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TOPICAL METAL CHELATION THERAPY AMELIORATES OXIDATION-INDUCED TOXICITY IN DIABETIC CATARACT

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Oxidative stress plays a critical role in cataractogenesis, the leading cause of blindness worldwide. Since transition metals generate reactive oxygen species (ROS) formation, metal chelation therapy has been proposed for treatment of cataracts. However, the effectiveness of most chelators is limited by low tissue penetrability. This study is the first to demonstrate that the topically applied divalent metal chelator ethylenediamine tetraacetic acid (EDTA) combined with the carrier and permeability enhancer methyl sulfonyl methane (MSM) ameliorates both oxidation-induced lens opacification and the associated toxic accumulation of protein-4-hydroxynonenal (HNE) adducts. Both in vitro (rat lens culture) and in vivo (diabetic rats), EDTA–MSM (1) significantly reduced lens opacification by about 40–50%, (2) significantly diminished lens epithelial cell proliferation and fiber cell swelling in early stages of cataract formation in vivo, and (3) notably decreased the levels of protein–HNE adducts. These findings have important implications specifically for the treatment of cataract and generally for other diseases in which oxidative stress plays a key pathogenic role.

Cataract is the leading cause of blindness and visual impairment globally, accounting for about 48% of blindness (Congdon et al., 2004). The prevalence of mature cataract in Americans over 40 years of age was estimated at 20.5 million (17.2%) in 2000, and is projected to rise to 30.1 million by 2020. The incidence of cataract increases with age, with more than two-thirds of individuals over 80 years old having mature cataracts. Diabetes mellitus is a major risk factor for cataract, with cataractogenesis being one of its earliest secondary complications (Kyselova et al., 2004). The age-adjusted prevalence for cataracts in subjects older than 50 years of age is higher among those with diabetes (31.8%) than among those without diabetes (21.2%) (Saaddine et al., 2004).

There is a need for novel interventions that will maintain the transparency of the lens. It is estimated that a delay of 10 years in cataract formation may reduce the prevalence of cataract by 45% (Kupfer, 1985). Such a delay would enhance the quality of life for much of the world’s older and diabetic population and considerably diminish the economic burden of disability and surgery. Currently, surgery is the sole treatment option and is primarily indicated when the cataract reduces visual function to a level that interferes with the daily activities of the patient. Therefore, surgery primarily occurs for late-stage cataracts, leaving many patients...
Metal chelation therapy was proposed for delaying cataract, based on the hypothesis that metal-catalyzed oxidation of biological macromolecules occurs in the lens and contributes to cataract formation (Garland, 1990). Some anti- cataract drugs possess metal-chelating action and potentiation of this property could drive future drug design (Woollard et al., 1990). Aldose reductase inhibitors delay diabetic cataracts by functioning as metal chelators in addition to their primary function of inhibiting the sorbitol pathway (Ou et al., 1996). Advanced glycosylation end products interact with lens crystallins and redox-active copper (Cu) that catalyzes ascorbate oxidation and suggest that chelation therapy may be efficacious (Saxena et al., 2000). Increasing levels of iron (Fe) associated with aging are involved in a broad range of ocular diseases including cataract, and therefore the therapeutic potential of limiting metal-induced oxidative damage is high (He et al., 2007). Lipid peroxidation, catalyzed by Fe^{2+}, generates high levels of alkoxyl radicals that degenerate into saturated and unsaturated aldehydes such as 4-hydroxynonenal (HNE). Three functional groups in HNE provide unusually high reactivity—the α,β-unsaturated carbon–carbon double bond, the aldehyde group, and the hydroxyl group. HNE can readily conjugate to various amino acids to form protein–HNE adducts (Esterbauer et al., 1991). Protein–HNE adducts can intercalate cell membranes and change membrane fluidity (Chen & Yu, 1994; Subramaniam et al., 1997).

