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Investigation of Novel Nanoplatforms for Delivery of an Anticataract Drug in Whole Lens Cultures

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Abstract

Cataracts are a leading cause of blindness, but the only available treatment is surgery, which may not be a viable option for many patients. Instead, pharmacological interventions that target cataractogenesis at the molecular level represent an attractive method for preventing or delaying cataract formation. Antioxidant drugs like N-(2-mercaptopropionyl)glycine may interfere with oxidative processes that lead to age-related nuclear cataracts. Nevertheless, achieving therapeutic concentrations in the lens has proven challenging. Novel delivery strategies such as functionalized nanodiamonds offer several advantages over conventional platforms in terms of stability and customizability. Therefore, we investigated the effects of three different types of functionalized nanodiamonds on the uptake and efficacy of a potential anticataract agent in a whole organ culture model of oxidative insult in the lens. Lenses treated with sodium selenite exhibited detrimental morphological changes and significantly deteriorated redox status. Lenses treated with the neat drug showed marked improvements. However, only hydroxylated nanodiamonds appeared to improve drug uptake, and their effects on lens glutathione and cysteine were modest. This work represents a critical step in understanding the anticataract effects of N-(2-mercaptopropionyl)glycine, and it suggests that other drug delivery strategies may be warranted to realize these effects *in vivo*.

Keywords

Oxidation, Nanoparticles, Models, Rats, Therapy, Ophthalmology

1. Introduction

Cataracts are the leading cause of blindness worldwide [1], and their prevalence is expected to increase as the global population ages [2] [3] [4]. Cataract sur-

gery is generally routine and effective but can be at a higher-risk procedure in patients with pre-existing conditions such as uncontrolled diabetes, glaucoma, and retinal degeneration [5]. Moreover, socioeconomic factors limit access for many patients in developing nations [1] [2]. Even without these considerations, surgically removing the lens may negatively impact long-term ocular health. The lens plays a vital and active role as an oxygen sink and antioxidant reservoir for surrounding ocular tissues [6]. As a result, there is a high demand for a non-invasive, economical approach to preserve the transparency of the native lens for as long as possible [7] [8]. In the U.S., forestalling cataract surgery by only ten years would cut the number of cataract surgeries in half [3].

Age-related nuclear (ARN) cataract arises when accumulated oxidative damage renders crystallin proteins less soluble and prone to aggregation [4] [7] [9]. The resulting aggregates scatter light, producing the characteristic cloudy appearance of cataractous lenses. In young, healthy lenses, very high concentrations of endogenous antioxidants, especially glutathione (GSH), prevent or repair this damage by scavenging radicals and reducing oxidized crystallin residues that would otherwise lead to aggregation. However, levels of GSH decrease significantly with age, leaving crystallins vulnerable to damage [2] [9]. Pharmacological approaches to cataract prevention often target oxidative modifications or remediate loss of antioxidant capacity as the lens ages [2]. Directly replenishing lenticular GSH via topical or systemic administration has proven challenging [8], as it does not easily pass through lipophilic membranes without a specific transporter [7] [10]. Instead, when GSH levels are low, thiol-containing drugs with more favorable pharmacokinetic profiles may be taken up more readily by ocular tissue, where they can protect crystallins by functioning similarly to GSH: forming adducts with electrophilic reactive species, undergoing thiol-disulfide exchange, and reducing radical species [10] [11] [12]. One such drug is tiopronin, or N-(2-mercaptopropionyl)glycine (MPG). Like GSH, it is an antioxidant, radioprotectant, metal chelator, radical scavenger, and thiol-disulfide exchanger [12]. It is already FDA-approved for the treatment of other conditions and has exhibited anticataract effects in multiple *in vivo* studies [13] [14] [15]. Accumulation in the lens through systemic administration would require high doses, thus increasing the risk of side effects. Local, topical delivery would circumvent these issues, but >95% is quickly washed away in tear fluid [7] [16]. Increasing MPG absorption with a poloxamer gel significantly delayed cataract onset and reduced severity compared to neat MPG [13]. However, polymer-based delivery strategies tend to be unstable with respect to sterilization and storage conditions needed for eyedrop formulation and distribution [16].

