Protective Effects of Glucosamine on Oxidative-Stress and Ischemia/Reperfusion-Induced Retinal Injury

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Purpose: To investigate the protective effects of glucosamine (GlcN) using oxidative stress and rat models of ischemia-reperfusion (I/R) injury and to determine the antiapoptotic and anti-inflammatory mechanisms of GlcN treatment.

Methods: We determined the effects of GlcN and the levels of O-linked N-acetylglucosamine (O-GlcNAc) in in vitro retinal ganglion cells (RGCs) treated with or without H2O2. The survival and apoptosis rates of RGCs were compared after the addition of GlcN, glucose, or O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAC). Retinal I/R injury was induced in Sprague-Dawley rats by elevating the IOP to 110 mm Hg for 60 minutes. An intraperitoneal injection of GlcN (1000 mg/kg) or normal saline was administered in the different groups, including a control group, a GlcN group, an I/R group, a GlcN + I/R group (1000 mg/kg GlcN 24 hours before I/R injury), and an I/R + GlcN group (7-day period of 1000 mg/kg GlcN 24 hours after I/R injury). The rats were killed 7 days after the I/R injury, and the retinas were collected from each rat for thickness measurements. Quantitative analysis of RGC survival was further determined using labeling with FluoroGold.

Results: The GlcN increased levels of O-GlcNAc in a dose-dependent manner in the RGCs treated with or without H2O2. The GlcN resulted in increased cell survival and reduced apoptosis in the RGCs under oxidative stress conditions. In the rat model of I/R injury, GlcN significantly protected against I/R-induced retinal thinning and suppressed the I/R-induced reductions in α- and β-wave amplitudes of the ERG. In terms of RGC survival, significant incremental density of RGCs was found in the I/R + GlcN group compared with the I/R group. Notably, the use of GlcN in the rat retina decreased apoptosis and the formation of reactive oxygen species (ROS) after I/R injury. We also found that mitogen-activated protein kinase signal pathways played a critical role in the GlcN-mediated attenuation of ROS-induced damage in vitro and I/R injury in vivo.

Conclusions: Glucosamine treatment provides multiple levels of retinal protection, including antiapoptotic, anti-inflammatory, and antioxidative benefits. More research on the role of GlcN as a potential agent for the prevention and treatment of glaucoma is warranted.

Keywords: glucosamine, oxidative-stress injury, ischemia/reperfusion injury, retina

Glaucome is the second-leading cause of blindness after cataracts and represents a significant global health care problem. Glaucome is a clinical disorder characterized by IOP-associated optic nerve damage. This damage is characterized by optic neuropathy that is consistent with excavation and underrining of the optic disc, the eventual death of retinal ganglion cells (RGCs), and visual field loss.1 The death of RGCs in glaucomatous eyes occurs via apoptosis, as has been demonstrated in different species.2 The rapid increase in IOP elicited by acute glaucoma contributes to a series of retinal cell insults, including the disturbance of retinal blood flow, ischemia-reperfusion injury,3 metabolic derangements,4 over-load of reactive oxygen species (ROS) and inflammatory cytokines,5,6 apoptosis of RGCs,7,8 impaired retrograde transport of neurotrophins,9 and increased vascular permeability.10 Emerging evidence indicates that ROS-induced oxidative stress plays an important role in the pathophysiological mechanism of glaucoma.11,12 Such stress was found to induce p38 mitogen-activated protein kinase (MAPK)-mediated TNF production and stimulated nuclear factor (NF)-κB to initiate a cytokine cascade through Toll-like receptors.13,14 Oxidative stress overload was also reported to activate the intrinsic apoptosis pathway, leading to the destruction of the integrity of the cell membrane and altered permeability of calcium channels.15-18 Notably, a number of cellular responses and inflammatory mediators were found to be induced due to hypoxic-ischemic insult following high IOP, such as VEGF, nitric oxide synthase, TNF-α, IL-1β, adhesion molecule-1(ICAM-1), and vascular cell adhesion molecule-1.19-21 Although understanding of the mechanisms of glaucoma has increased, there
are no available and effective therapeutic strategies to prevent and reduce glaucomatous optic nerve apoptosis.

