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Metal chelator combined with permeability enhancer ameliorates oxidative stress-associated neurodegeneration in rat eyes with elevated intraocular pressure

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Abstract

Since as many as half of glaucoma patients on intraocular pressure (IOP)-lowering therapy continue to experience optic nerve toxicity, it is imperative to find other effective therapies. Iron and calcium ions play key roles in oxidative stress, a hallmark of glaucoma. Therefore, we tested metal chelation by means of ethylenediaminetetraacetic acid (EDTA) combined with the permeability enhancer methyl sulfonyl methane (MSM) applied topically on the eye to determine if this non-invasive treatment is neuroprotective in rat optic nerve and retinal ganglion cells exposed to oxidative stress induced by elevated IOP. Hyaluronic acid (HA) was injected in the anterior chamber of the rat eye to elevate the IOP. EDTA-MSM was applied topically to the eye for 3 months. Eyeballs and optic nerves were processed for histological assessment of cytoarchitecture. Protein-lipid aldehyde adducts, and cyclooxygenase-2 (COX-2) were detected immunohistochemically. HA administration increased IOP and associated oxidative stress and inflammation. Elevated IOP was not affected by EDTA-MSM treatment. However oxidative damage and inflammation were ameliorated as reflected by decrease in formation of protein-lipid aldehyde adducts and COX-2 expression, respectively. Furthermore, EDTA-MSM treatment increased retinal ganglion cell survival and decreased demyelination of optic nerve compared with untreated eyes. Chelation treatment with EDTA-MSM ameliorates sequelae of IOP-induced

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toxicity without affecting IOP. Since most current therapies aim at reducing IOP and damage occurs even in the absence of elevated IOP, EDTA-MSM has the potential to work in conjunction with pressure-reducing therapies to alleviate damage to the optic nerve and retinal ganglion cells.

Keywords

Glaucoma; Intraocular pressure; Retinal ganglion cell; Glaucomatous neurodegeneration; Oxidative stress; Reactive oxygen species; Oxidative damage; Inflammation; Hyaluronic acid; Ocular Hypertension; Methylsulfonylmethane (MSM); Ethylenediaminetetraacetic acid (EDTA); Metal chelation; Chelation therapy

Introduction

Glaucoma, a leading cause of irreversible blindness, affects over 60 million people worldwide and 2.2 million in the US. It is the second leading cause of global blindness and projections estimate that by 2020, 11 million people world-wide will be blinded by glaucoma [1]. Progressive loss of optic nerve axons and retinal ganglion cells results in characteristic optic nerve atrophy and visual field defects in glaucoma patients. Important risk factors for glaucoma include family history, increased intraocular pressure (IOP), cupping of the optic nerve, myopia, exfoliation syndrome, pigment dispersion syndrome, retina vein occlusion, and systemic diseases such as diabetes mellitus and changes in blood pressure. Elevated IOP is an especially important risk factor, and is the sole target for most current glaucoma therapies.

Elevated IOP triggers the initiation and progression of oxidative stress-induced toxicity resulting in programmed retinal ganglion cell death and optic nerve degeneration [2]. Oxidative stress is characterized by an overproduction of reactive oxygen species (ROS) such as superoxide, hydroxyl and oxygen free radicals. The generation of most ROS is catalyzed by redox-active metal ions such as copper and iron [3]. These ions, released from storage proteins, contribute to increased oxidative stress by a variety of mechanisms including Fenton and Haber-Weiss reactions. Though metals in general and iron specifically are known to be involved in many eye diseases [4], the exact role of iron in glaucoma is unknown. However, a recent study shows that ingestion of high levels of iron and calcium supplements elevates the risk of glaucoma [5], implying that elevated levels of these metals are an important risk factor for glaucoma. In addition, it has been shown that the iron-regulating proteins transferrin, ceruloplasmin and ferritin are up-regulated at both the mRNA and the protein levels in glaucoma in cynomolgus monkeys and humans [6]. Transferrin and ferritin are major members of the retinal proteome. Each accounts for as much mRNA in the eye as the visual pigment rhodopsin. Perhaps because of the free-radical inducing effects of UV light in the eye, the retina seems to have a particularly high need for iron regulating proteins.

Glaucoma shares features with other well-known neurodegenerative diseases such as Alzheimer and Parkinson diseases [7]. Neurodegenerative diseases of diverse etiologies share common features including dysregulation of mitochondrial function leading to increased calcium-induced stress, glial activation, and inflammation. In addition, the

abundance of poly-unsaturated fatty acids in neural tissues makes them especially vulnerable to iron-catalyzed lipid peroxidation. The resulting formation of lipid-derived aldehydes includes 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE), and malonaldehyde (MDA [8]). These extremely reactive aldehydes conjugate with proteins in the cell membranes and cause tissue damage [9–16]. The resulting protein-aldehyde adducts cause changes in membrane fluidity [17, 18], resulting in calcium influx [19, 20] that can activate caspases, induce apoptosis, and contribute to glaucomagenesis [21]. Calcium overload and dysregulation have been found in both trabecular meshwork [22] and lamina cribrosa [23] from human glaucomatous eyes. The oral calcium antagonist, nifedipine, has been shown to slow visual field loss in normal tension glaucoma [24], further implicating calcium dysregulation in glaucoma.

