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Glutathione Improves the Antioxidant Activity of Vitamin C in Human Lens and Retinal Epithelial Cells: Implications for Vitreous Substitutes

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ABSTRACT

Purpose: Tissues in the eye are particularly susceptible to oxidative damage due to light exposure. While vitamin C (ascorbic acid) has been noted as a vital antioxidant in the vitreous humor, its physiological concentration (1–2 mM) has been shown to be toxic to retinal and lens epithelial cells in *in vitro* cell culture. We have explored adding vitamin C to hydrogel vitreous substitutes as a potential therapeutic to prevent oxidative damage to intraocular tissues after vitrectomy. However, vitamin C degrades rapidly even when loaded at high concentrations, limiting its long-term effectiveness. Glutathione, another antioxidant found abundantly in the lens at concentrations of 2–10 mM, was proposed to be used in conjunction with vitamin C.

Methods: Cell viability and reactive oxygen species activity of human retinal and lens epithelial cells treated with various combinations of vitamin C, glutathione, hydrogen peroxide, and a hydrogel vitreous substitute were determined using CellTiter-Glo luminescent cell viability assay and dichlorofluorescein assay, respectively. The vitamin C remaining in hydrogel vitreous substitute or glutathione-vitamin C solutions was determined using a microplate reader at 265 nm wavelength, compared against standard solutions with known concentrations.

Results: Glutathione protected the lens and retinal cells from the negative effect of vitamin C on cell viability and prolonged the antioxidant effect of vitamin C *in vitro*. While the detected reading of pure vitamin C solution decreased rapidly from 100% to 10% by 3 days, glutathione provided a significant extension to vitamin C stability, with 70% remaining after 14 days when the glutathione was used at physiological concentrations found in the lens (2–10 mM).

Conclusions: These results indicate glutathione might be an effective addition to vitamin C in intraocular implants, including potential vitreous substitutes, and warrants additional studies on the effectiveness of the vitamin C – glutathione combination in preventing oxidative stress post-vitrectomy.

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Introduction

The vitreous humor is a fragile hydrogel composed of water, collagen fibers, and hyaluronic acid that occupies 80% of the volume of the eye.[1] Due to its proximity to the lens and retina, the vitreous humor has important roles in protecting other ocular tissues. One of its functions is to create and maintain an oxygen gradient with a high level of oxygen near the retina and a low level of oxygen near the lens, protecting the lens from oxidative damage. Tissues in the eye are particularly susceptible to oxidative damage, especially the lens.[2,3] Common ocular complications such as retinal detachments and tears typically require vitrectomy, or complete surgical removal of the vitreous. With the removal of the vitreous and replacement of the tissue with current vitreous substitutes such as saline solutions and silicone oil, the lens is no longer protected from oxidative damage that might cause cataract formation. In fact, up to 95% of patients require cataract extraction within 2 years post-vitrectomy.[4–8] There is a clear need for a new generation of vitreous substitutes that can replace not only the physical roles but also the biochemical functions of the natural vitreous humor.

One of the goals for new vitreous substitutes should be to restore and maintain the natural oxygen gradient in the vitreous chamber. Vitamin C (VC), or ascorbic acid, has been hypothesized to be one of the factors that creates the oxygen gradient in the vitreous humor.[3] The concentration of vitamin C is between 1 and 2 mM in the human eye, about 40 times higher than the physiological concentration in the blood. Vitamin C, which accounts for 75% of the antioxidant potential in the aqueous humor,[9,10] was shown to consume oxygen in the vitreous and was hypothesized to be one of the agents that protects the lens and trabecular meshwork from reactive oxygen species (ROS). When the natural vitreous is removed and replaced with silicone oil, depleting the natural reservoir of vitamin C, the incidence of cataract and glaucoma increases.[3,10-12]

Vitamin C is known to be actively transported into the vitreous humor by the ciliary body and retinal epithelial cells.[13–15] However, the vitamin C level in the eye was shown to be low during the first two postoperative weeks after vitrectomy before increasing to normal levels by the fourth week.[16,17] We therefore speculate that vitamin C should be introduced

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with current vitreous substitutes to replenish the natural vitamin C removed with the vitreous humor during the first two to four weeks post-vitrectomy. We have explored adding vitamin C to hydrogel vitreous substitutes as a potential mechanism to prevent oxidative-stress-induced complications post-vitrectomy, including cataract. However, vitamin C degraded rapidly to 0% after approximately 5 days even when loaded at a high initial concentration of 2 mM, limiting its long-term effectiveness.[18] Vitamin C has been shown to degrade rapidly in aqueous solutions due to light exposure, pH, level of dissolved oxygen, presence of metal ions, and storage temperatures.[19–22]