Detonation nanodiamonds (ND) offer several advantages for the ophthalmic delivery of thiol antioxidants over conventional delivery platforms: they are highly stable and biocompatible, with a tunable surface chemistry and release profile [17]. Additionally, ND reportedly exhibit intrinsic antioxidant effects [18] and may stimulate endocytosis [19] [20] [21], making them excellent candidates to deliver MPG to the lens. Our group recently characterized the loading and release pro-

files of MPG with three different types of surface-functionalized ND: carboxylated (ND-COOH), hydroxylated (ND-OH), and aminated (ND-NH_2) [22]. However, the ability of ND to improve the uptake of small-molecule antioxidant drugs in ocular tissues is unknown. To determine whether functionalized ND could improve lenticular uptake of MPG, we employed a whole organ culture model of selenite-induced oxidative insult, and we treated lenses with neat MPG and MPG loaded onto ND-COOH, ND-OH, or ND-NH_2. We then evaluated the ability of each treatment to protect against oxidative damage by examining lens morphology, MPG uptake, and thiol redox status.

2. Materials and Methods

N-(2-mercaptopropionyl)glycine (MPG), glutathione (GSH), glutathione disulfide (GSSG), L-cysteine (Cys), cystine, Tris-HCl, L-serine, boric acid, diethylenetriaminepentaacetic acid, tris(carboxyethyl)phosphine (TCEP), and sodium selenite (Na_2SeO_3), were purchased from MilliporeSigma (St. Louis, MO). All other reagents were purchased from Fisher Scientific (Fair Lawn, NJ) unless otherwise indicated. Stock solutions were generated in sterile Type 1 water prepared in-house with a Millipore Simplicity 185 System or passed through 0.22 μm nylon membrane filters under sterile conditions prior to formulating treatment media. Lenses were cultured in ATCC-formulated Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B (100 IU/mL) (Thermo Fisher Scientific) in a humidified incubator with 5% CO_2/95% air at 37˚C.

Whole eyes were extracted from male Wistar rats (4 weeks old, approximately 100 g) and shipped overnight in PBS in a chilled, insulated container (BioIVT, Westbury, NY). Eyes were dissected, and lenses were excised immediately upon arrival using a posterior approach. Lenses were then placed in complete medium (cEMEM) composed of EMEM supplemented with FBS and penicillin/streptomycin. Lenses were incubated for 2 hours at 5% CO_2 and 37˚C in a 24-well plate (1 mL in each well). Opaque and otherwise visibly damaged lenses were excluded from analysis. Intact lenses were randomly assigned to treatment groups and then gently transferred into 1 mL aliquots of cEMEM with additives corresponding to the treatment conditions shown in **Table 1**. Concentrations for Na_2SeO_3, MPG, and ND were selected based on preliminary experiments and previous literature [7] [23] [24] [25]. Separate stock suspensions of each functionalized ND loaded with MPG were generated by agitating each type of ND overnight at 4˚C in aqueous MPG solution, yielding ND-COOH: MPG, ND-OH: MPG, and ND-NH_2: MPG, as described previously [22]. For selenite-exposed groups, incubation media contained 0.1 mM Na_2SeO_3. Incubation media contained 5 mM MPG for MPG-treated groups, and incubation media with MPG loaded onto ND contained 50 μg/mL of functionalized ND and a total concentration of 5 mM MPG. Lenses were incubated at 37˚C and 5% CO_2 for 72 hours. After the incubation

Table 1. Experimental groups for sodium selenite exposure and treatment with neat MPG or MPG adsorbed onto ND.