Since transition metals and calcium ions are integral parts of neurodegenerative processes including glaucoma, reduction or removal of these ions can potentially provide both antioxidative and anti-neurodegenerative protection. Ethylenediaminetetraacetic acid (EDTA) is a chelator of divalent metal ions such as copper, iron, calcium, and zinc. EDTA chelation can theoretically prevent metal ions from participating in the generation of ROS, lipid peroxidation, and detrimental calcium-dependent cascades. However, topically applied EDTA does not penetrate effectively through the epithelial cells of the cornea [25]. A recent study from our laboratory demonstrates that the permeability of EDTA can be enhanced by the addition of methyl sulfonyl methane (MSM [26]). MSM is a widely used dietary supplement in the nutraceutical market and has no known toxicity. Being amphipathic, MSM possesses unique solubilizing properties that facilitate the entry of EDTA into tissue. The purpose of this study is to determine the efficacy of EDTA-MSM in inhibiting pathological symptoms associated with elevated IOP. Intracameral injection of hyaluronic acid was used in rats to induce elevated IOP by blocking the aqueous humor outflow pathway [27]. EDTA-MSM was applied topically to the eyes of rats to ameliorate the sequelae of oxidative stress and neurodegeneration induced by chronically elevated intraocular pressure.

Material and methods

Chemicals

Hyaluronic acid (HA), kanamycin solution, EDTA, and MSM were purchased from Sigma Aldrich Inc. (St. Louis, MO). Phosphate-buffered saline (PBS) solution, dimethyl sulfoxide (DMSO), and staining reagents were purchased from Invitrogen Corporation (Carlsbad, CA). Proparacaine hydrochloride ophthalmic solution USP was obtained from Bausch & Lomb Inc. (Rochester, NY). Antibody against COX-2 was purchased from Abcam (Cambridge, MA). Protein-HNE, protein-HHE, and protein-MDA, antibodies were obtained from Cayman Chemicals (Ann Arbor, MI).

Animals

The animal studies were approved by the University Animal Care and Use Committee and met the guidelines of both the National Institutes of Health's Guide for the Care and Use of the Laboratory Animals (Department of Health, Education, & Welfare publication no.

85-23, revised 1985, USA) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male adult Sprague Dawley rats (Harlan Laboratories, Houston TX), weighing between 170–190 g, were housed in the University of Texas Medical Branch Animal Research Center in a standard animal room with food and water *ad libitum* under controlled conditions of humidity and temperature ($21 \pm 2^\circ\text{C}$). Elevated intraocular pressure (IOP) was induced by weekly intracameral injection of HA [27]. Rats were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (5 mg/kg) administered intraperitoneally. Topical anesthesia with 0.5% proparacaine hydrochloride ophthalmic drops and ocular surface rinse with 0.5% kanamycin solution were given before HA injection. Using an insulin syringe, 25 μl of 15 mg/ml HA was injected into the anterior chamber of one eye of anesthetized rats, while an equal volume of vehicle (PBS) was injected into the fellow (control) eye. The eyes were observed during the procedure using a Bausch & Lomb binocular dissecting microscope. After injection, 0.5% kanamycin eye drop was given immediately to prevent infection.

Application of eye drops and IOP assessment

Four rats were treated in both eyes (one eye injected with HA and one eye injected with PBS) with 0.86% EDTA and 0.8% MSM solution in the conjunctiva sac, while four other rats were similarly treated with PBS. Treatments were continued twice daily on weekdays and once daily on weekends for 3 months. Each week, 24 hours after injection at the same time of day under same illumination, IOP readings were performed with the TonoLab tonometer (Colonial Medical Supply, Franconia, NH; [28]). Pressure readings were performed by one operator who was blind with respect to group and treatment. Before IOP assessment, rats were given both general and topical anesthesia for more reliable readings. Each tonometer reading required six measurements. The internal software eliminates high and low readings, providing four machine-generated readings for each eye that were used to determine the mean IOP for that time point. Data for IOP are expressed as the mean $\pm$ SEM. Statistical analysis of results was made by one-way or two-way analysis of variance (ANOVA) using GraphPad Prism software (GraphPad Software Inc., LaJolla, CA.) A *p*-value of <0.05 was considered statistically significant.

Histopathological Evaluation

After 3 months of injection and eye drop application, animals were euthanatized by CO_2 asphyxiation. Eyeballs with optic nerve segments extending 2 mm from the back of the globe were quickly enucleated after death. Separate optic nerves sections from >2 mm from the back of the eyeball were also collected. Eyes and nerves were fixed with 4% paraformaldehyde solution for 48 hours at 4°C , then washed in ice-cold PBS twice and kept in 70% alcohol at 4°C until they were embedded in paraffin. Before embedding, the nasal and temporal poles of the eyeball and the optic nerve were marked with tissue dye. The horizontal central plane of the eyeball was oriented with these 3 marks. Slices were cut parallel to this plane to insure access to the optic nerve head and its central position. Horizontal sections of eyeballs and transverse sections of optic nerves (both $5\mu\text{m}$ -thickness) were cut and stained with hematoxylin & eosin (H&E) and Luxol-fast blue/cresyl violet following standard protocols. Optic nerve and retina were observed by bright field light microscopy. For retinal ganglion cell counts, one horizontal section that passed through the

optic nerve head and included both nasal and temporal retina was selected from 3 different eyes from three treatments (control, HA, HA + EDTA-MSM). The nucleus of every ganglion cell across the whole retina was counted by independent observers using light microscopy.