Besides the active transport of vitamin C, oxidized vitamin C can also be recycled inside the eye by other antioxidants such as glutathione (GSH). Glutathione is an antioxidant found at high concentrations in the ocular lens (2–10 mM).[23–25] Glutathione can regenerate oxidized vitamin C molecules through the glutathione-ascorbic acid cycle and potentially protect the lens from oxidized products of vitamin C.[26–29] In fact, it is known that in many forms of cataract, glutathione levels are unusually low.[30–34]

While numerous studies have investigated the role of vitamin C and glutathione as vital antioxidants in the vitreous humor and lens, respectively,[3,10,12,32,35-42] much less is known about their complementary interaction, especially in hydrogel vitreous substitutes. Additionally, the effects of vitamin C or glutathione on lens or retinal cells have usually been investigated separately. Due to the proximity and interconnectedness of the vitreous humor to the lens and retina, it is imperative to study the effects of glutathione and vitamin C on both lens and retinal epithelial cells systematically, particularly for loading in hydrogel vitreous substitutes. Considering the potential shortcomings of vitamin C (lens protein crosslinking, toxicity, instability, etc.), we hypothesize that glutathione can protect both human lens and retinal epithelial cells from the toxicity of vitamin C, prolong its antioxidant activity, and improve its stability in an aqueous environment, including in hydrogel vitreous substitutes.

Materials and methods

Materials

Poly(ethylene glycol) methacrylate (PEGMA, average molecular weight (MW) 360), poly(ethylene glycol) diacrylate (PEGDA, average MW 575), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), ascorbic acid (ACS reagent, ≥ 99%), glutathione (BioReagent, ≥98.0%), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Dialysis tubing with molecular weight cut off of 12–14 kDa, Dulbecco's Modified Eagle's/Nutrient Mixture F-12 Ham's Medium (DMEM/F12), Dulbecco's Modified Eagle's Medium (DMEM), DMEM without phenol red, fetal calf serum (FCS), Penicillin-Streptomycin (Pen Strep), and hydrogen peroxide (certified ACS, 30%) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and used as received. Human retinal pigment epithelial cells (ARPE-19 ATCC CRL-2302) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Immortalized SRA 01/04 human lens epithelial cells (LEC) were originally provided by Dr. Venkat N. Reddy, University of Michigan and shared by Dr. Marlyn P. Langford, Louisiana State University. The cell line was produced by transfection of human lens epithelial cells with plasmid vector DNA containing a large T antigen of SV40.33.[43] CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega (Madison, WI, USA). Dichlorofluorescein (2,7-Dichlorodihydrofluorescein diacetate, DCF) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Preparation of hydrogel vitreous substitute

The PEGDA-co-PEGMA hydrogel was formed by free radical polymerization as previously published.[18] Briefly, PEGMA and PEGDA monomers (1:1 ratio by weight) were dissolved in deionized water (3% polymer by weight) and extensively purged with nitrogen gas to remove oxygen molecules that might terminate the reaction prematurely. APS aqueous solution (10% w/v) and TEMED were added as free radical initiator and accelerator at 1:200 and 1:800 v/v, respectively. The solution was polymerized for 12 hours. The hydrogel was purified against deionized water for 7 days in dialysis tubing to remove unreacted monomers and low molecular weight polymer chains.

Cell culture

ARPE-19 and LEC (passages 5 to 10) were seeded in 96-well plates at 1×10^4 cells per well in DMEM/F-12 and DMEM, respectively, supplemented with 10% FCS and 1% Pen Strep for 24 hours at 37°C in 5% CO_2 humidified atmosphere.[18,44,45] To prevent any potential interference with the assays or differential interactions between the testing conditions, the normal culture medium (DMEM/F12 or DMEM) in each well was removed after 24 hours and replaced with DMEM without phenol red, FCS, or Pen Strep. The hydrogel vitreous substitute was sterilized in 70% ethanol for 1 hour, submerged in deionized water three times for 1 hour each to remove the residual ethanol, and mixed well with serum-free and phenol-red-free DMEM at a hydrogel concentration of 10% w/w.[18,46-49]

Screening of hydrogen peroxide, vitamin C, and glutathione concentrations using cell viability assay

