorescein sodium was injected through the hypoglossal vein of rats. Anesthesia and pupil dilation were done as mentioned above.

Histology Paraffin-embedded tissues were sectioned at 3μm thickness. Eyes were cut from cornea to the optic nerve head along the vertical meridian, then stained with hematoxylin and eosin. Axioskop microscope (Zeiss, Thornwood, NY) was used to capture the images.

Autofluorescence of Flatmount For preparation of flat mounts, one eye of each animal was enucleated. After fixation, anterior part of the eye, as well as cornea, lens and sensory retina were gently removed and the remaining eye cup was washed in PBS. Four cuts were made from edge to center which helps to flatten the eye cup onto a glass slide. The autofluorescence of RPE in the flatmount was studied and captured on a confocal microscope (Zeiss LSM510; Zeiss, Thornwood, NY) using an Argon laser (wavelength 488nm).

ARPE–19 Cells Culture ARPE-19 cell line was purchased from ATCC Company (American Type Culture Collection, Manassas, VA). It was incubated in 37℃, 50mL/L CO_2 condition. Growth medium was composed of 1∶1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 1.2g/L sodium bicarbonate, 2.5mmol/L L-glutamine, 15mmol/L HEPES, 0.5mmol/L sodium pyruvate and 100g/L fetal bovine serum (All purchases from Invitrogen Corporation, Carlsbad, CA). Confluent cultures were released by digestion with 2.5g/L trypsin-0.2g/L EDTA (Sigma-Aldrich, St. Louis, MO).

Cell Proliferation Assay ARPE-19 cells were grown in 96-well tissue culture plates overnight. Medium was then replaced by fresh medium or various concentrations of $NaIO_3$ (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100mg/L). After incubation for 48 hours, cells were washed with Dulbecco's phosphate-buffered saline one time and further incubated with 100g/L 3-(4,5-dimethythiazol-2-yl)-2, 5diphenyl tetrazolium bromide (MTT) 100μL for exactly 4 hours. Media was then removed by aspiration, 100μL DMSO was added into each well and dishes were shaken for 2 minutes to dissolve cells. Light absorbency in each well was read at 570nm using a Spectra Count plate reader (Packard BioScience, Meridan, CT). Proliferation rate was valued by $NaIO_3$ treated cells comparing to normal cells. So the proliferation rate of normal cells was assigned as 100%.

Statistical Analysis Both eyes of each animal were used in the experiments. Cell culture was repeated 6 times and 6 wells were used each time for one group. Student's *t* test was used for statistical analysis. One-tail *t*-test was used for *in vivo* experiment and two-tail *t*-test was used for *in vivo* part.

RESULTS

ERG Recordings After AC-ERG recording, we measured a-wave from baseline to the first negative trough; maximum b-wave amplitude (Vbmax) from the first negative trough (a-wave) to the first positive peak of the b- wave (Figure 1A). For the DC-ERG recording (Figure 1B), the second positive peak followed b-wave was c-wave. The amplitude of which was measured from the trough after b-wave (which was after potential, AP) to the peak of c-wave. The amplitude of FO was measured from c-wave peak to the FO trough while LP was measured from the FO trough to the LP maximum [18]. As shown in Figure 1C, ERG b-wave disappeared in 60mg/kg $NaIO_3$ group from 28 days treatment, which decreased gradually in 40mg/kg $NaIO_3$ group from 3 days and vanished after 2 months. Thirty mg/kg $NaIO_3$ could decrease almost all the ERG waves at 7, 14 and 28 days (Figure 1D-H). Twenty mg/kg $NaIO_3$ decreased the b-wave