Immunohistochemical studies

Paraffin-embedded sections were deparaffinized, rehydrated, and washed. Endogenous peroxidase activity was blocked with 3% H₂O₂, and then sections were rinsed in PBS twice and incubated with blocking buffer (2% BSA, 0.1% Triton X-100, 2% normal rabbit IgG, and 2% normal goat serum) overnight at 4°C. Sections were incubated with antibodies against 1) protein-HNE rabbit polyclonal antibody (diluted 1:500); 2) protein-HNE rabbit polyclonal antibody (diluted 1:1000); and 3) protein-MDA rabbit polyclonal antibody (diluted 1:500) for 1 hour at room temperature. Slides were incubated in secondary antibody, stained with peroxidase (LSAB+System HRP; DakoCytomation, Carpinteria, CA), and counterstained with hematoxylin. The sections were examined by bright-field light microscopy (EPI-800 microscope; Nikon, Tokyo, Japan) and photographed with a camera (Nikon) fitted to the microscope.

Some optic nerve sections were used for immunofluorescence analysis. After initial processing as above, sections were incubated with antibodies against 1) COX-2 rabbit polyclonal antibody (diluted 1:250), and 2) protein-MDA rabbit polyclonal antibody (diluted 1:250). Sections processed for anti-COX-2 were incubated with the secondary antibody, goat anti-rabbit IgG conjugated with Alexa Fluor 594 green (diluted 1:400.) Sections processed for anti- protein-MDA were incubated with the secondary antibody, goat anti-rabbit IgG conjugated with Alexa Fluor 594 red (diluted 1:400.) Sections were counterstained with DAPI (diluted 1:1000). Subsequently, the slides were mounted with FluorSave reagent (Calbiochem, San Diego, CA). Fluorescence images were recorded and analyzed with a Nikon E800-UIC Upright Epifluorescence Microscope with a Roper Coolsnap FX 12-bit Digital Cooled CCD camera.

Quantification of the immunohistochemical Images

Positive immunoreactivities of HHE, HNE, MDA, and COX2 were quantified on photomicrographs of optic nerve or retinal photoreceptor layer by using the NIH ImageJ program. Briefly, the immunostained sections were visualized and photographed by using an Olympus BX53 digital microscope. For micrographs of peroxidase staining, quantification was performed by converting the image to 8-bits, adjusting the threshold to distinguish the structure of interest from others, outlining the region of interest and measuring area and integrated density. For micrographs of immunofluorescence staining, quantification was carried out by outlining the region of interest followed by measuring the area and RGB integrated density via “Plugins-Analyze” orders. Measured integrated density was divided by measured area and expressed as integrated density per unit area (per 6 mm² for optic nerve longitudinal sections; per 1 mm² for retina photoreceptor layer and optic nerve transverse sections). From each animal, at least one stained section was photographed and quantified. All values from the same animal were averaged to represent that animal. Group data (n = 3) are presented as mean ± SEM. One-way ANOVA with Tukey’s post test was

used to determine differences among the three groups. Differences were considered significant at $p < 0.05$.

Results

Effect of EDTA-MSM on HA-induced glaucoma model

To determine the effects of EDTA-MSM on HA-induced elevated IOP in the rat eye, we measured IOP weekly for 12 weeks. Figure 1 shows the average IOP of rats injected with HA (in one eye) and vehicle (in the other eye). There was no difference between the groups before injection (mean IOP 9.94 ± 0.16). In PBS- injected eyes, treatment with EDTA-MSM compared to PBS treatment did not significantly affect IOP across treatment [$F(1,6) = 0.51$, $p = 0.50$], time [$F(11,66) = 1.14$; $p = 0.35$], or interaction [$F(11,66) = 1.24$; $p = 0.28$] by repeated measures two-way ANOVA. These eyes were pooled into one group for the subsequent analyses. With two-way ANOVA, we found significant direct effects of treatment [$F(2,13) = 191.95$, $p < 0.0001$] and time [$F(12,156) = 8.85$, $p < 0.0001$] on IOP. We also found a significant interaction between IOP and time [$F(24,156) = 4.09$, $p < 0.0001$]. Treatment accounted for 58.6% of the total variation and time accounted for 12.9% of total variation. Post-hoc Bonferroni multiple comparisons reveal that beginning with the first injection of HA, IOP was significantly elevated in HA-injected eyes compared with the PBS injected eyes for the remainder of the 12 weeks. At week one, the IOP of the HA+EDTA-MSM treated eyes was significantly higher than that of the HA+PBS eyes ($p < 0.001$). Two weeks after injection and later, IOP of eyes treated with EDTA-MSM were not significantly different from eyes without EDTA-MSM treatment.