Glucosamine (GlcN), a naturally occurring amino monosaccharide, exerts a certain degree of immunosuppressive effects in vitro and in vivo and is used widely as an alternative therapeutic regimen for rheumatoid arthritis and osteoarthritis.\textsuperscript{22–24} In our previous study, GlcN inhibited TNF-\(\alpha\)- or IFN-\(\gamma\)-induced the expression of the ICAM-1 gene in human retina or retinal pigment epithelial cells.\textsuperscript{25} In addition, GlcN, the main precursor of glycosylation by posttranslational modification, exhibited a regulatory role in the activation of the hexosamine biosynthetic pathway.\textsuperscript{26} The posttranslational attachment of O-linked N-acetylglucosamine, or O-GlcNAC, to serine and threonine residues of nuclear and cytoplasmic proteins is increasingly recognized as a key regulator of diverse cellular processes. For example, the production of O-GlcNAC was shown to be essential for cell survival, and increased cellular O-GlcNAC levels are cytoprotective.\textsuperscript{27} One of the major focuses of the study of glucosamine in the past decade had been to investigate the nature of its anti-inflammatory, antioxidative, and antiapoptotic effects.\textsuperscript{28–30}

Much remains to be understood regarding the biological activities of GlcN in glaucoma, as this subject remains largely uninvestigated. Therefore, the purpose of the present study was to investigate the protective effects of GlcN on RGCs via the O-GlcNAC posttranslational modification in vitro and in vivo.

\section*{Methods}

\subsection*{Cell Culture}

An RGC line (RGC5) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in 1 mg/mL glucose Dulbecco's modified Eagle's medium-F-12 (Invitrogen-Gibco, Grand Island, NY, USA) supplemented with 4 mM L-glutamine, 10% fetal bovine serum (Invitrogen-Gibco), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin (Sigma-Aldrich Corp., St. Louis, MO, USA). The cells were grown in a humidified 37°C/5% CO\(_2\) incubator. The culture medium was replaced twice weekly.

\subsection*{Induction of Acute Glaucoma in the Animal Model}

All of the animal experiments were approved and conducted under the guidance of the Institutional Animal Care and Use Committee (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International), National Defense Medical Center, Taipei, Taiwan. All of the animals used in the study were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eight-week-old male Sprague-Dawley (SD) rats weighing 250 to 300 g were used in this study. These SD rats were housed in a temperature- and humidity-controlled animal room under a 12-hour light/12-hour dark cycle, with food and water provided ad libitum.

All of the surgical procedures for induction of acute glaucoma-induced ischemia-reperfusion injury were performed under aseptic conditions. General anesthesia was induced via intraperitoneal injection of a mixture of 50 mg/kg ketamine and 2 mg/kg xylazine. Corneal analgesia was administered using a drop of topical 0.5% proparacaine hydrochloride ophthalmic solution, and pupillary dilatation was maintained with 0.5% tropicamide and 0.5% phenylephrine. Rats were placed on a temperature-controlled heated table to maintain body temperature at 37°C. After analgesia and dilatation of the pupil, the anterior chamber of the left eye was cannulated with a 50-gauge needle connected to a saline reservoir at 150 cm above the eye, leading to a high IOP of 110 mm Hg. The presence of retinal ischemia was examined by the fundus. After removing the infusion needle from the anterior chamber for a period of 60 minutes, the IOP returned to normal. Antibiotic ophthalmic gel with tobramycin was topically applied to the eye before and after the procedure. Only the left eye of the SD rats was used in all of the experiments.\textsuperscript{31,32}

\subsection*{Group Assignment and Drug Administration}

The SD rats were randomly allocated to the control group or the treatment group. The SD rats were divided into the following experimental groups: (1) control group, neither surgical procedures for the induction of acute glaucoma-induced ischemia-reperfusion (I/R) injury nor administration of GlcN were performed; (2) GlcN group, the SD rats received intraperitoneal injection of 1000 mg/kg (Sigma-Aldrich Corp.) based on the pharmacokinetics of GlcN\textsuperscript{33}; (3) I/R group, the SD rats were subjected to acute glaucoma-induced I/R injury and intraperitoneal injection of normal saline rather than GlcN; (4) GlcN + I/R group, the SD rats were treated with a single-dose intraperitoneal injection of 1000 mg/kg GlcN 24 hours before the induction of acute glaucoma-induced I/R injury; and (5) I/R + GlcN group, the SD rats were intraperitoneally injected over a 7-day period with 1000 mg/kg GlcN 1 hour after the induction of acute glaucoma-induced I/R injury. The rats were killed 7 days after I/R injury to observe the long-term effect of GlcN. The retinas were collected from each rat, and the thicknesses of the retinas were recorded.