Group	Description	Incubation Media	Abbreviation
1	Control	cEMEM + PBS	C
2	MPG only	cEMEM + neat MPG	M
3	Selenite only	cEMEM + Na_2SeO_3	S
4	Selenite + MPG	cEMEM + Na_2SeO_3 + neat MPG	S + M
5	Selenite + MPG adsorbed onto carboxylated ND	cEMEM + Na_2SeO_3 + ND-COOH: MPG	S + COOH: M
6	Selenite + MPG adsorbed onto aminated ND	cEMEM + Na_2SeO_3 + ND-NH_2: MPG	S + NH_2: M
7	Selenite + MPG adsorbed onto hydroxylated ND	cEMEM + Na_2SeO_3 + ND-OH: MPG	S + OH: M

period, lenses were carefully removed and gently rinsed with PBS. Excess non-lenticular tissue was gently removed (e.g. ciliary body and retina fragments), and lenses were weighed, promptly snap-frozen in microcentrifuge tubes, and kept at −80˚C until analysis.

Serine-borate buffer (SBB) was used for homogenization and derivatization of free thiols. SBB contained 100 mM Tris-HCl, 5 mM L-serine, 10 mM boric acid, and 1 mM diethylenetriaminepentaacetic acid dissolved in Type 1 water, with a pH adjusted to 7.0 with dilute aqueous NaOH. Lenses were homogenized using a Bullet Blender Storm Pro (Next Advance, Troy, NY) at −20˚C. Cold SBB and 0.5 mm ZrO_2 beads were added to the microcentrifuge tubes with each lens according to the manufacturer's instructions. Lens homogenates were diluted with additional cold SBB and centrifuged at 10,000 ×g for 15 minutes to remove insoluble particulates. Supernatants were promptly removed and placed into separate vials for subsequent analysis of free and total GSH, free and total Cys, and MPG levels. Free and total GSH and Cys were analyzed in lens homogenates according to a sensitive HPLC method developed in our lab [26]. Determination of total Cys and total GSH was performed using 1.25 mM TCEP as a reducing agent. Levels of MPG and 2-mercaptopropionic acid were determined according to the method described by Beltz *et al.* [27]. Analyte concentrations in lens tissues were normalized by soluble protein levels determined according to the method described by Bradford [28].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad, San Diego, CA, USA). All values reported represent the mean ± SEM for 5 - 7 lenses. Statistically significant differences between groups were determined by one-way ANOVA followed by Tukey's or Dunnett's multiple comparison tests. Values of $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. Effects of Functionalized ND on MPG Uptake, Lens Morphology, and Mass

Our primary objective was to determine whether functionalized ND (ND-COOH, ND-OH, or ND-NH_2) could improve the uptake and antioxidative effects of MPG in whole lens cultures exposed to sodium selenite. Therefore, we examined lens morphology, water retention, MPG uptake, and endogenous thiol redox status in lenses after treatment. Sodium selenite is a widely used, well-established cataractogenic agent *in vivo*, and it is also employed to generate oxidative insult in the lens *ex vivo* [7] [23] [29]. We hypothesized that the lenses treated with MPG would exhibit fewer of the detrimental changes observed in the untreated selenite-exposed lenses. If functionalized ND improved MPG uptake, we anticipated that ND: MPG groups would exhibit higher levels of MPG accompanied by improvements in lens weight and redox status compared to the neat MPG-treated group. Although ND: MPG would not typically be delivered directly to the lens surface *in vivo*, ND are reportedly endocytosed by target tissues [19] [20] [21], and uptake of the loaded ND by the cornea and passage into the aqueous humor is nevertheless a possible route for delivery to the lens.