Effect of EDTA-MSM on optic nerve lesion induced by elevated IOP

To evaluate the optic nerve lesion caused by elevated IOP, cytoarchitecture of the optic nerves was observed under bright field microscopy. Serial longitudinal sections of the optic nerve initial segment including the back of the eyeball were stained with hematoxylin and eosin (Fig. 2). Within the eyeball itself, no marked thinning of the nerve fiber layer of the retina or optic nerve head excavation was observed. Optic nerves from the control group were observed to have a regular texture of nerve fibers and a typical narrow neck as the fibers cross the scleral plane, the rat equivalent of the human lamina cribrosa (Fig. 2A). This narrow neck was widened in the untreated HA eye (Fig. 2B).

In nerves from HA rat eyes treated with EDTA-MSM, the narrowed neck observed in controls was apparent (Fig 2C). Axonal fibers were regular and dense in optic nerves from control eyes (Fig. 2A), but loosely packed and disorganized in HA-injected eyes (Fig 2B). Additionally, highly focal swelling of nerve fibers in the optic nerve was detected. In EDTA-MSM treated HA-injected eyes, axonal organization resembled the regular and dense packing of control nerves (Fig. 2C). Disorganization of glial nuclei along with increased cellularity were detected in optic nerves from untreated HA nerves compared to control (Fig 2B). Vacuolation was also apparent in untreated HA nerves. EDTA-MSM treatment ameliorated these defects (Fig 2C). Edema of the optic nerve from untreated HA-injected eyes compared to PBS-injected eyes can readily be observed in cross-sections of the optic nerve (Fig. 3A, B.) Treatment with EDTA-MSM ameliorated this edema (Fig 3C.)

Additional longitudinal sections of optic nerve were stained with Luxol fast blue to evaluate the effect of increased IOP on myelination in the optic nerve. In sections from control nerves, axons are densely stained with Luxol fast blue indicating robust myelination typical of the optic nerve caudal to the lamina cribrosa (Fig. 4A.) As shown in Fig. 4B, the intensity of Luxol blue stain in the optic nerve from eyes with HA injection and no EDTA-MSM treatment is reduced showing that demyelination has occurred. Though not as robust as in the control optic nerve, myelin staining in EDTA-MSM treated nerves is darker than seen in untreated nerves (Fig 4C). This implies that EDTA-MSM treatment partially ameliorates demyelination.

Effect of EDTA-MSM treatment on oxidative stress and inflammatory markers in eye and optic nerve from rats with elevated IOP

Oxidative stress induces lipid peroxidation in polyunsaturated fatty acids that generates a great diversity of cytotoxic aldehydes *in vivo* as well as *in vitro*. These toxic aldehydes interact with proteins and form adducts. We examined the effect of EDTA-MSM on the protein adduct expression of three different toxic lipid derived aldehydes, 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE), and malonaldehyde (MDA). Retina and optic nerve from control rats showed little evidence of protein-HHE adducts (Fig. 5A, D). Staining for protein-HHE adducts was robust in the optic nerves and photoreceptor layer of the retina in HA-injected eyes (Fig. 5B, E). Treatment with EDTA-MSM nearly eliminated protein-HHE adducts in the optic nerves and greatly reduced them in the retinas of HA-injected eyes (Fig 5C, F).

Protein HNE-adducts staining was observed in the optic nerves and the photoreceptor layer of the retina in all groups of eyes (Fig. 6). However, the staining was much more intense in the optic nerves and photoreceptor layer of eyes injected with HA than in the other two groups (Fig. 6B, E). Treatment with EDTA-MSM reduced protein-HNE adducts in the optic nerves and retinas of HA-injected eyes, particularly in the photoreceptor layer (Fig 6C, F).

Protein-MDA adducts were also present in all groups (Fig. 7). However, protein-MDA staining was stronger in the outer nuclear layer, with very little in the photoreceptor layer, in contrast to protein HHE and protein HNE staining. In HA-injected eyes, there was increased aggregated staining in optic nerve, outer nuclear layer, and ganglion cells (Fig. 7B, E). EDTA-MSM treatment reduced protein-MDA staining to near control levels (Fig. 7C, F). Optic nerve cross-sections stained fluorescently also showed an increase in protein-MDA. Counterstaining the optic nerve with DAPI revealed that glial cell nuclei appeared to be shrunken and condensed compared to controls (Fig. 7G, H). EDTA-MSM treated nerves appeared similar to control nerves (Fig. 7G, I).

Increases in oxidative stress as well as changes to membrane properties induced by lipid peroxidation can lead to influx of Ca^{2+} . Such influx turns on the expression of a number of proteins of the inflammation pathway, including cyclooxygenase (COX), the rate-limiting enzyme in the synthesis of prostaglandins. Prostaglandins are involved in the regulation of normal IOP, and their analogues are used to manage glaucoma [29]. Therefore, we next examined COX-2, the generally inducible isoform. In optic nerves from HA-injected eyes, the expression of COX-2 was increased compared to control nerves (Fig. 8A, B).