\section*{Western Blot and OxyBlot Analysis}

After the treatments, RGCs were rinsed with ice-cold PBS and detached by scraping. The cells were pelleted at 1000g, resuspended, and sonicated in cold lysis buffer (50 mM Tris-HCl [pH 7.5], 2% SDS, 1 mM phenylmethysulfonyl fluoride, and 10 \(\mu\)L/mL protease inhibitors). The lysates were centrifuged at 14,000g for 20 minutes at 4°C, and the clear supernatant was removed into fresh Eppendorf tubes. The concentration of total protein was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), with BSA as the standard. After 5 minutes of boiling, the samples (30 \(\mu\)g of lysate) were resolved with one-dimensional SDS/10% polyacrylamide gel, separated electrophoretically, and transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% (wt/vol) milk in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 0.05% Tween-20 for 1 hour at room temperature on a shaking table. The blots were incubated overnight at 4°C with primary antibodies directed against the following proteins: p38, C-Jun N-terminal kinase (JNK), phosphorylated JNK, extracellular signal regulated protein kinase (ERK) 1, ERK2, phosphorylated ERK1/2, and phosphorylated AKT, all of which were purchased from BD Pharmingen (San Diego, CA, USA). Antibodies against phosphorylated p38, caspase 3, and cleaved caspase 3 were obtained from Cell Signaling Technology (Beverly, MA, USA). The Bid, NF-\(\kappa\)B, and IxB-zantibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); superoxide dismutase-1 (SOD-1), glutathione peroxidase-1 (GPX-1), and catalase antibodies were obtained from Genetex (San Antonio, TX, USA). The Bcl-2 antibody was purchased from eBioscience (San Diego, CA, USA). The membranes were washed and blotted with horseradish peroxidase-conjugated secondary antibody (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour at room temperature. The proteins were visualized on X-ray film using the standard enhanced
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chemiluminescence procedure, and the mean protein levels were determined densitometrically with ImageJ 1.46a (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Oxidation proteins were measured by Western blots. The quantification of total oxidized proteins was performed using the OxyBlot Protein Oxidation Detection Kit (Millipore Corp.), as previously described.34

**Flow Cytometry**

Flow cytometric identification with BD Phosflow reagents (BD Biosciences, San Jose, CA, USA) and quantification of viable cells were performed to examine the effects of O-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAC) and GlcN treatment following hydrogen peroxide (H2O2)-induced oxidative stress in RGC5 cells. The procedures were performed according to the manufacturer’s protocol. Confluent cultured cells were preincubated with or without 0.5 mM H2O2 for 1 hour and further incubated with 1 mM GlcN for 24 hours, 2.5 mM GlcN for 24 hours, or 0.1 mM PUGNAC for 1 hour at 37°C. After incubation, the cells were washed twice in PBS before analysis with a FACScan flow cytometer (Becton, Dickinson, & Co., Sunnyvale, CA, USA).

**Cell Viability Assay**

The MTT (3-[4, 5-cimethylthiazole-2-yi]-2, 5-diphenyl tetrazolium bromide) assay was used to assess cell viability. The cells were plated in 180 μL medium at the appropriate seeding density into 96-well microtiter plates. After 1 mM MTT stock solution was added to each culture, the samples were incubated in the dark at 37°C with shaking for 3 hours. The MTT formazan crystals were then resolubilized by adding 200 μL 100% dimethyl sulfoxide to each well. The absorbance or optical density was quantitated at 540 nm using a scanning multiwell spectrophotometer (Titertek Biosciences, San Jose, CA, USA) and quantification of viable cells were performed to examine the effects of O-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAC) and GlcN treatment following hydrogen peroxide (H2O2)-induced oxidative stress in RGC5 cells. The procedures were performed according to the manufacturer’s protocol. Confluent cultured cells were preincubated with or without 0.5 mM H2O2 for 1 hour and further incubated with 1 mM GlcN for 24 hours, 2.5 mM GlcN for 24 hours, or 0.1 mM PUGNAC for 1 hour at 37°C. After incubation, the cells were washed twice in PBS before analysis with a FACScan flow cytometer (Becton, Dickinson, & Co., Sunnyvale, CA, USA).

**Calcium Assay**

Intracellular Ca2+ is a key second messenger in cellular signaling processes, especially in the contexts of apoptotic and necrotic cell death. A membrane-permeable dye, Calcium Sensor Dye eFluor 514 (eBiosciences), was applied to monitor intracellular free Ca2+ concentrations using fluorescence microscopy. The calcium assay was conducted according to the manufacturer’s instruction.

**The TUNEL Assay**

The TUNEL assay is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids that results from apoptotic signaling cascades. To explore whether the use of GlcN reduced I/R-induced apoptosis, we performed a TUNEL assay on retinal cross sections. The TUNEL assay was performed on retinal cryosections according to the manufacturer’s instruction (TUNEL Apoptosis Detection Kit; Millipore Corp.).

**Electroretinography**

Retinal functions were examined by ERG 7 days after I/R injury. After dark adaptation for 4 hours, these SD rats were anesthetized by intraperitoneal injection of ketamine 1 mL/kg and 1% xylazine under dim red illumination. Their pupils were dilated by instillation of 0.5% tropicamide and 0.5% phenylephrine. The ERG responses were recorded using stainless-steel wire loops placed on the cornea to act as the corneal electrode after topical application of Vidisic ophthalmic gel to maintain corneal hydration. The reference and ground electrodes were placed subcutaneously on the temporal canthus and the tail, respectively. The cone-isolated responses were recorded by means of a light flash (2.5 cd s/m²) from a photic stimulator.