Despite the rationale supporting the use of metal chelation therapy for cataract, its utility for therapeutic purposes has been limited by the problem of bioavailability (Liu et al., 2006). Permeability barriers such as cell membranes and blood–brain or blood–retinal barriers limit availability of chelators to tissues. Ethylenediamine tetraacetic acid (EDTA) binds Fe and Cu ions and makes it particularly attractive for preventing metal-catalyzed oxidative reactions underlying cataract. Methyl sulfonyl methane (MSM) was successfully used as a permeability enhancer to deliver EDTA into the lens (Zhang et al., 2009). MSM is an organosulfur compound that is widely used as a dietary supplement in the nutraceutical market with no apparent known toxicity. The studies reported here were conducted to determine if topically applied EDTA–MSM prevents or ameliorates oxidation-induced lens opacification and toxic accumulation of protein–HNE adducts.

MATERIALS AND METHODS

Materials

Ethylenediaminetetraacetic acid, MSM, streptozotocin (STZ), ferrous ammonium sulfate, ferric chloride, adenosine 5′-diphosphate (ADP), ascorbic acid, and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO). All cell culture medium components were obtained from Invitrogen Corporation (Carlsbad, CA). Protein–HNE antibodies were purchased from Cayman Chemicals (Ann Arbor, MI). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (Ig) G was purchased from BioRad (Hercules, CA). Biotinylated secondary antibody was from the LSAB+ System-HRP kit (Dako North America, Inc., Carpinteria, CA).

Animals

Male Sprague-Dawley rats were obtained from Harlan Laboratories (Houston, TX). Rats
weighing 200–250 g were used for the in vitro lens culture studies. Rats weighing approximately 100 g were used for the in vivo diabetic cataract study. National Institutes of Health (NIH) guidelines and the Society of Toxicology's “Guiding Principles in the Use of Animals in Toxicology” were strictly followed to ensure the welfare of the animals.

**In Vitro Lens Organ Culture**

Rat lenses were dissected with the capsule intact, and washed with 1% penicillin/streptomycin in sterile phosphate-buffered saline (PBS), pH 7.4. The lenses were cultured in TC-199 medium that contained 0.1% gentamicin at 37°C in a 5% CO₂ humidified atmosphere. Lenses were exposed to oxidants in the absence and presence of EDTA (0.5 mM) and MSM (4 mM). Oxidants used were 50 mM glucose and 1 mM ascorbate and 100 µM H₂O₂. To mimic a diabetes-like state, 50 mM glucose was included as an oxidant. The medium was changed every 24 h for up to 7 d. The lenses were visualized under a Nikon Eclipse 200 microscope (Nikon Instruments, Inc., Melville, NY), and photographs were taken using the Multi Dimensional Imaging System (Multi Dimensional Imaging, Austin TX). The transparency of the lenses was quantified by digital image analysis using the Digital Images System (Buckinghamshire, UK). Two lenses were measured in each group, and the mean percent transmittance was calculated. After 5 d in culture, the rat lens epithelia along with the capsules were removed under a surgical microscope, mounted on glass slides, fixed, and processed for immunohistochemistry as described later.

**In Vivo Diabetic Study**

Based on previous in vitro and in vivo experiments (Zhang et al., 2009), a molar ratio of 1:8 for EDTA to MSM was found to be effective. Lower and higher doses of EDTA with a 1:4 molar ratio were also investigated. Five experimental groups of 6 rats each were used:

- Group 1—Diabetic administered PBS.
- Group 2—Diabetic administered 0.1% EDTA + 0.1% MSM.
- Group 3—Diabetic administered 0.25% EDTA + 0.5% MSM.
- Group 4—Diabetic administered 0.5% EDTA + 0.5% MSM.
- Group 5—Nondiabetic control administered PBS.

To induce diabetes, 100 g rats received intraperitoneal (ip) injections of STZ (60 mg/kg) in 0.1 M citrate buffer, pH 4.5, as previously described (Srivastava & Ansari, 1988). Streptozotocin is toxic to insulin-producing beta cells of the pancreas and produces hyperglycemia within a few days (Mansford & Opie, 1968). Control rats were injected with an equivalent volume of citrate buffer. Three days post STZ administration, blood glucose levels were assessed using a glucose meter. A distal tail snip generated the 5-µl quantity of blood necessary for analysis. Weekly glucose levels were determined at 9 a.m. by removing the scab formed on the tail. Ten days after the STZ injection, treatment with eyedrops was begun. Diabetic and nondiabetic rats were administered PBS or various doses of EDTA–MSM twice daily in the form of a 10-µl eye drop. Fresh solution was made weekly and stored at 4°C. Lenses were examined in vivo 0, 40, and 60 d after the beginning of eyedrop treatment. The pupils were dilated with 1% atropine sulfate ophthalmic solution (Alcon Laboratories, Inc., Fort Worth, TX) and the lens was examined using an ophthalmoscope (Welch Allyn halogen ophthalmoscope, Skaneateles Falls, NY). Score of lens opacity was determined on a scale of 0 to 10, where 0 represents a clear lens and 10 an opaque lens demonstrating mature cataract.