Application of EDTA-MSM decreased the levels of this inflammatory marker to control levels (Fig. 8C). This demonstrates that treatment with EDTA-MSM prevented the increase in COX-2 caused by elevated IOP, and possibly decreased damaging inflammation in general.

Quantification of the immunohistochemical Images

Image analysis of the immunostained slides was conducted (described in the Methods section) for a quantitative comparison of immunoreactivities among all the three groups of animals (Figure 9). Immunoreactivity of HHE in the optic nerve head and photoreceptor layer increased by 5.9- and 16.6-fold as compared to the control, respectively. Treatment with EDTA+MSM brought about a 40% and 64% reduction of the increase, respectively. Immunoreactivity of HNE in the optic nerve head and photoreceptor layer increased by 4.2- and 2.6-fold as compared to the control, respectively. Treatment with EDTA+MSM brought about a 50% and 40% reduction of the increase, respectively. Immunoreactivity of MDA in the optic nerve head (Longitudinal section), photoreceptor layer and optic nerve head (transverse section) increased by 3.38, 3.24- and 3.10-fold as compared to the control, respectively. Treatment with EDTA+MSM brought about a 59%, 55% and 72% reduction of the increase, respectively. Immunoreactivity of Cox-2 in the central optic nerve increased by 3.43-fold as compared to the control and treatment with EDTA+MSM brought about a 55% reduction of the increase.

EDTA-MSM amelioration of retinal ganglion cell loss induced by elevated IOP

In humans, the death of retinal ganglion cells is generally thought to be the reason for vision loss [30]. We compared total number of retinal ganglion cell somata in cross section of eyes in the different eye treatment groups ($n = 2-3$ eyes/group; Fig. 10). Ganglion cell counts were as follows: 1) control 521.3 ± 14.5 ; 2) untreated with HA injection 445.7 ± 27.2 ; and 3) HA-injection with EDTA-MSM treatment 576.5 ± 20.5 . The results show a statistically significant drop in the number of ganglion cells in the HA group as compared to the control. Treatment with EDTA+MSM prevented the HA-induced drop in the ganglion cells. The p-values of control vs. HA was 0.035, control vs. HA+ EDTA-MSM was 0.053 and HA vs. HA+ EDTA-MSM was 0.021 by one-way ANOVA with Tukey's post test.

Discussion

Metal chelation can hypothetically intervene at various levels of biochemical cascades to ameliorate oxidative damage. *Upstream*, chelation can inhibit production of ROS by binding transition metals and inhibiting Fenton and Haber-Weiss reactions. *Midstream*, it can reduce metal-catalyzed lipid peroxidation reducing the formation of highly toxic lipid-derived aldehydes. *Downstream*, chelation can inhibit the calcium-dependent caspases and kinases and thus ameliorate inflammation and apoptosis.

Metal chelation therapy has been suggested for diseases with a strong oxidative stress component [31–34]. Within ophthalmic diseases, recent studies have shown that oral and systemic administration of the metal chelator deferiprone (DFP) is effective in ameliorating signs of retinal degeneration in transgenic mouse models of age-related macular degeneration [35, 36]. Combination free-radical scavenger/metal chelator compounds have

been shown to mitigate retinal damage induced by light [37]. We have previously shown that chelation by EDTA-MSM is effective in reducing oxidative stress-induced toxicity in diabetic cataract and in an *in vitro* model of neurodegenerative disease [16, 38]. Surprisingly, chelation therapy has not been investigated for glaucoma. This study is the first to demonstrate the efficacy of chelation in the form of topically administered EDTA-MSM in reducing pathology in a rat model of glaucoma.

Animal models of glaucoma have been created by experimentally elevating IOP using several methods. Commonly used methods induce elevated IOP by decreasing aqueous humor outflow and include injecting hypertonic saline into the episcleral vein to sclerose the trabecular meshwork [39], and laser photocoagulation of the trabecular meshwork [40]. Cautery of the episcleral vein [41] or vortex veins [42] is also used to increase IOP, but these methods induce congestion of the uveal vasculature and result in less optic nerve and RGC damage. These methods also require special equipment and techniques, and need some time to develop stabilized ocular hypertension. Another method utilizes repeated periodic injection of foreign particles into the anterior chamber to increase IOP by blocking outflow through the trabecular meshwork. Particles include autologous ghost red blood cells [43], latex microspheres [44], and viscoelastic extracellular matrix materials such as hyaluronic acid (HA; [27]). Since previous studies demonstrate that repeated intracameral HA injection in rats can effectively increase IOP and induce glaucomatous changes in retina and optic nerve, we used this method to induce ocular hypertension in rats.

Several different methods to chronically increase IOP in rats have been shown to result in elevated oxidative stress [27, 45, 46]. Using acutely elevated IOP, Liu et al. (2007) showed increase in the markers of oxidative stress starting one hour of increased IOP in mice. In human glaucoma patients, oxidative DNA and protein damage has been detected in both aqueous humor and serum [47, 48]. Although EDTA-MSM treatment did not affect IOP in our study, it did alleviate signs of glial changes and markers of oxidative stress that may be the more damaging aspect of glaucoma. It has been previously reported that free radical scavengers increased ganglion cell survival in eyes with elevated IOP [45] or oxidative stress induced by NMDA intraocular injection [49]. In our system, EDTA-MSM can be regarded as an indirect free radical scavenger since by chelating transition metals, it acts to decrease the generation of ROS and thus, oxidative stress-induced damage.

