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# Chitosan oligosaccharides prevented retinal ischemia and reperfusion injury via reduced oxidative stress and inflammation in rats

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ABSTRACT

The purpose of the present study was to investigate the protective effect and mechanism of chitosan oligonucleotides (COS) on retinal ischemia and reperfusion (I/R) injury. Rats pretreated with PBS, low-dose COS (5 mg/kg), or high-dose COS (10 mg/kg) were subjected to retinal ischemia by increasing their intraocular pressure to 130 mm Hg for 60 min. The protective effect of COS was evaluated by determining the electroretinograms (ERGs), morphology of the retina, and survival of retinal ganglion cells (RGCs). The oxidative damage was determined by imuunohistochemistry and ELISA, respectively. The expressions of inflammatory mediators (TNF-α, IL-1β, MCP-1, iNOS, ICAM-1) and apoptotic-related proteins (p53, Bax, Bcl-2) were quantified by PCR and Western blots. The detection of NF-κB p65 in the retina was performed by immunofluorescence. The protein levels of IκB and phosphorylated mitogen-activated protein kinases [MAPK; viz. extracellular signal-regulated protein kinases (ERK), c-Jun N-terminal kinases (JNK) and p38] and the NF-κB/DNA binding ability were assessed by Western blot analysis and EMSA. We found that pretreatment with COS, especially a high dosage, effectively ameliorated the I/R-induced reduction of the b-wave ratio in ERGs and the retinal thickness and the survival of RGCs at 24 h. COS decreased the expression of inflammatory mediators, p53 and Bax, increasing Bcl-2 expression and thereby reducing retinal oxidative damage and the number of apoptotic cells. More importantly, COS attenuated IκB degradation and p65 presence in the retina, thus decreasing NF-κB/DNA binding activity after I/R. In addition, COS decreased the phosphorylation levels of JNK and ERK but increased the phosphorylation level of p38. Pretreatment with p38 inhibitor (SB203580) abolished the protective effect of COS on retinal oxidative damage, as indicated by increased retinal 8-OHdG stains, and significantly increased the expression of inflammatory mediators (TNF-α, MCP-1, iNOS, ICAM-1) in I/R-injured rats. In conclusion, COS prevented retinal I/R injury through its inhibition of oxidative stress and inflammation. These effects were achieved by blocking the activation of NF-κB, JNK, and ERK but promoting the activation of p38 activation.



## 1. Introduction

Retinal ischemia/reperfusion (I/R) injury is critically involved in the pathogenesis of several major vision-threatening diseases, including diabetic retinopathy, hypertensive retinopathy, acute glaucoma, and retinal vascular occlusion (Tso and Jampol, 1982; Stefansson et al., 1992; Levine, 2001). These diseases are major causes of blindness worldwide and usually result in vision loss due to irreversible damage to the retinal neurons. Retinal damage induced by I/R injury is caused by the depletion of adenosine triphosphate during the ischemia status (Yokota et al., 2011) and by the generation of reactive oxygen species and proinflammatory mediators during the reperfusion status (Szabo et al., 1991; Laskowski et al., 2000).

Chitosan oligosaccharides (COS), the hydrolyzed product of chitosan, is a mixture of oligomers of β-1,4-linked D-glucosamine residues and is abundant in the exoskeletons of crustaceans and the cell walls of fungi and insects (Pae et al., 2001). COS is known to have various biological activities, including antitumor,

*Abbreviations:* COS, chitosan oligonucleotides; I/R, ischemia and reperfusion; ERG, electroretinogram; RGCs, retinal ganglion cells; TUNEL, TdT-dUTP terminal nick-end labeling; ROS, reactive oxygen species.

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antimicrobial, anti-inflammation, anti-oxidative, and anti-apoptotic effects (Pangestuti and Kim, 2010; Qin et al., 2002; Chen et al., 2006; Joodi et al., 2011). COS has good solubility in water and is easily absorbed in the intestine, which makes it an attractive ingredient in many healthy foods or dietary supplements. We have previously shown that COS effectively attenuated oxidative-stress related retinal degeneration in rats. However, whether COS is able to exert protective effects on an animal model of retinal I/R injury remains unknown.

Transiently raising the intraocular pressure is a well-established animal model of retinal I/R injury. Interruption of the blood supply to the retina results in a wide variety of metabolic derangements, and the process of reperfusion itself is deleterious to injured retinal cells through the generation of free radicals and inflammatory cytokines. This model induces an extensive loss of retinal ganglion cells and the inner nuclear layer and an increase in apoptotic cells in the inner retina (Lam et al., 1999; Wu et al., 2004), both of which closely resemble the pathological changes observed in patients suffering from retinal ischemic insults, severe diabetic retinopathy, and acute glaucoma (Zheng et al., 2007).