Selenite-induced cataractogenesis is multifactorial with several pathways contributing to opacification and other morphological changes to the lens [29]. Studies suggest that it involves GSH loss, followed by calcium influx and calpain activation [4] [23] [29]. These changes affect lens osmotic balance and crystallin solubility, leading to decreases in transparency. The selenite-only lenses differed from controls upon gross morphological examination, particularly with respect to cortical vacuolization and the appearance of a slight pinkish tint, as documented previously [30] [31] (**Figure 1**). However, dense nuclear cataracts seen *in vivo* are rarely observed in shorter-term lens culture models [4] [30] [31]. MPG-treated selenite-exposed lenses, in general, appeared to exhibit fewer of these features than selenite-only lenses.

Lens wet weights were also compared amongst the treatment groups. Some studies report changes in lens water retention or weight after selenite exposure as a result of compromised membrane integrity and electrolyte imbalance, but others report no significant differences in these metrics [30] [31]. For the purposes of the present study, we hypothesized that treatment with MPG would mitigate changes observed in the selenite-only lenses. Selenite-only lenses weighed more than either control or MPG-only lenses on average, but the difference was not significant. MPG alone does not appear to impact lens wet weight, as no significant difference between control and MPG-only lenses was observed. However, in lenses exposed to selenite, treatment with MPG, ND-OH: MPG, or ND-COOH: MPG significantly increased lens weight compared to control and MPG-only groups ($p < 0.05$, **Figure 1**). MPG may be ineffective at inhibiting certain pathways responsible for selenite-induced water retention, although this effect was minimal under the conditions used here. For example, as a chain-breaking

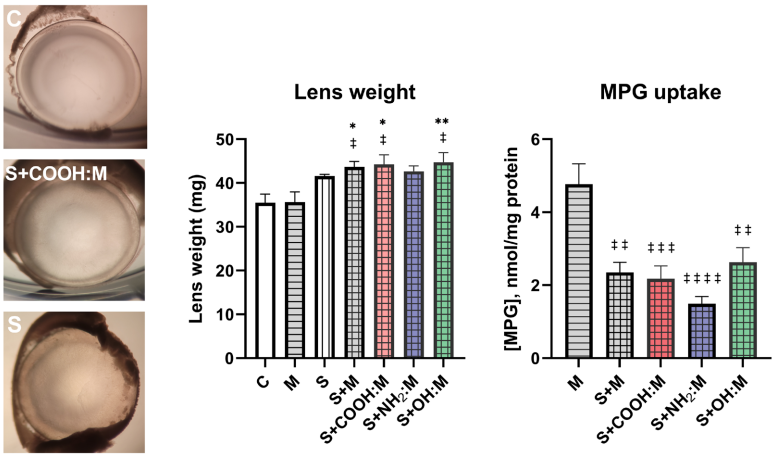

Figure 1. Gross morphological lens examination, lens weight, and MPG uptake. (Left) Representative photographs of lenses from control group (top left), selenite + ND-COOH: MPG group (middle left), and selenite-only group (bottom left) after 72 h incubation. Dark areas surrounding each lens correspond to tissue remaining attached after lens extraction. (Middle) Lens wet weight after 72 h incubation for each treatment group. (Right) MPG uptake by lenses after 72 h incubation. Bars represent mean ± SEM (n = 5 - 7), * $p < 0.05$ compared to control group, ** $p < 0.01$ compared to control group, ‡ $p < 0.05$ compared to MPG-only, ‡‡ $p < 0.01$ compared to MPG-only group, ‡‡‡ $p < 0.001$ compared to MPG-only, ‡‡‡‡ $p < 0.0001$ compared to MPG-only group. See **Table 1** for abbreviated treatment group names.

antioxidant and thiol-disulfide exchanger, MPG may not be able to directly prevent calcium influx and calpain activation. However, MPG has been shown to address GSH loss and oxidative modifications to crystallins, processes that feature prominently in both human ARN and selenite cataracts [4]. Weighing lenses before *and* after incubation may have provided more robust support for selenite-induced changes in lens mass. However, it was necessary to handle lenses as little as possible to avoid surface damage prior to incubation in treatment media.