Lipid peroxidation, a particularly damaging result of oxidative stress, is known to occur in glaucoma. Lipid-derived aldehydes commonly measured as biomarkers of lipid peroxidation include HNE, HHE and MDA [8]. MDA-thiobarbituric acid reactive substances (TBARS) have been reported in the aqueous humor of human glaucoma patients [50] and in the retinas of rats injected with HA [27]. MDA has also been measured spectrophotometrically in rat retina [45]. HNE was found to be elevated in mouse retina after only 1 hour of increased IOP [51]. HHE has not been reported in glaucoma, though it has been reported to be present in other eye diseases [52, 53]. In our study, protein-HNE, -HHE and -MDA adducts in retina were reduced by EDTA-MSM treatment. Lipid aldehyde disruption of plasma membranes allows abnormal influx of Ca^{2+} that can result in neuroinflammation. One sequel of this inflammation is glial activation. Oxidative injury to astrocytes in the pre-laminar optic nerve head has been observed in human glaucoma [54]. Loss of the support functions of glia

contributes to glaucomatous damage in axons traversing the lamina cribrosa. Additionally, glial cells exposed to ROS are potent inducers of T-cell activation, leading to amplification of inflammatory cascades [55]. One protein of the inflammation pathway important in glaucoma is cyclooxygenase (COX), the rate limiting enzyme in the synthesis of prostaglandins. Prostaglandins are involved in the regulation of normal IOP and their analogues are used to manage glaucoma [29]. Increased oxidative stress and inflammation are often observed together, and it has been suggested that the COX-2 isozyme acts as a bridge between these states [56].

In various glaucoma models, COX-2 expression has shown differing patterns. COX-2 staining is negative in optic nerve of normal human eyes, and is only sparsely detected in late stage or severe glaucoma [57, 58]. On the other hand, COX-2, constitutively expressed in the ciliary body of normal human eye, is reduced in patients with primary open-angle glaucoma [59]. In spontaneously glaucomatous dog eyes, COX-2 expression is low in normal eye, but elevated in glaucoma [60]. These varying results may be explained by the varying causes of IOP. In studies where ischemia/reperfusion dominates other factors, the COX-2 signal becomes stronger. Increased COX-2 expression has been detected in rat retina at 6 to 24 hours [61] and at 2 and 7 days after ischemia/reperfusion injury [62]. Repeated HA injections result in transient and repeated elevations of IOP and thus share features with ischemia/reperfusion injury. During our experiment, ischemia of the iris was observed and regarded as a sign of successful injection. Therefore, it is not surprising that COX-2 expression in our study was upregulated in the post laminar region of the optic nerve. This enhanced expression of COX-2 was inhibited by EDTA-MSM, implying that EDTA-MSM ameliorates inflammation caused by ischemia/reperfusion injury. Chelation of excess free Ca^{2+} that stimulates inflammatory cascades is a possible mechanism for this reduction.

The death of retinal ganglion cells, possibly the last degenerative step in glaucoma [63], is generally thought to be the reason for vision loss. Ganglion cell loss has been previously shown in rodent models of glaucoma [49, 64]. In our study, ganglion cell loss was significantly prevented by EDTA-MSM. Since the EDTA-MSM treatment decreased biomarkers of oxidative stress without decreasing IOP, this implies that a decrease in ROS formation and subsequently decreased lipid peroxidation may play a more important role in prevention of glaucomatous neurodegeneration and blindness than injury caused directly by ocular hypertension.

Paradoxically, EDTA-MSM improved histological symptoms of glaucoma without reducing IOP. Though this may initially appear to be counterintuitive, the actual relationship between elevated IOP and glaucomatous neurodegeneration is tenuous. More than 50% of human patients with glaucoma do not show elevated IOP [65]. Furthermore, typical optic nerve lesions and visual field defects are found in some patients without elevated IOP [66–68]. Even more baffling, some patients with normal-tension glaucoma benefit from further lowering of IOP, although it is not clear what proportion of patients benefit from this therapy [69]. Conversely, as many as half of the patients on IOP-lowering therapy continue to experience neurodegeneration [70]. Despite this poor correlation, the lowering of IOP is currently the treatment of choice for glaucoma. The present results indicate that a metal chelator co-applied with the permeability enhancer MSM may be an effective therapeutic

strategy for protecting the retina and optic nerve against oxidative damage in glaucoma. The fact that EDTA-MSM ameliorated symptoms without decreasing IOP can be seen as a positive sign. Since most current therapies aim at reducing IOP and damage occurs even in the absence of elevated IOP, EDTA-MSM has the potential to work in conjunction with pressure-reducing therapies to alleviate damage to the optic nerve and retinal ganglion cells. EDTA-MSM may break the vicious cycle of oxidative stress→inflammation→neurodegeneration and allow endogenous antioxidant systems to eliminate accumulated ROS, repair tissue damage, and prevent neuronal death and blindness.