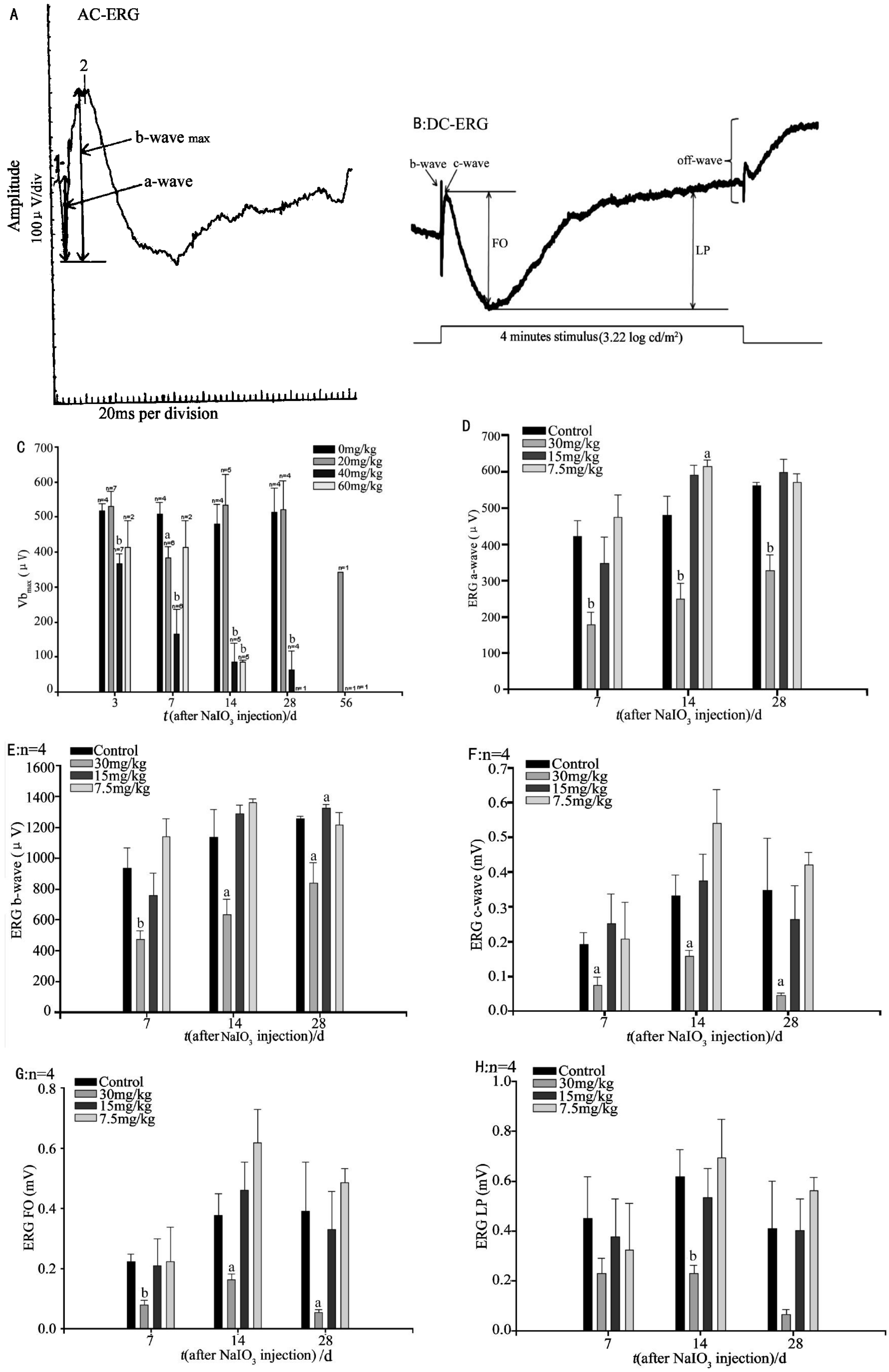

Figure 1 ERG waves after NaIO3 injection to BN rats $^aP<0.05$, $^bP<0.01$ *vs* Control

at 7 days but recovered afterward. Fifteen and 7.5mg/kg $NaIO_3$ didn't suppress any ERG wave (Figure 1D-H).