Because oxidative stress and inflammation are contributing factors in the pathogenesis of retinal I/R injury, we hypothesized that COS may display anti-inflammatory and anti-oxidative stress effects in protecting retinal cells from I/R injury. In view of the crucial role of NF-κB and mitogen–activated protein kinase (MAPK) in regulating retinal inflammation and oxidative stress during retinal I/R injury, we evaluated the effects of COS on NF-κB activation and MAPK phosphorylation in a rat model of retinal I/R injury induced by transiently raising the intraocular pressure.

## 2. Material and methods

### 2.1. Reagents

Chitosan oligosaccharide was purchased from Sigma–Aldrich (St. Louis, MO, USA). The DNA fragmentation detection kit (TUNEL) was obtained from Calbiochem (La Jolla, CA, USA). Green Fluorescent Protein (GFP) antibody was purchased from BioVision (Mountain View, CA, USA). Mounting medium with 4′, 6-diamidino -2-phenylindole (DAPI) and phycoerythrin Streptavidin antibodies was obtained from Vector Laboratories (Burlingame, CA, USA). Anti-p65 antibodies were purchased from Rockland (Gilbertsville, PA, USA).

### 2.2. An animal model of retinal ischemia/reperfusion injury

Sprague–Dawley (SD) rats weighting 150–200 g were used in all subsequent experiments. All animal experiments in this study were carried out in strict accordance with the recommendations in the guide for the care and use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the committee on the Ethics of Animal Experiments of the National Taiwan University. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The anterior chamber of the right eye was cannulated with a 27-gauge infusion needle connected to a bottle containing normal saline. The intraocular pressure was raised to 130 mmHg for 60 min by elevating the saline reservoir. Retinal ischemia and reperfusion were confirmed by the whitening of the fundus and restoration of the retinal blood flow. Sham-procedure right eyes were treated similarly but without the elevation of the bottle; thus, normal ocular tension was maintained.

### 2.3. Animal grouping and treatment

SD rats were randomly divided into four groups:

Group 1: intraperitoneal injection of phosphate-buffered saline (PBS) and then cannulation only without elevation of the bottle, serving as normotensive control (control).
Group 2: intraperitoneal injection of PBS before inducing retinal ischemia/reperfusion injury (PBS-treated group).
Group 3: intraperitoneal injection of a low dose of COS (5 mg/kg) before inducing retinal ischemia/reperfusion injury (low-dose COS group).
Group 4: intraperitoneal injection of a high dose of COS (10 mg/kg) before inducing retinal ischemia/reperfusion injury (high-dose COS group).

### 2.4. Electroretinogram (ERG) recordings

The ERG was performed at 24 h and 7 days after retinal ischemia/reperfusion injury. The rats were dark-adapted for 1 h before performing the ERG. All manipulations were performed under dim red light illumination. After being anesthetized, the rats were place on a heating pad. A recording electrode was placed on the cornea after the application of 0.5% methyl cellulose. A reference electrode was attached to the shaved skin of the head, and a ground electrode was clipped the animal's tail. A single flash light (duration, 100 ms) 30 cm from the eye was used as the light stimulus. Responses were amplified with a gain setting of ±500 μV and filtered with low 0.3 Hz and high 500 Hz from an amplifier. The pattern of b-waves was recorded. The b-wave ratio was defined as the b-wave amplitude of the right eye/the b-wave amplitude of the left eye. The fold of the b-wave ratio represented the b-wave ratio at 24 h or day 7/the b-wave ratio at day 0.

### 2.5. Histological study and retinal thickness

For all control and experimental animals, both eyes were collected at 24 h and at day 7 after treatment. The specimens were fixed with 4% paraformaldehyde in PBS. Sections (1 μm) were cut along the vertical meridian of each eye and passed through the optic nerve head for staining with hematoxylin and eosin (H&E). We measured different layer thicknesses to quantify the ischemic damage in the retina at day 7. The total retinal thicknesses (from the inner limiting membrane to the pigment epithelium), the outer nuclear layers (ONL), the inner nuclear layer (INL), and the inner plexiform layer (IPL) were measured. Alterations in the thickness of the retinal layers were measured for each eye in the same topographic region of the retina (1 mm from the optic nerve head) under ×200 magnification.

### 2.6. Neu N stain in flat-mounted retinas and counting of NeuN-positive cells

The density of retinal ganglion cells (RGCs) was evaluated by immunofluorescence staining with NeuN at 24 h after retinal I/R injury. Briefly, the retina was cryoprotected overnight in 30% sucrose, followed by three freeze–thaw cycles and overnight incubation with monoclonal FITC-conjugated NeuN antibody (Millipore, Billerica, MA, USA). Finally, the retina was flat-mounted and viewed with a Leica TSL AOBS SP5 confocal microscope (Leica Microsystems, Exton, PA, USA). The number of Neu N-positive cells was counted in 4 selected retinal areas located at the same eccentricity (approximately 1.5 mm from the optic disk) in the four retinal quadrants. The cell number was quantified with image-analysis