For histological studies, a second set of diabetic and nondiabetic rats were treated with and without the optimal concentration of 0.25% EDTA + 0.5% MSM. These rats were sacrificed by CO₂ inhalation after 10 d or 20 d of eyedrops treatment. These times were used to monitor the early changes in lens epithelium and fibers that precede the formation of mature cataract. As the cataract matures,
it becomes difficult to obtain artifact-free sections. Eyeballs were fixed, sectioned, and processed for immunohistochemistry as described next.

**Immunohistochemistry**

The slides of the lens epithelia generated in the in vitro were fixed for 10 min in 4% paraformaldehyde, transferred in 75% alcohol, and stored at 4°C. Slides were first treated with 0.5 mg/ml proteinase XXIV in PBS, and incubated for 5 min in a 37°C water bath. After three 10-min washes, 5% goat serum in PBS was added and slides were incubated for 1 h at room temperature. The primary antibody used was anti-protein–HNE antibody produced in rabbits (1:250 dilution). After incubation with the primary antibody for 1 h at room temperature, slides were incubated with secondary antibody for 1 h at room temperature. The secondary antibody used for the in vitro study was goat anti-rabbit IgG conjugated with FITC (1:750 dilution). The slides were mounted with FluorSave reagent (Calbiochem, San Diego, CA).

In the in vivo studies, eyeballs were fixed overnight in methacarn solution (60% absolute methanol, 30% chloroform, and 10% glacial acetic acid) at 4°C. Eyes were sectioned at 4 µm. Sections were incubated in primary antibody (anti-protein HNE diluted 1:250) for 30 min at room temperature. The secondary antibody was biotinylated anti-rabbit IgG from the LSAB+System-HRP kit. The slides were visualized under a Nikon Eclipse 800 upright microscope using either epifluorescence or bright-field imaging. Subsequent image capture and analysis was performed using Metamorph software (MDS Analytical Technologies, Toronto, Canada).

**Data Analysis**

The data are expressed as the mean ± SEM. Statistical comparisons were made using two-way analysis of variance (ANOVA), followed by post hoc evaluation of the treated versus untreated groups with the Bonferroni analysis using Prism software (Graph Pad Software, Inc., La Jolla CA). The criterion for significance was set at $p < .05$.

**RESULTS**

**EDTA–MSM Ameliorates Opacification and Oxidation-Induced Toxicity in Rat Lens Cultures**

Rat lenses were cultured for up to 7 d with or without oxidants (glucose or ascorbate and H$_2$O$_2$), in the presence or absence of EDTA–MSM. As shown in Figure 1, A and D, lenses cultured for up to 7 d without any added oxidants remained clear, while those cultured with 1 mM ascorbate + 100 µM H$_2$O$_2$ (Figure 1B) or 50 mM glucose (Figure 1E) developed nearly complete opacification. Lenses treated with oxidants in the presence of EDTA–MSM were less opaque as compared to those cultured with the oxidants alone (Figure 1, C and F). The overall transparency was greatly improved, and there remained an opaque ring that consisted of vacuolization in the outer cortex of the lens (Figure 1, C and F). The transmittance of the lenses was quantified by digital image analysis. The 100% transmittance of the control lenses was decreased to approximately 30% by the oxidants (Figure 1, B and E). With EDTA–MSM treatment, transmission was improved to approximately 70% (Figure 1, C and F).

Subsequently, rat lens epithelia along with capsules were processed for immunohistochemistry to qualitatively examine protein–HNE adduct formation. As shown in Figure 2A, the epithelium of control lenses showed very little protein–HNE immunostaining. Staining markedly intensified in the lenses incubated with 50 mM glucose (Figure 2B), and this rise in immunoreactivity was ameliorated by EDTA–MSM (Figure 2C).