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Abbreviations

COX-2	cyclooxygenase-2
DFP	deferiprone
EDTA	ethylenediaminetetraacetic acid
HA	hyaluronic acid
HHE	4-hydroxyhexenal
HNE	4-hydroxynonenal
IOP	intraocular pressure
MDA	malonaldehyde
MSM	methyl sulfonyl methane
ROS	reactive oxygen species
TBARS	thiobarbituric acid reactive substances

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Highlights

- Topical applied EDTA reduces signs of oxidative stress and inflammation in glaucoma
- Chelation reduces neurodegeneration in glaucomatous retina and optic nerve
- Oxidative stress and inflammation are relieved without lowering the IOP
- Chelation plus IOP lowering therapies could be effective treatment for glaucoma

Figure 1.

EDTA-MSM treatment had no effect on IOP. Graph of IOP measured weekly over the 12 weeks of this experiment. There was no difference between the groups before injection (week 0). There were no statistical differences between the PBS-injected eyes treated with PBS or EDTA-MSM at any time point (repeated measures two-way ANOVA), so these values were pooled for further statistical analysis. Both HA-injected groups had significantly higher IOP values than the PBS injected group from the first week onward. In week one, the IOP of the HA+EDTA-MSM treated eyes was significantly higher than that of the HA+PBS eyes ($p < 0.001$). At later time points, there was no significant difference between the HA-injected eyes treated with PBS or EDTA-MSM.

Figure 2.

EDTA-MSM ameliorated cytoarchitectural disruption of the optic nerve induced by HA injection. Longitudinal sections of paraffin-embedded rat eyeballs including the optic nerve head and initial segment were stained with hematoxylin and eosin. Upper panel: 100X magnification, Lower panel: 200X magnification. N: Typical narrow neck as fibers cross the scleral plane, the rat equivalent of the lamina cribrosa in humans. *Center of optic nerve. (A) PBS-injected eye. Fibers in optic nerves from control eyes had a dense and regular texture. (B) HA injected eye. Nerve fibers were loosely packed and disorganized in HA-injected eyes. Highly focal swelling of nerve fibers was observed in these eyes. The narrowed neck of the optic nerve as it passes through the sclera was widened. Disorganization of astrocytic nuclei along with increased cellularity was seen in nerves from HA-injected eyes as compared to control and HA+EDTA-MSM, while swelling was much less in PBS and HA+EDTA-MSM eyes. (C) HA injected eye treated topically with EDTA-MSM. Fibers in optic nerves had a dense and regular texture similar to that seen in control eyes. The optic nerve fibers as they cross the sclera plane resembled the normally narrow neck. Organization and cellularity of glial nuclei also appeared normal. Within the eyeball itself, no significant thinning of the nerve fiber layer of the retina or optic nerve head excavation was observed.

Figure 3.

EDTA-MSM ameliorated the edema of optic nerve under HA-induced ocular hypertension. Transverse frozen section of optic nerve, stained with hematoxylin and eosin. Upper panel: 40X magnification; Lower panel: 200X magnification. (A) Optic nerve of control eye without elevated IOP. The texture of the nerve is regular with densely packed fibers. (B) Optic nerve from HA-injected eyes. Edema of the optic nerve in these eyes as compared to control eyes can readily be observed in cross-sections of the optic nerve. Vacuolization is apparent at the higher magnification. (C) Optic nerve of HA-injected eyes treated with EDTA-MSM. Optic nerve fibers are regular and dense with no apparent edema or vacuolization.

Figure 4.

EDTA-MSM treatment mitigates demyelination in the optic nerve associated with increased IOP. Longitudinal sections of the optic nerve are stained with Luxol fast blue and counterstained with cresyl violet. 200X magnification. (A) Control optic nerve from eye injected with PBS. Axons are densely stained indicating robust myelination typical of the optic nerve caudal to the lamina cribrosa. (B) Optic nerve from eye injected with HA. The intensity of Luxol blue stain in optic nerve from untreated eyes with is greatly reduced compared with control, suggesting that demyelination has occurred in these nerves. (C) Optic nerve from eye injected with HA and treated with EDTA-MSM. Though not as robust as in the control optic nerve, myelin staining in EDTA-MSM treated nerves is darker than seen in untreated nerves.

Figure 5.

Treatment with EDTA-MSM reduced protein-HHE adducts in the optic nerves and retinas of HA-injected eyes. Anti-protein-HHE immunohistochemistry counterstained with hematoxylin. Upper panel: optic nerve head and longitudinal section of initial segment of optic nerve, 100X magnification; lower panel: cross-section of retina, 600X magnification. (A, D) Control optic nerve and retina. Staining for protein-HHE adducts is not visible in control optic nerve or in retina. (B, E) Aggregates of HHE-protein adducts are visible within the optic nerve from HA-injected eyes. In the retina, protein-HHE aggregated in photoreceptor layer (arrow), with some staining in the outer nuclear layer and in retinal ganglion cells. (C, F) EDTA-MSM treated optic nerve and retina. Treatment with EDTA-MSM nearly eliminated these protein adducts in the optic nerves of treated HA-injected eyes. EDTA-MSM also ameliorated accumulation of protein-HHE adducts in the photoreceptor layer, where it was weak in treated HA-injected eyes. Some staining remained in the outer nuclear layer and in ganglion cells.