**Immunohistochemistry**

For histology and immunohistochemistry, the eyes were enucleated immediately after death and immersion-fixed in 10% buffered formalin for at least 24 hours until processing. The left eye of each animal was immersed in Davidson’s solution for 24 hours and then transferred to 70% ethanol until processing. Davidson’s solution included two parts formaldehyde (37%), three parts 100% ethanol, one part glacial acetic acid, and three parts water.35,36 The whole eyes were processed according to the following schedule: 70% ethanol for 120 minutes, 80% ethanol for 120 minutes, 95% ethanol for 120 minutes, 99% ethanol for 24 hours, xylene for 30 minutes, xylene for 30 minutes, xylene for 30 minutes at 62°C, embedding. The globes were embedded sagittally, and 4-μm sections were cut using a microtome. The sections were stained with hematoxylin and eosin for histopathologic analysis.

**Retinal Flatmount Imaging and Retrograde FluoroGold Labeling of RGCs**

Retinal ganglion cells were labeled with FluoroGold (FG) (Sigma-Aldrich Corp.) by injecting the FG solution into the superior colliculi using a stereotaxic device (Stoelting, Wood Dale, IL, USA), as described previously.37,38 After the rats were anesthetized with the mixture of ketamine and xylazine, the skin over the cranium was incised to expose the scalp. Two vertical holes approximately 1 mm in diameter were drilled on both sides of the skull with a dentist’s drill 6 mm posterior to the bregma and 1.5 mm lateral to the midline; 2 μL 3% FG solution was delivered by a micropipette at depths of 3.8, 4.0, and 4.2 mm from the bone surface.39

After the rats were killed, the eyes were enucleated. The retinas were dissected and fixed in 4% paraformaldehyde (Sigma-Aldrich Corp.) for 1 hour. After washing with PBS, retinal flatmounts were performed by making four radial incisions and placing the retinas on slides in 10% glycerol (Sigma-Aldrich Corp.) in PBS. The numbers of RGCs were counted under fluorescence microscopy (Olympus BX-50; Olympus Optical, Tokyo, Japan) using UV excitation (330–385 nm) and a barrier filter (420 nm). Each retina was divided into quadrants (superior, inferior, nasal, and temporal). Quadrants were further divided into central (0.8–1.2 mm from the optic disc), middle (1.8–2.2 mm from the optic disc), and peripheral regions (0.8–1.2 mm from the retinal border). At each region, 50% fields (200 x 200 μm²) were counted at 62°C in each group of animals was presented as the number of RGCs per square millimeter of the counted retina area (RGCs/mm²).39

**Statistical Analysis**

Descriptive results are expressed as the means ± SD for continuous variables and as the number and percentage for categorical variables. The differences between the groups were examined by the χ² test or Student’s t-test, as appropriate. One-way ANOVA was used for comparisons involving three or more group means. All of the statistical assessments were two-sided and evaluated at the 0.05 significance level. Statistical analyses were performed with the SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA).
RESULTS

GlcN-Induced Levels of the O-GlcNAC Posttranslational Protein Modification in RGCs Under Oxidative Stress

A growing number of studies have indicated that the anti-inflammatory effect of GlcN is associated with the attenuation of the lipopolysaccharide-induced increase in NF-κB signaling, intracellular TNF-α, and ICAM-1 expression by increasing levels of the O-GlcNAc protein modification. To examine the levels of the O-GlcNAc protein modification induced by GlcN treatment in RGCs under oxidative stress, we treated RGCs with or without H2O2, GlcN, PUGNAc, or glucose (Fig. 1). PUGNAc, a 1,5-hydroximolactone that inhibits a variety of N-acetylhexosaminidases, was long thought to increase levels of O-GlcNAc in human cells. In Figure 1, the Western blot results show that GlcN increased the level of the O-GlcNAc modification in a dose-dependent manner in RGCs treated with or without H2O2. The exposure of H2O2 significantly decreased the levels of O-GlcNAc in RGCs treated with GlcN, PUGNAc, or glucose (Fig. 1). PUGNAc, a 1,5-hydroximolactone that inhibits a variety of N-acetylhexosaminidases, was long thought to increase levels of O-GlcNAc in human cells.