**Topical EDTA–MSM Ameliorates Development of Diabetic Cataract in Rats**

In order to model diabetic cataract, hyperglycemia was induced by injecting rats with
FIGURE 1. EDTA–MSM ameliorates oxidation-induced opacification in rat lens organ cultures. Representative rat lenses cultured for 7 d. (A, D) Without oxidants. (B, C) With 1 mM ascorbate and 100 µM H$_2$O$_2$. (E, F) With 50 mM glucose. (A, B, D, E) No EDTA–MSM; (C, F) in the presence of 0.5 mM EDTA and 4 mM MSM. Lenses treated with oxidants are qualitatively much darker than control. Those treated with EDTA–MSM are less opaque. The dark ring seen in the EDTA–MSM-treated lens is residual vacuolization in the outer cortex of the lens.

FIGURE 2. EDTA–MSM ameliorated oxidation-induced toxicity in cultured rat lens epithelium. After 7 d in culture without or with glucose, in the presence or absence of EDTA–MSM, lens epithelia along with capsules were fixed and processed for immunohistochemistry using primary antibodies against protein–HNE. (A) Control epithelium without glucose. (B) Epithelium incubated in 50 mM glucose. (C) Epithelium incubated in 50 mM glucose with 0.5 mM EDTA and 4 mM MSM. Lens epithelia oxidatively stressed with glucose exhibit high levels of protein–HNE conjugates that are reduced in the presence of EDTA–MSM (400× magnification).

STZ. By 3 d after injection, 80% of the rats showed an increase in blood glucose, and by 10 d, all rats showed elevated blood glucose (Figure 3A). EDTA–MSM treatment did not affect the growth of the rats, as body weight did not vary significantly among the groups (Figure 3B). Nondiabetic rats did not exhibit cataract formation (Figure 4A). Topical EDTA–MSM significantly ameliorated the development of diabetic cataract. As shown in Figure 4D, diabetic rats treated with 0.25% EDTA + 0.5% MSM had significantly less lens opacification measured on d 40 and 60 as compared to diabetic rats treated with PBS. Lens opacity was reduced by about 50% on d 60. Diabetic rats treated with 0.1% EDTA + 0.1% MSM had significantly less lens opacification measured on d 40 as compared to diabetic rats treated with PBS. Representative images of diabetic rat lens show that EDTA–MSM decreased lens opacity after 60 d of treatment (Figure 4C).
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FIGURE 3. Hyperglycemia was induced by injecting rats with STZ 10 days before the initiation of EDTA–MSM treatment at d 0. Graphs show mean ± SEM. (A) STZ alone. (B) EDTA–MSM treatment plus STZ.

FIGURE 4. Topically applied EDTA–MSM ameliorated development of diabetic cataract in rats. EDTA–MSM or PBS was applied topically onto the eyes of diabetic rats twice per day. Lens opacification, evaluated over 60 d using an ophthalmoscope, was graded on a scale of 0–10, where 0 = clear and 10 = opaque. (A–C) Representative images of diabetic rat lens after 60 d of treatment with PBS or EDTA–MSM. (A) Normal rat showing no cataract development. (B) Diabetic rat's eye treated with PBS showing severe cataract development. (C) Diabetic rat eye treated with 0.25% EDTA and 0.5% MSM showing amelioration of the cataract phenotype. (D) Graph showing mean ± SEM of the four groups of rats (n = 6 rats per group). Asterisk indicates significantly different from diabetic rat (p < .05).

EDTA–MSM Ameliorates Oxidative Stress-Induced Toxicity in Diabetic Rats Lens

Since the 0.25% EDTA + 0.5% MSM dose was the most effective in ameliorating cataract in the in vivo study already reported, this dose was used to examine early histological changes in the lens that are associated with later cataract formation. Diabetic rats were treated twice daily with PBS or 0.25% EDTA + 0.5% MSM administered topically onto the eyes. Histological changes were examined at 10 and 20 d of EDTA–MSM treatment. Early changes in the eyes of diabetic rats that did not receive EDTA–MSM included damage in lens epithelial and fiber cells that can be observed in hematoxylin and eosin-stained lens sections. Figure 5 shows that treatment with EDTA–MSM markedly reduced epithelial cell proliferation as well as fiber cell swelling and damage, qualitatively observed at two early stages of cataract (10 and 20 d). The rescuing effects of EDTA–MSM were particularly noticeable at 20 d (Figure 5F), although differences in epithelial...
cell proliferation could already be seen at d 10 (Figure 5E). Eyeball sections were also processed with immunohistochemistry to qualitatively examine protein–HNE adduct formation. As illustrated in Figure 6, diabetes increased protein–HNE immunoreactivity in lens epithelium plus outer cortex. These cytotoxic changes became apparent as a diffuse staining under the epithelial cell layer at 10 d (Figure 6B). By 20 d, staining was pronounced and formed aggregates (Figure 6C). Treatment with EDTA–MSM decreased protein–HNE levels in diabetic rat lenses (Figure 6, E and F).