Figure 6.

Treatment with EDTA-MSM reduced protein-HNE adducts in the optic nerves and retinas of HA-injected eyes. Anti-protein-

HNE immunohistochemistry counterstained with hematoxylin. Upper panel: longitudinal section of optic nerve, 100X magnification; lower panel: cross-section of retina, 600X magnification. (A, D) Control optic nerve and retina. Protein HNE-adducts were observed in the optic nerves and the photoreceptor layer of the retina in all groups of eyes, including controls. (B, E) Protein-HNE staining was much more intense in the optic nerves and retina of eyes injected with HA than in controls.

Staining was particularly intense in the photoreceptor layer (arrows). (C, F) Application of EDTA-MSM ameliorated accumulation of protein-HNE adducts, particularly in the photoreceptor layer. In the optic nerve, protein-HNE staining was reduced to approximately the same level seen in control nerves.

Figure 7.

Treatment with EDTA-MSM reduced protein-MDA adducts in the optic nerves and retinas of HA-injected eyes. Upper and center panels, Anti protein-MDA immunohistochemistry counterstained with hematoxylin. Upper panel: longitudinal section of optic nerve, 100X magnification; Center panel: cross-section of retina, 600X magnification. Lower panel: fluorescent staining of anti-protein-MDA (red) counterstained with DAPI (blue). Transverse sections of optic nerve, 600X magnification. (A, D, G)

Control optic nerve and retina. Protein MDA-adducts were observed in the optic nerves and in all layers of the retina in all groups of eyes, including controls. (B, E, H) Protein-MDA staining was strongly aggregated in optic nerve of eyes injected with HA. In the retina, staining was observed in all layers, but was strongest in the inner nuclear layer. Glial cell nuclei in optic nerves (H: DAPI stain) appear shrunken and condensed compared to control. (C, F, I) Application of EDTA-MSM ameliorated accumulation of protein- MDA adducts, such that staining level is similar to that in control eyes. In the optic nerve, protein-MDA staining was also reduced to approximately the same level seen in control nerves. Glial cell nuclei resembled those seen in control eyes.

Figure 8.

EDTA-MSM treatment decreased the activity of COX-2 in HA-injected eyes. Optic nerve cross-section. Upper panel: COX-2 fluorescent immunohistochemistry (green). Lower panel: COX-2 fluorescence (green) co-stained with DAPI (blue). (A, D) Control optic nerve from eye injected with PBS. Little fluorescence from COX-2 was visible in control optic nerve. (B, E) Optic nerve from eye injected with HA. Increased fluorescence corresponding to the increased levels of COX-2 was observed in the optic nerve from HA-injected eyes. (C) Optic nerve from eye injected with HA and treated with EDTA-MSM. Fluorescence levels in treated nerves resembled that of control nerves.

Figure 9.

Quantification of positive immunoreactivity of HHE, HNE, MDA, and COX2 (shown in Figs. 5–8) in retinal photoreceptor layer, optic nerve head, or central optic nerve. Immunoreactivity is expressed as the integrated density per unit area (per 1 mm² for retinal photoreceptor layer and optic nerve transverse sections; per 6 mm² for optic nerve longitudinal sections) as measured by NIH Image. (A) Quantification of protein- HHE expression in optic nerve head and retina photoreceptor layer shown in Fig 5. (B) Quantification of protein-HNE expression in optic nerve head and retina photoreceptor layer shown in Fig 6. (C)

Quantification of protein-MDA expression in retina photoreceptor layer, optic nerve (longitudinal section), and optic nerve (transverse sections) shown in Fig 7. (D) Quantification of COX2 expression in central optic nerve shown in Fig 8. Statistical significance was determined using one-way ANOVA with Tukey's post test. * p < 0.05, vs. corresponding control; #, p < 0.05, vs. HA alone.

Figure 10.

EDTA treatment ameliorated retinal ganglion cell loss in eyes with elevated IOP. Bars show the average of total number of RGC somata in cross sections of eyes from different treatment groups ($n = 2-3$ eyes/group). Ganglion cell counts were as follows: control without elevated IOP, 521.33 ± 25.15 ; untreated (HA only) 445.66 ± 47.06 ; and treated (HA + EDTA-MSM) 576.5 ± 28.99 . There is a significant increase in RGC loss in the HA injected group as compared to the control group (one-way ANOVA with Tukeys post test, $p = 0.035$). EDTA-MSM treatment significantly decreased RGC loss in eyes with elevated IOP (treated) compared to eyes with elevated IOP and no treatment (untreated; $p = 0.026$). RGC loss in the HA + EDTA-MSM was not significantly different from the control group ($p = 0.053$). * $p < 0.05$, vs. corresponding control; ** $p < 0.05$, vs. HA alone