Next, we compared lenticular MPG levels to determine if ND improved MPG uptake. The ocular lens possesses no direct blood supply. Therefore, high systemic doses are required to achieve adequate drug concentrations in surrounding tissues. However, side effects are noted at high systemic doses (grams/day, per os) [32] [33]. In contrast, ophthalmic solutions are tolerated at millimolar concentrations when applied locally [13]. Even when applied locally, several barriers impede uptake of the neat drug. Any drug accumulating in surrounding tissues (e.g. aqueous or vitreous humor) via local delivery must first pass through the capsular membrane [8]. Under this membrane, a layer of epithelium covers the anterior surface of the lens. Passive diffusion through the epithelium may be challenging for hydrophilic molecules like MPG. Studies indicate that drug molecules may extensively traverse the surface of the lens prior to entry, and drug concentrations in the aqueous humor may exceed those in the lens by several-fold [7] [8]. Active transport mechanisms similar to the lenticular glutamate-cystine exchanger could facilitate uptake of small, charged amino acid derivatives like MPG [34], but this

has not yet been reported in the literature. Additionally, it may be necessary to overcome dysfunctional transport systems for GSH and other substances that have been observed in ARN cataract [35].

For selenite-exposed groups, comparing neat MPG-treated lenses to ND: MPG-treated lenses yielded no significant indication that any of the three functionalized ND increased MPG uptake. The selenite + ND-OH: MPG group exhibited the highest MPG uptake, but the difference was slight ($p > 0.05$) compared to the selenite + neat MPG group. Interestingly, MPG-only lenses exhibited the highest levels of MPG. Lower levels of MPG in all of the selenite-exposed groups suggest that MPG itself may be consumed as a result of selenite exposure. However, it is unlikely that MPG was oxidized to its disulfide, as MPG was measured after a reduction step that converts MPG disulfides to free thiols that can react with the derivatizing agent. Further, the thiol-containing metabolite of MPG, 2-mercaptopropionic acid [27], was not detected in the lens homogenates (the other is glycine). It is possible that the product of MPG oxidation exhibits a higher oxidation state (>−1) and is thus not reducible to the free thiol by TCEP. Another possibility is that MPG was metabolized to other products besides 2-mercaptopropionic acid and glycine, or it may have formed mixed disulfides with proteins that were precipitated prior to analysis. Thus, the fate of MPG in selenite-treated lenses requires further investigation. Even though none of the functionalized ND appeared to improve MPG uptake significantly, it was still necessary to investigate their effects on thiol redox status of the treated lenses.

3.2. Effects of Functionalized ND and MPG on Thiol Redox Status in Whole Lens Cultures

Glutathione, glutathione disulfide, and GSH/GSSG ratio

Along with ascorbate, GSH is the primary antioxidant in the lens, and high GSH levels are critical to maintaining lens and overall ocular health [2] [6] [9]. A precipitous decline in lenticular free GSH levels following selenite exposure has been reported in numerous studies [7] [23] [29]. In control lenses, free GSH levels were 5.8 ± 1.0 nmol/mg protein, comparable to levels reported in other studies using similar models [36] (**Figure 2**). In contrast, free GSH in lenses exposed to selenite alone dropped significantly to 1.8 ± 0.2 ($p < 0.0001$, compared to controls). However, lenses treated with neat MPG exhibited the highest free GSH of the selenite-exposed lenses ($p < 0.01$, compared to selenite-only) and were not significantly different from the controls. ND: MPG-treated groups exhibited intermediate levels of free GSH; these were not significantly higher than the selenite-only group, but they were not significantly lower than the controls. GSSG is the oxidation product of two molar equivalents of GSH. Higher levels of GSSG are associated with disruption to redox homeostasis and are considered toxic in many cases [37]. Determination of GSSG revealed an approximately fourfold increase in the selenite-only lenses compared to controls ($p < 0.0001$). GSSG levels in all other groups were statistically similar to the controls, albeit slightly elevated in selenite + MPG and selenite + ND: MPG groups. These findings