**DISCUSSION**

This study demonstrates that the metal chelator EDTA combined with the membrane permeability enhancer MSM reduces the cataractogenic effects of oxidants/hyperglycemia and accumulation of toxic protein–HNE adducts in both in vitro and in vivo cataract models. Increased generation of oxidants involving metal catalysts plays a critical role in the pathogenesis of cataracts of various etiologies, including senile and diabetic cataract (Garland, 1990; Srivastava et al., 1990; Spector, 2000; Truscott, 2005). Indeed, metal ions such as Cu and Fe are found in higher concentrations in cataractous versus normal human lenses (Garland, 1990; Garner et al., 2000). Metals participate at various levels of the oxidation-induced cytotoxic cascade that damages biomacromolecules and results in lens opacification (Garland, 1990; Xiao et al., 2004). *Upstream*, transition metal ions catalyze production of reactive oxygen species (ROS) including free radicals via Fenton-like reactions (Winterbourn, 1995). *Midstream*, metals participate with ROS in formation of toxic aldehydes via lipid peroxidation (Pryor & Porter, 1990). HNE is one of the most abundant and toxic of these (Esterbauer et al., 1991; Esterbauer, 1993) and was found to induce lens opacification (Ansari et al., 1996). HNE readily conjugates to certain amino acids leading to elevated levels of protein–HNE...
adducts (Esterbauer et al., 1991; Xiao et al., 2004). Downstream, protein–HNE adducts intercalate cell membranes and produce membrane fluidity changes, leading to calcium influx, and activation of caspases inducing apoptosis that contributes to cataractogenesis (Choudhary et al., 2002). Protein–HNE adducts have been associated to cataract formation in animal models with diminished aldehyde dehydrogenase activity (Lassen et al., 2007).

The body’s solution to controlling oxidative damage is to control the concentration of components necessary to produce them, specifically, ROS and metal ions. ROS are detoxified by enzymatic systems such as catalase, peroxidase, and superoxide dismutases (Michiels et al., 1994). Most metal ions are bound to protein for metabolic purposes such as for hemoglobin and myoglobin or for transportation purposes such as for transferrin and lactoferrin. In the cell, ferrous ions are oxidized and stored in the ferritin storage protein complex (He et al., 2007). However, in disease states and even in normal aging, antioxidant and metal binding capabilities can be compromised (Stohs & Bagchi, 1995).

Oxidative stress is an attractive target for pharmacological intervention because it is a common pathway for various etiologies of cataract formation, including aging, ultraviolet radiation, and diabetes. Targeted metal chelation therapy may have a greater net potency compared to antioxidant therapies in development for cataract. Antioxidants, including vitamin E, vitamin C, and carotenoids, showed early promise for limiting the risk of cataract in many epidemiological studies (Kyselova et al., 2004), although interventional trials have been less encouraging (Chiu et al., 2007). These antioxidants, unlike metal chelators, act only at the upstream level of ROS by either (1) preventing its formation via direct reduction of oxidizing species, or (2) eliminating created ROS (i.e., free radical intermediates) via scavenging, trapping, and quenching (Sies, 1993). Antioxidants are generally either lipophilic (e.g., vitamin E, carotenoids) or hydrophilic (i.e., vitamin C) (Sies, 1997), and thus may not necessarily cross membrane and vascular barriers to reach the site of ROS generation (Garland, 1990). Similarly, oral antioxidants’ lack of effectiveness may be due to the fact these agents did...
not reach the site of disease action (Christen, 1994). Most existing antioxidant therapies are currently not available as topical applications that can be applied close to the site of damage. In contrast to the single upstream level of action of antioxidants, chelation may act at multiple levels of the oxidation-induced cytotoxic cascade that result in opacification (Garland, 1990; Kyselova et al., 2004; Xiao et al., 2004). Metal chelation would be expected to have upstream (reduction of ROS) (Stohs & Bagchi, 1995), midstream (reduction of protein–HNE adduct production) (Xiao et al., 2004), and downstream effects (change in calcium influx, reduction of caspase activation and apoptosis) (Choudhary et al., 2002).