GlcN Increases Cell Survival and Inhibits Oxidative Stress–Induced RGC Apoptosis

An in vitro H2O2-induced apoptosis model was used to mimic glaucomatous ischemia reperfusion. For this analysis, RGCs were treated with H2O2 (0.5 mM) for 60 minutes, and cell viability was measured using MTT (Fig. 2A) assays and flow cytometry (Fig. 2B). In Figure 2A, the cell survival in the H2O2-treated groups is seen to be significantly lower than in the control group. Following the co-incubation with 2.5 mM GlcN or 0.1 mM PUGNAc, the survival rates of cells treated with H2O2 plus GlcN or with H2O2 plus PUGNAc were significantly higher than in the H2O2-only group. As shown in Figure 2B, the apoptotic rates of the H2O2-treated groups and control groups were 1.08% and 10.56%, respectively. After treatment with 2.5 mM GlcN and PUGNAc, the apoptotic rate in the H2O2-treated groups decreased to 2.67% and 3.08%, respectively. The findings revealed that GlcN and PUGNAc both had a protective effect against H2O2-induced RGC apoptosis.

GlcN Reduces H2O2-Induced Apoptosis by Modulating Calcium Mobilization and ROS Generation

To investigate the effects of GlcN on ROS formation, 2', 7'-dichlorofluorescin (DCF) was used as a detection reagent...
Figure 3. The use of GlcN attenuated H_2O_2-induced apoptosis in RGCs. (A) Generation of ROS was detected by fluorescence imaging microscopy in RGCs without or with 0.5 mM H_2O_2. These cells were exposed to 1 mM GlcN for 24 hours, 2.5 mM GlcN for 24 hours, or 0.1 mM PUGNAc for 1 hour. (B) Calcium mobilization was assessed using Calcium Sensor Dye eFluor 514 in the RGCs without or with 0.5 mM H_2O_2. These cells were exposed to 1 mM GlcN for 24 hours, 2.5 mM GlcN for 24 hours, or 0.1 mM PUGNAc for 1 hour.

(Fig. 3A). The H_2O_2 increased the intracellular ROS content in RGCs, and 1 mM or 2.5 mM GlcN inhibited the production of ROS in H_2O_2-treated RGCs. Similarly, PUGNAc significantly attenuated the production of ROS in H_2O_2-treated RGCs.

Previous studies have reported that both apoptosis and the overproduction of superoxide anion (O_2^•-) and H_2O_2 during the reperfusion of ischemic tissue are associated with cellular Ca^{2+} overload via the opening of the mitochondrial permeability transition pore.41 These results prompted us to investigate whether GlcN impaired apoptosis-induced calcium release using calcium sensor dyes. As shown in Figure 3B, a significant decline in the fluorescence intensity was observed when the concentration of GlcN increased from 1.0 mM to 2.5 mM. The fluorescence intensity in the PUGNAc-treated group was significantly lower than in the H_2O_2-only group. Taken together, these findings indicate that GlcN improved cell survival by diminishing ROS formation and apoptosis-induced calcium release in H_2O_2-treated RGCs.

GlcN Protects Against I/R-Induced Retinal Thinning and Dysfunction In Vivo

Photomicrographs of the retina in the five groups are shown in Figure 4A. After 60 minutes of high IOP, histopathologic features of ischemic damage were seen in the I/R group, the GlcN-I/R group, and the I/R+GlcN group. The thickness of the entire retina in the I/R group was decreased significantly compared with that in the control group. In the GlcN-I/R group, a nonsignificant improvement in overall retinal thickness was recorded compared with the control group. The retinal thickness in the I/R+GlcN group was thicker than that in the I/R group, and this difference was significant.

To focus on the protective effects of GlcN on the RGCs after I/R injury, quantitative analysis of RGC survival was determined using labeling with FG (Fig. 4B). In the I/R group, the density of RGCs was significantly lower than that in the control group or GlcN group. Furthermore, the significant incremental density of RGCs was found in the I/R+GlcN group compared with the control group. The above-mentioned findings indicated that the administration of GlcN can protect RGCs from I/R injury in vivo.

Electroretinograms were recorded in all groups 7 days after I/R injury (Fig. 4C). In the control and GlcN groups, a normal typical ERG was observed. In the I/R group, the retinal ischemia contributed to substantial decrease in the a- and b-wave amplitudes. The difference between the a- and b-wave amplitudes in the I/R group declined significantly compared with the control and GlcN groups. Seven days after I/R insult, improvement in the difference between the a- and b-wave amplitudes in the GlcN-I/R group did not reach statistical significance. Notably, there was a significant recovery in the difference between the a- and b-wave amplitudes in the I/R+GlcN group. The application of GlcN reversed the amplitude reduction elicited by retinal ischemia 7 days after I/R injury, especially in the I/R+GlcN group.