Fundus Photos and Fluorescein Angiography Rats from different groups were chosen to take ocular fundus pictures

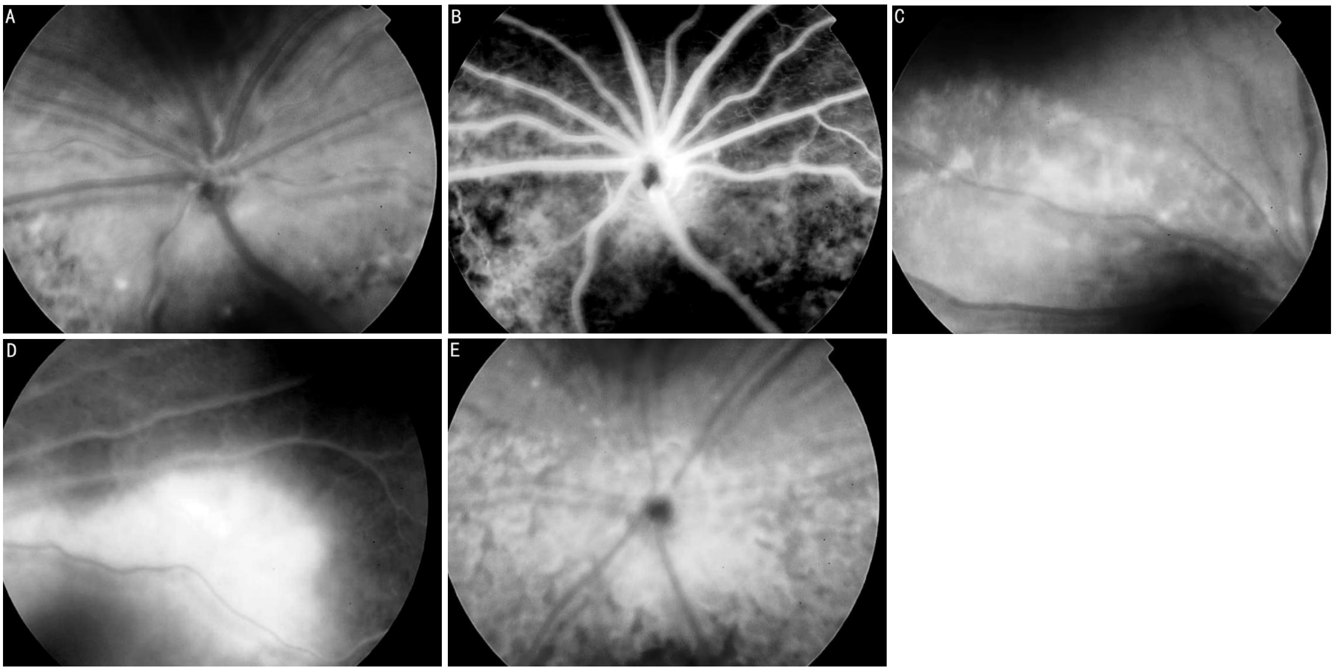

Figure 2 Ocular fundus and fluorescein angiogram on $NaIO_3$ treated BN rats A: retinograph of 30mg/kg $NaIO_3$ treated BN rat at day 28; B: fluorescein angiogram of 30mg/kg $NaIO_3$ treated BN rat at day 28; C: retinograph of 40mg/kg $NaIO_3$ treated BN rat at day 7; D: fluorescein angiogram of 40mg/kg $NaIO_3$ treated BN rat at day 7; E: retinograph of 40mg/kg $NaIO_3$ treated BN rat at day 8

and fluorescein angiography (FA). For FA, hyperfluorescence in the whole retina were seen in 60mg/kg $NaIO_3$ group as early as at 3 days, even no obvious changes were seen in fundus pictures at the time. Partial retinal hyperfluorescence could be seen at 3 days in both 40mg/kg and 30mg/kg $NaIO_3$ groups; the former is much more severe. Hypofluorescence could be seen from peripheral retina with a longer time period. Yellow dots or scars could be seen as early as at 7 days in all three groups from peripheral to the central retina, which was related to the dose of $NaIO_3$ (Figure 2). In 20mg/kg $NaIO_3$ group, changes were not obvious till 28 days.

Histology RPE cells fell off and photo cells decrease could be seen at 60mg/kg $NaIO_3$ from 3 days, and 40 mg/kg $NaIO_3$ from 7 days. The melanin disappearance in RPE cells could be seen at 30mg/kg $NaIO_3$ from 7 days. No obvious changes were seen in 20mg/kg $NaIO_3$ groups (Figure 3).