Our previous studies showed that the carrier MSM enhances permeability and facilitates the delivery of the chelator EDTA into the various eye tissues (Zhang et al., 2009). EDTA–MSM penetrated as far as the retina and vasculature of the choroid. This pharmacokinetic profile putatively allows EDTA–MSM to target the lens, and ameliorate metal-catalyzed oxidative damage of biomolecules implicated in age-related and diabetic cataractogenesis (Garland, 1990; Srivastava et al., 1990; Christen, 1994; Spector, 2000; Kyselova et al., 2004). In this current study, opacification of lenses was markedly reduced by EDTA–MSM treatment in two models—in vitro exposure to potent oxidants or in vivo STZ leading to marked hyperglycemia (sustained blood glucose levels of >500 mg/dl). In vitro, EDTA–MSM diminishes oxidation-induced opacification of cultured rat lens by about 40%. In these experiments, culture media contained Fe that may arguably have been chelated by EDTA. However, the in vivo STZ-induced hyperglycemic model did not use exogenous Fe. This leads us to conclude that EDTA–MSM is acting within the lens to chelate the available metal ions there, thus preventing opacification in situ.

In vivo, topical application of EDTA–MSM to diabetic rat eyes significantly reduced lens epithelial cell proliferation and fiber cell swelling in early stages of cataract formation, and significantly decreased opacification by about 50% after 60 d of daily treatment. Histological examination of PBS only treated lenses showed lens epithelial cell proliferation and lens fiber cells swelling and damage, confirming that opacification was occurring within the lens. In addition, the appearance of protein–HNE adducts implicated oxidative stress and lipid peroxidation in this opacification. In both models of oxidative stress, treatment with EDTA–MSM markedly decreased protein–HNE adduct formation. This would presumably protect epithelial cell membranes from fluidity changes (Chen & Yu, 1994; Subramaniam et al., 1997) and hinder downstream apoptotic events contributing to cataractogenesis (Xiao et al., 2004).

Finally, the findings presented here suggest diverse potential applications for EDTA–MSM beyond preventing cataract. Metal-catalyzed oxidation reactions have been implicated in a wide range of diseases and aging, for which metal chelation therapy might be beneficial. In ophthalmology, there is accumulating evidence that Fe may play a role not only in cataract, but also in age-related macular degeneration (AMD), glaucoma, and conditions producing intraocular hemorrhage, such as proliferative diabetic retinopathy (Christen, 1994). Systemic, local, or topical Fe chelation was suggested as having clinical potential for these disorders (He et al., 2007). Furthermore, loss of homeostasis of Fe and Cu was implicated in age-related neurodegeneration in Alzheimer’s and Parkinson’s diseases (Zecca et al., 2004). Accordingly, several reports suggest that metal chelation may help in the treatment of these and other neurological disorders (Zheng et al., 2005). In fact, metal chelation was proposed for many illnesses, including atherosclerosis (Kruszewski, 2004), cardiovascular complications in diabetes (Cooper et al., 2005), cancer (Buss et al., 2004), and inflammatory/autoimmune diseases such as multiple sclerosis (van Meeteren et al., 2005) and rheumatoid arthritis (Omoto et al., 2005). Our data suggest that further preclinical and clinical studies of EDTA–MSM in these various conditions are indicated. To date, there are no noninvasive treatments against retinal
disorders such as AMD, diabetic retinopathy, etc. It appears that EDTA–MSM eyedrops used in this study to treat diabetic cataract may be especially valuable in treating retinal diseases since EDTA–MSM is shown to penetrate ocular tissues and reach the retina (Zhang et al., 2009). Given this finding, EDTA–MSM treatment may offer hope to delay cataract of various etiologies as well as other ocular diseases in humans.

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