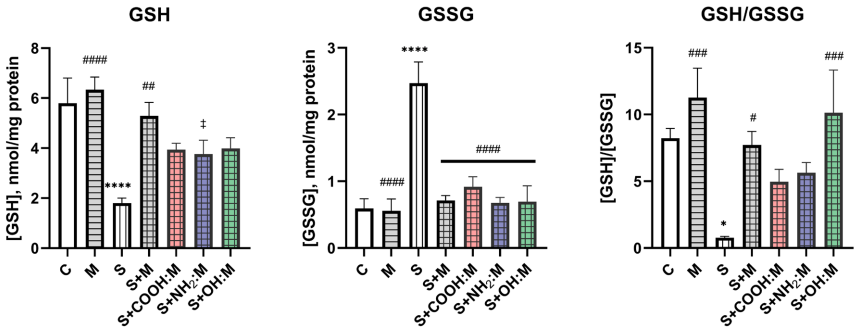

Figure 2. Free GSH, GSSG, and GSH/GSSG ratio in selenite-exposed lenses treated with neat MPG or MPG loaded onto ND. Bars represent mean ± SEM (n = 5 - 7). GSH and GSSG levels are reported in nmol/mg soluble protein. * $p < 0.05$ compared to control, **** $p < 0.0001$ compared to control, ‡ $p < 0.05$ compared to MPG-only, # $p < 0.05$ compared to selenite-only, ## $p < 0.01$ compared to selenite-only, ### $p < 0.001$ compared to selenite-only, #### $p < 0.0001$ compared to selenite-only. See **Table 1** for abbreviated treatment group names.

corroborate the results of GSH analysis, and they suggest that MPG protects lens GSH from selenite-induced oxidation. This process would otherwise quickly overwhelm endogenous antioxidant defenses, as evidenced by our findings in the selenite-only group. More than absolute levels of either analyte alone, tissue GSH/GSSG ratio is a well-established indicator of tissue redox status; lower values correspond to increased oxidative stress. Here, we observed a significant decrease in GSH/GSSG for selenite-only lenses ($p < 0.05$ compared to controls). In contrast, selenite-exposed lenses that were treated with neat MPG or ND-OH: MPG exhibited GSH/GSSG ratios very similar to those of the controls and significantly higher than those in selenite-only lenses ($p < 0.05$ and $p < 0.001$, respectively). Interestingly, detonation ND-OH exhibit a more positive ζ potential and therefore may experience stronger electrostatic attraction to negatively charged phospholipids present on the surface of the lens epithelium [22]. We speculate that this interaction may have improved MPG uptake, leading to improved redox status. However, further investigations are needed to elucidate the mechanism, as the ND-OH: MPG-treated lenses exhibited GSH/GSSG ratios that were only slightly higher than those of neat-MPG treated lenses. GSH/GSSG levels in ND-COOH: MPG and ND-NH_2: MPG lenses were slightly elevated compared to selenite-only lenses, but the difference was not significant.

Cysteine, cystine, and cysteine/cystine ratio

Free cysteine (Cys) is a nonessential amino acid normally present in healthy human lens tissues at concentrations well above the K_m for γ-glutamyl cysteine ligase (GCL), the enzyme that catalyzes the rate-limiting step of GSH synthesis. Therefore, under normal conditions, cysteine availability is not rate-limiting [10] [38], but under oxidative stress, GSH synthesis is upregulated via Nrf2 activation and transcription of GCL subunits, potentially depleting intracellular Cys pools [37]. Compared to controls, all lenses exhibited lower levels of free Cys, which were statistically significant for the selenite-only, MPG-only, selenite + ND-COOH: MPG, and selenite + ND-OH: MPG (**Figure 3**). Cystine levels in the