Autofluorescence of Flatmount Autofluorescence of RPE cells could be seen in flatmount (Figure 4) induced by laser with measurement of confocal microscope. After treatment with 30 mg/kg $NaIO_3$ or higher, small holes increased from 3 days, which indicated necrosis of RPE cells. In 20mg/kg $NaIO_3$ group, small holes in flatmounts were found from 7 days, which were less as compared with larger dose groups.

Cell Proliferation The cell rate decreased after 48 hours treatment with 5, 30 and 100mg/L $NaIO_3$ (Figure 5). Lower concentrations of $NaIO_3$ had no effect.

Effects of Hydralazine We measured ERG c-wave of rats after administration of 35mg/kg $NaIO_3$ for 4 weeks. It was found that ERG c-wave fell markedly to 31% of normal group in $NaIO_3$ group ($P<0.01$). In 10g/L hydralazine+ $NaIO_3$ group, ERG c-wave fell down to 50% of normal group ($P<0.05$) which was a significant reversal to 61% of suppression of $NaIO_3$ group ($P<0.01$).

DISCUSSION

It was found in this study that the retina of BN rat was easily damaged by high doses of $NaIO_3$, from neural retina layers to photoreceptor and RPE cell layers, which was not reversible (Figure 1C). From morphological data we could also note that 60mg/kg $NaIO_3$ induced every severe retina toxicity. In lower doses, such as 20mg/kg $NaIO_3$ or lower, not so much histology changes were found. Even though the functional decrease was observed at the beginning, which could be recovered later. So neither too high nor too low doses of $NaIO_3$ could be suitable for the treatment of dry-AMD animal model. Accordingly, 30mg/kg to 40mg/kg $NaIO_3$ would be optimal to be used in this animal model. As far as action mechanism is concerned, larger doses of $NaIO_3$ could destroy all layers of retina whereas lower doses only influence photoreceptors and RPE cells (Figure 3C). Only 30mg/L $NaIO_3$ and higher concentration could suppress RPE

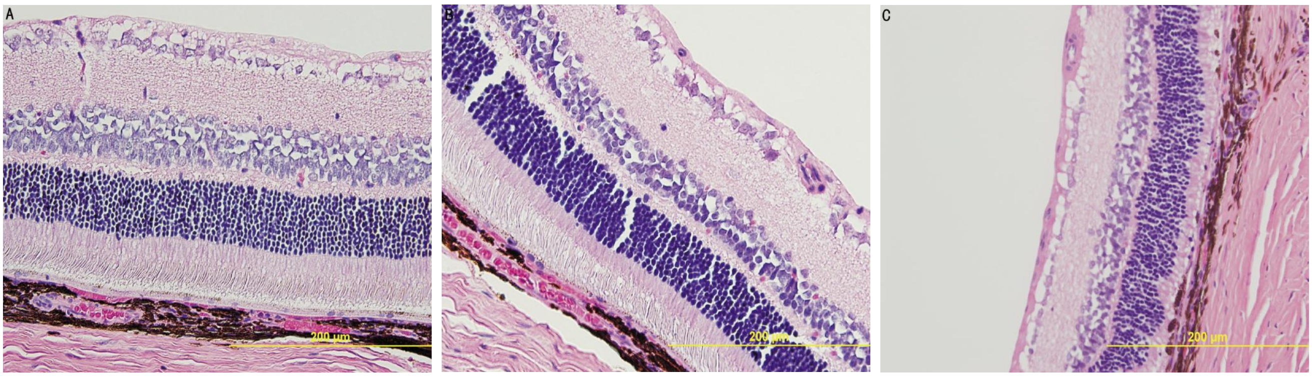

Figure 3 Histology of retina on $NaIO_3$ treated BN rat eyes A: 20mg/kg $NaIO_3$ at 28 days;B: 30mg/kg $NaIO_3$ at 28 days;C: 40mg/kg $NaIO_3$ at 14 days