The Effect of GlcN on the DNA Apoptosis in the Retina

Photomicrographs of TUNEL-positive cells in the retina are presented in Figure 5A. The number of TUNEL-positive cells was 37.81 ± 26.79 /mm^2 in the I/R group, 35.45 ± 27.06 /mm^2 in the GlcN-I/R group, and 18.09 ± 13.28 /mm^2 in the I/R+GlcN group. The results showed that the induction of I/R triggered apoptosis in the retina, whereas GlcN reduced apoptosis in the retina of I/R-induced rats.

In chemical-induced apoptosis, the cleavage of Bid by caspase-8 causes the release of cytochrome c from the mitochondria and activates caspase-3, leading to apoptosis. This apoptotic pathway had been shown to be negatively regulated by antiapoptotic proteins such as Bcl-2 through the suppression of cytochrome c release. Moreover, the effect of GlcN on the expression of Bid and Bcl-2 in the retina of I/R-subjected rats was studied using Western blot analyses.32 As shown in Figure 5B, the Bid/Bcl-2 ratio in the I/R group was significantly reduced compared to the control group. Notably, the Bid/Bcl-2 ratio in the GlcN-I/R group and in the I/R+GlcN group was significantly reduced compared to the I/R group, suggesting that the GlcN treatment activated Bcl-2 and suppressed Bid. The decline in the Bid/Bcl-2 ratio in the I/R+GlcN group was larger than in the I/R+GlcN group, and this difference was significant.

GlcN Prevents I/R-Induced ROS Formation In Vivo

In vitro experiments showed that GlcN treatment inhibited the production of ROS and had a protective effect in H_2O_2-treated RGCs. Therefore, we explored whether ROS formation occurs in I/R-induced SD rats and examined the effect of GlcN on ROS formation and the activity of antioxidant enzymes, including GPX-1, SOD-1, and catalase. We observed an increase in intracellular ROS content in the I/R group, and the administration of 1000 mg/kg/d GlcN after I/R injury significantly attenuated this increase (Fig. 6). Figure 6A depicts the 5-fold increase in SOD-1 production that was observed in the I/R+GlcN group compared with the control group. With respect to GPX-1 activity, the I/R group had lower antioxidant enzyme activity than the control group, but this did not reach statistical
significance. Notably, the I/R insult resulted in a significant 2-fold increase in the activity of catalase compared with the control group. This increase in the activity of catalase was diminished in both the I/R+GlcN and GlcN+I/R groups after the administration of GlcN.

**GlcN Decreased I/R-Induced Inflammation In Vivo**

We next determined whether treatment with GlcN in SD rats subjected to I/R injury attenuated ICAM-1 expression. As shown in Figure 7A, ICAM-1 expression levels were substantially elevated in the I/R and the GlcN+I/R groups. In the I/R+GlcN group (lane 5), GlcN treatment after I/R injury significantly reduced ICAM-1 levels. Moreover, to test whether the inhibitory effect of GlcN on I/R-induced ICAM-1 expression was mediated via the degradation of NF-kB and activation of IxBs, the expression of these factors was examined in these groups (Fig. 7B). Consistent with previous results, the level of NF-kB in the I/R group was significantly higher than in the control group. Expression of NF-kB in the I/R+GlcN group decreased significantly compared with the control group. The data showed that treatment with GlcN resulted in a decrease of I/R-mediated NF-kB activation (Fig. 7B). With respect to the IxBs (Fig. 7B), there was a decrease of expression in the I/R group. Compared with GlcN+I/R group, IxB expression was significantly increased and NF-kB levels were significantly decreased in the I/R+GlcN group.
The Effect of GlcN on MAPK Signaling Pathways in ROS-Induced Damage and I/R Injury In Vitro and In Vivo

At least three distinct groups of MAPKs, including ERK1/2, JNK, and p38 MAPK, play important roles in ROS-induced injury. We further addressed whether MAPK signaling was involved in ROS damage in vitro and I/R injury in vivo. In RGCs treated with or without H$_2$O$_2$, the effects of GlcN and PUGNAc on the expression of ERK1/2, p38 MAPK, and JNK were investigated (Figs. 8A–C). Treatment with GlcN (2.5 mM) for 24 hours or PUGNAc (0.1 mM) for 60 minutes significantly blocked H$_2$O$_2$-induced phosphorylation of p38 MAPK. However, treatment with GlcN or PUGNAc failed to attenuate the phosphorylation of ERK1/2 or JNK, respectively.

In the rat model of I/R injury, we observed a sustained increase in the phosphorylation of p38 MAPK and JNK in the I/R groups (Figs. 8D, 8E). With respect to the p38 MAPK pathway, GlcN treatment before I/R injury did not alter the increased phosphorylation of p38 MAPK that was induced by I/R injury. The use of GlcN after I/R injury significantly inhibited the phosphorylation of p38 MAPK. With respect to JNK, the levels of phosphorylated JNK in the I/R+GlcN group and the GlcN+I/R group were both markedly suppressed compared with those in the I/R group. Taken together, our data indicated that MAPK signaling was involved in the GlcN-mediated retinal protection in the in vitro oxidative stress and in vivo I/R injury models.