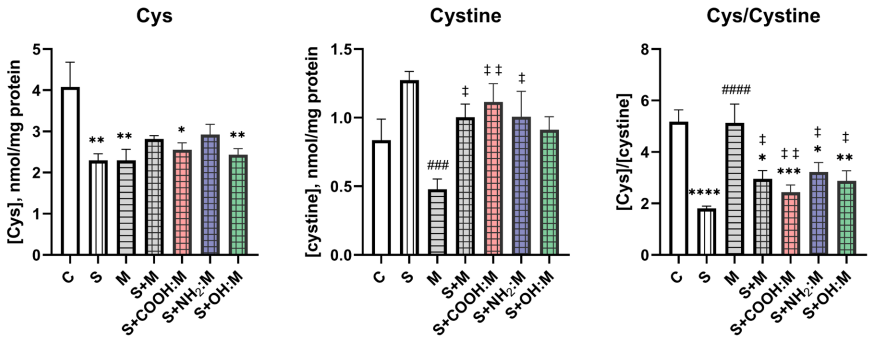

Figure 3. Free cysteine, cystine, and Cys/cystine ratio in selenite-exposed lenses treated with neat MPG and ND: MPG. Bars represent mean ± SEM (n = 5 - 7). Cys and cystine levels are reported in nmol/mg soluble protein. * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control, **** $p < 0.0001$ compared to control, ‡ $p < 0.05$ compared to MPG-only, ‡‡ $p < 0.01$ compared to MPG-only, ‡‡‡ $p < 0.001$ compared to MPG-only, ‡‡‡‡ $p < 0.0001$ compared to MPG-only, # $p < 0.05$ compared to selenite-only, ## $p < 0.01$ compared to selenite-only, ### $p < 0.001$ compared to selenite-only, #### $p < 0.0001$ compared to selenite-only. See **Table 1** for abbreviated treatment group names.

MPG-only group were the lowest, even lower than controls, albeit not significantly. MPG may have participated in thiol-disulfide exchange with cystine in the lens cultures; this reaction underpins MPG's ability to solubilize cystine kidney stones in cystinuria patients. However, we would then expect higher levels of free cysteine unless it had been used to synthesize GSH. This is possible, given the slightly elevated levels of free GSH in the MPG-only group ($p > 0.05$ compared to controls), but further investigations are needed to elucidate the fate of lenticular cysteine after treatment with MPG with or without selenite exposure. Like GSH/GSSG, the Cys/cystine ratio is indicative of changes in redox status [39]. However, the relationship between Cys oxidation and redox status is not as clear-cut as with GSH: intracellular levels of free cysteine are orders of magnitude lower than levels of GSH in the lens, and they are continually used for protein synthesis in metabolically active cells, such as those in the epithelium and lens cortex [38]. Here, all selenite-exposed groups exhibit significantly lower Cys/cystine than either control or MPG-only lenses. While MPG-treated lenses appear to exhibit some improvement over selenite-only lenses, the difference was not significant. We speculate that GSH synthesis may have increased in selenite-exposed lenses, consuming free cysteine, and MPG appears to mitigate this to some extent. Additional investigation of Nrf2 activation and GCL expression may be warranted to confirm or rebut this conjecture.

4. Conclusion

In summary, this study reports the protection of whole lens cultures by the antioxidant drug MPG, with minimal improvement offered by the use of a drug delivery nanoplatform. Further investigations are needed to probe the ability of MPG to provide comprehensive protection to other lens components *in vivo*,

especially crystallins, lipids, and UV filters. The work presented here clearly indicates that improvements should be made to a drug delivery strategy before undertaking animal or human studies. Ultimately, antioxidant drugs like MPG may be a viable approach to addressing oxidative damage that causes cataracts in the lens, but optimization is needed to realize this goal in human patients.

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Authors' Contributions

AC, AP, and NE contributed to the conception/design of the research. AC and AP conducted the experiments. All authors contributed to the analysis of data and interpretation of the studies, as well as editing of the manuscript. All authors agree to be fully accountable for the accuracy of the work and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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