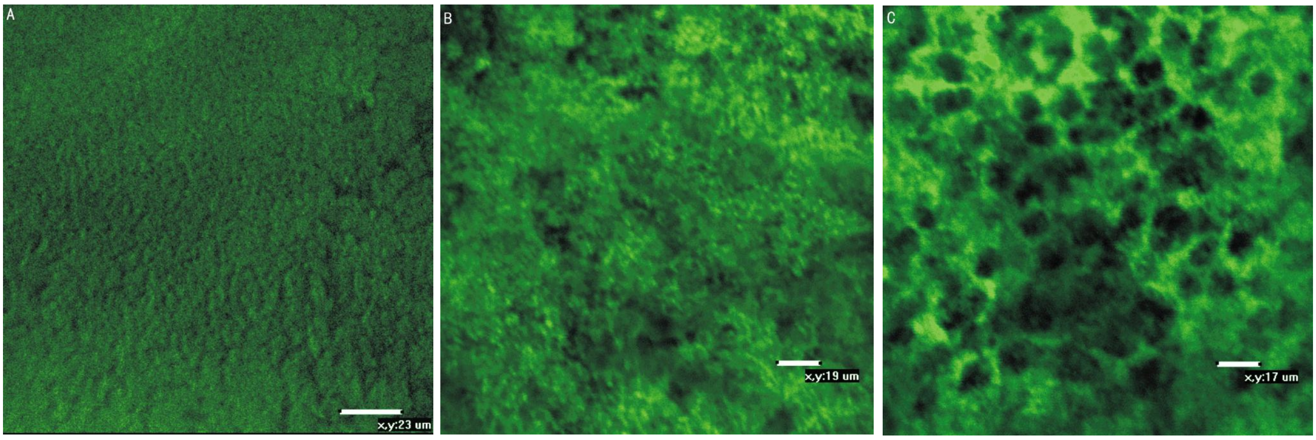

Figure 4 Autofluorescence of RPE cells in flatmount induced by laser and measured with confocal microscope A: normal RPE cells; B: 30mg/kg $NaIO_3$ at 7 days; C: 40mg/kg $NaIO_3$ at 7 days

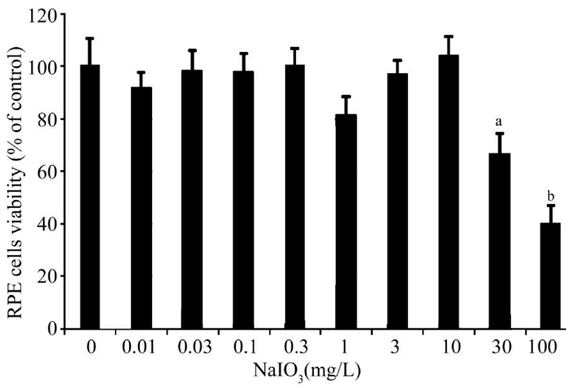

Figure 5 ARPE-19 cells proliferation rate after 48-hour treat-ment with $NaIO_3$ $^aP<0.05$, $^bP<0.01$, *vs* Control

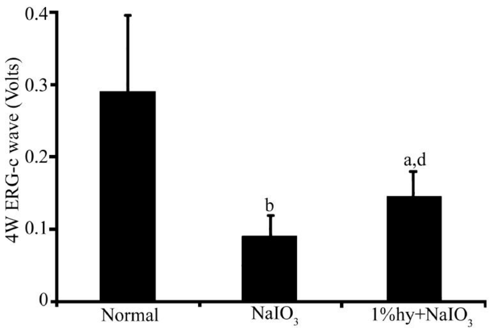

Figure 6 Effects of hydralazine eyedrops on 35mg/kg $NaIO_3$-induced RPE degeneration in rat eyes $^aP<0.05$, $^bP<0.01$, *vs* Normal, $^dP<0.01$, *vs* $NaIO_3$

cells (Figure 5), which might indicate that melanin is important for the toxicity of $NaIO_3$ in RPE cells. Since not much melanin exists in the cell cytoplasm, low dose of $NaIO_3$ has no effect on APRE-19 cells growth.

Since hydralazine can cause choroidal vasodilatation and increase choroidal blood flow in the eyes, retina can receive more blood and oxygen. We found that for rats treated with 10g/L hydralazine after $NaIO_3$ injection, the ERG c-wave didn't fall as markedly as in $NaIO_3$ group. This action of hydralazine might postpone the development of non-exudative age-related macular degeneration and could be used to treat non-exudative age-related macular degeneration in the future.

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