**DISCUSSION**

To the best of our knowledge, the effects of GlcN on RGCs and mechanisms underlying its effects have not been previously described. Herein, we examined the protective effect of GlcN on RGCs under oxidative stress in vitro. The findings indicated that treatment with GlcN in the RGCs increased cell viability by decreasing both oxidative stress and apoptosis. In a previous study, GlcN was observed to increase levels of O-GlcNAc, attenuate NF-$\kappa$B reporter activity, and inhibit the expression of ICAM-1 in TNF-$\alpha$-treated ARPE-19 cells. In our study, GlcN increased O-GlcNAc protein levels in RGCs under oxidative stress. To further investigate the effect of GlcN in glaucomatous retinal damage in vivo, I/R injury was induced by means of a continuous infusion of normal saline into the anterior chamber of the left eye to create a higher IOP of 110 mm Hg for 60 minutes. The retinal I/R injury model mimicked acute glaucoma and has been a well-known animal model for studying retinal cell damage after ischemic insult. Our study demonstrated that the use of GlcN provided protection against the production of ROS as well as retinal cell apoptosis and inflammation after I/R injury. In the experimental model of I/R injury, GlcN treatment not only alleviated RGC apoptosis, attenuating the reduction of retinal thickness, but also preserved retinal function to restore the ERG response. Because the estimated average half-life of GlcN elimination is 15 hours, only a single-dose intraperitoneal injection of 1000 mg/kg GlcN was used before induction of the model to elucidate the effect of treatment before and after I/R injury.
FIGURE 6. Effects of GlcN on the generation of ROS. (A) Effects of GlcN on the expression of SOD-1 protein in the five groups. The results are the means ± SEMs of three independent experiments. #P < 0.05 versus the I/R group. (B) Effects of GlcN on the expression of catalase protein in the five groups. (C) Effects of GlcN on the expression of GPX-1 protein in the five groups. (D) The schematic representation of western blot analysis. The results are the means ± SEMs of three independent experiments. **P < 0.01 versus the control group.

FIGURE 7. The use of GlcN attenuated the I/R-related inflammation. (A) The use of GlcN attenuated the expression of ICAM-1 in the I/R+GlcN group. The results are the means ± SEMs of three independent experiments. *P < 0.05, **P < 0.01 versus the control group; ###P < 0.001 versus the I/R group. (B) The effects of GlcN on the expression of NF-κB and IκBα among the five groups. The results are the means ± SEMs of three independent experiments. *P < 0.05 versus the control group; #P < 0.05 versus the I/R group.
In vitro, GlcN improved cell survival and decreased apoptosis in H$_2$O$_2$-treated RGCs by promoting the O-GlcNAc posttranslational protein modification. This result was consistent with a previous study, in which it was observed that increases in O-GlcNAc modification levels were associated with decreased cardiomyocyte apoptosis following I/R injury.\textsuperscript{17} Although the interactions between O-GlcNAc levels and retinal cell function are complicated and remain unclear, these pathways might play a critical role in the regulation of cell function and survival. Champananachai and colleagues\textsuperscript{48} reported that GlcN regulated apoptosis and protected neonatal rat ventricular myocytes against I/R injury via increased antiapoptotic Bcl-2 and O-GlcNAc modification levels. The apoptotic program is complex and involves both pro- and antiapoptotic proteins, such as Bcl-2, Bcl-xL, Bax, and Bad. In the pro- and antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-xL protect cells from apoptosis, whereas Bax and Bid have proapoptotic functions. These findings were in accord with the results of our studies. Compared with the I/R group, there was significant decline in the Bid/Bcl-2 ratio in the I/R+GlcN group.

In a study executed by Chen et al., it was shown that GlcN inhibits ICAM-1 expression by modulating the O-linked glycosylation of factors involved in NF-$\kappa$B signaling and by reducing the N-linked glycosylation of TNF-$\alpha$-induced ICAM-1 expression in ARPE-19 cells.\textsuperscript{25} Rajapakse et al.\textsuperscript{49} highlighted that GlcN exerts its anti-inflammatory effects by increasing O-GlcNAc protein levels and reducing TNF-$\alpha$-induced ICAM-1 expression in human umbilical vein endothelial cells. Our study supports these previous results, findings that GlcN protected RGCs from oxidative stress–induced injury via the modulation of protein O-GlcNAc glycosylation.

During I/R injury, ischemic retinal injury consists of a self-reinforcing destructive cascade that involves neuronal depolarization, calcium influx, and oxidative stress that is initiated by energy failure and increased glutamatergic stimulation.\textsuperscript{52} With respect to oxidative stress, disturbances of the antioxidant defense system have been demonstrated in the I/R injury, such as changes in the activities and expression of antioxidant enzymes. In the endothelial system under I/R injury conditions, GlcN may exert certain anti-inflammatory effects via endothelial adhesion molecule expression by enhancing O-GlcNAc levels as well as pro-oxidative functions.\textsuperscript{49} The results reported here did not support the findings of previous research, in which increasing GlcN-induced O-GlcNAc levels were found to attenuate H$_2$O$_2$-induced loss of the mitochondrial membrane potential.\textsuperscript{48} In the present study, the use of GlcN or PUGNAc diminished ROS formation in H$_2$O$_2$-treated RGCs. Rather than GPX-1 and catalase, there were significant increases in SOD-1 production in the I/R group. Notably, treatment with GlcN or PUGNAc blocked H$_2$O$_2$-induced increases in SOD-1 production in the I/R group.

### FIGURE 8.

The activation of MAPK pathway in the context of oxidative stress and I/R injury. (A–C) The activation of p38MAPK in RGCs treated with 0.5 mM H$_2$O$_2$. The results are the means ± SEMs of three independent experiments. $^*P < 0.05$ versus the group treated with only H$_2$O$_2$. (D, E) The activation of p38MAPK and JNK in the groups with I/R injury. The results are the means ± SEMs of three independent experiments. $^*P < 0.01$, $^**P < 0.001$ versus the control group; $^***P < 0.001$, $^#P < 0.01$, $^*P < 0.05$ versus the I/R group.

Our study indicated that the use of GlcN after I/R injury provided better retinal protection than treatment before I/R injury. In addition, GlcN suppressed inflammation by inhibiting the activation of the NF-$\kappa$B signaling pathway and subsequent upregulation of IkB.

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Emerging evidence supports a physiological role for ROS as “second messengers” in intracellular signaling cascades that control cell growth, proliferation, migration, and apoptosis.\textsuperscript{51} A number of cellular stimuli either induce ROS production or activate MAPK pathways; however, the exact pathophysiological mechanism behind this phenomenon has not been identified.\textsuperscript{52,53} In a study of the factors controlling MAPK activation, it was observed that H$_2$O$_2$ activates MAPK pathways via activation of growth factor receptors in several cell types.\textsuperscript{54} In line with these findings, MAPK pathways were activated in the H$_2$O$_2$-treated RGCs and rat retinas after I/R injury. Notably, treatment with GlcN or PUGNAc blocked H$_2$O$_2$-induced increases in SOD-1 production in the I/R+GlcN group.
phosphorylation of p38 MAPK in vitro. In addition, treatment with GlcN significantly inhibited the phosphorylation of p38 MAPK in vivo. Therefore, GlcN can downregulate H₂O₂- or I/R injury–induced MAPK pathway activation.

In our previous studies to explore the effect of GlcN in the mode of proliferative vitreoretinopathy (PVR) and endotoxin-induced uveitis (EIU), the intraperitoneal injection of 1000 mg/kg GlcN in the mouse model reduced the morphologic appearance of PVR and modulated the inflammatory reaction in EIU.25–56 In the present study, an intraperitoneal injection of GlcN (1000 mg/kg) in SD rats significantly protected against I/R injury. Based on the conversion formula of animal doses to human equivalent dose, the calculation results in the human equivalent dose was 9732 mg dose of GlcN for a 60-kg person.37 In the open-label Phase I study, total daily dose of 3 to 6 g GlcNac was administered orally as adjunct therapy for pediatric inflammatory bowel disease.58 No severe adverse effects were found. Although a high dose of GlcN not reasonably achievable through daily oral supplements, this concentration may be provided through a topical or subconjunctival control-release drug delivery system, such as carboxymethyl-hexanoyl chitosan or chitosan-gelatin–based hydrogel.59 We have undergone the studies about control-release drug delivery system of glucosamine in treatment of ocular inflammatory diseases.60,61 Based on our preliminary studies, the findings highlighted potential application and clinical usefulness of GlcN in treating human ocular diseases.

In conclusion, we provide convincing evidence to suggest that GlcN protects RGCs from oxidative-stress injury via the modulation of protein O-GlcNAc glycosylation. In the I/R injury rat model, GlcN treatment had several protective effects in the retina, including antiapoptotic, anti-inflammatory, and antioxidative benefits. Notably, the use of GlcN after I/R injury provided better retinal protection than treatment before I/R injury. Our findings highlight that GlcN is a potential drug for retinal protection from acute glaucoma-induced I/R injury.

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References