Various media prepared with vitamin C (0 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, and 10 mM), glutathione (0 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, and 10 mM), or vitamin C (1 mM) mixed with glutathione (0.1 mM, 2 mM, and 10 mM) in DMEM were added to each well (100 µL) and incubated for 24 hours. All media preparations for this study with both LEC and ARPE-19 used DMEM without phenol red. In a separate well-plate, cells were cultured in DMEM for 23.5 hours before hydrogen peroxide (0 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, and 0.6 mM) was added (10 µL) and incubated for 30 minutes. CellTiter-Glo luminescent cell viability assay was conducted according to the manufacturer's protocol. Briefly, the well plates were equilibrated to room

temperature for 30 minutes. CellTiter-Glo Reagent (100 μL) was added to each well, and the contents were mixed for 10 minutes using an orbital shaker. The well plates were incubated at room temperature for 10 minutes before the luminescent signal was measured using a Synergy HT multi-mode microplate reader (BioTek, Winooski, VT, USA).[50]

Antioxidant activity of vitamin C and glutathione on reactive oxygen species (ROS) activity using DCF assay

The ROS activity induced by hydrogen peroxide (0.2 mM for 30 minutes) of ARPE-19 and LEC treated with vitamin C and/or glutathione was determined using a DCF assay as previously published.[44,51] Briefly, LEC and ARPE-19 cells were cultured and treated as aforementioned. Cells cultured in normal culturing medium without hydrogen peroxide were used as negative controls. Cells cultured in normal culturing medium and treated with hydrogen peroxide were used as positive controls. The concentration and duration of hydrogen peroxide used (0.2 mM for 30 minutes) aimed to simulate acute oxidative damage on ocular tissues experienced during a vitrectomy.[44,51] DCF (100 μL, 20 μM final concentration) was added to each well, and the contents were incubated at room temperature for 30 minutes. The fluorescence signal was measured with excitation and emission wavelengths of 485 and 528 nm, respectively, using a Synergy HT multi-mode microplate reader (BioTek, Winooski, VT, USA).

Vitamin C stability study

Vitamin C solutions (2 mM) containing glutathione (1 mM, 2 mM, 4 mM, or 10 mM) were made in DPBS. The hydrogel was loaded with an excess amount of vitamin C (2.2 mM, prepared and replaced daily) in DPBS for 3 days to ensure equilibrium. A vitamin C concentration of 2.2 mM was chosen as the loading concentration to account for the rapid degradation of vitamin C. Control groups with no vitamin C and vitamin C alone (2 mM) in DPBS were included. All solutions were kept at 37°C throughout the stability studies. On days 0, 1, 2, 3, 4, 7, 8, 9, 10, 11, and 14, the samples were placed (0.2 mL) into a 96-well plate and measured using a Synergy HT multi-mode microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 265 nm. After two weeks, samples with higher concentrations of vitamin C were measured periodically until vitamin C was undetectable.

Statistical analysis

Data are expressed as mean ± standard error, unless noted otherwise. The cell viability of each group was normalized to the negative control group in normal culturing medium (no vitamin C, glutathione, or hydrogen peroxide). To account for all conditions tested (with/without hydrogen peroxide and with/without antioxidant), the ROS activity of each group treated with hydrogen peroxide was normalized twice, first to the corresponding negative control (e.g. cells treated with vitamin C and hydrogen peroxide were normalized to cells treated with only vitamin C) and then to the positive control group (cells treated with only hydrogen peroxide). Statistical analyses were implemented with Minitab software (version 18.1; Minitab, Inc., State College, PA). One-way ANOVA, with post hoc pairwise comparison using Tukey's test, was used to analyze cell viability and ROS activity of the lens and retinal epithelial cells and vitamin C stability between various groups. The null hypothesis stated that there was no difference between the groups for each test. An alpha value of 0.05 was used for statistical significance.

Results

Vitamin C had an adverse effect on cell viability for both lens and retinal pigment epithelial cells (Figure 1(a)). While low concentration vitamin C (0.1 mM and 0.5 mM) demonstrated greater than 70% cell viability, higher concentrations (1 mM, 2 mM, 4 mM, and 10 mM) significantly decreased cell viability ($p < .001$). Glutathione, even at the highest concentration evaluated (10 mM), did not decrease the cell viability to below 70% for both lens and retinal cells (Figure 1(b)). Lens cell viability was the lowest (81%, $p = .006$) when treated with glutathione at concentrations of 4 mM or 10 mM. Retinal cells had increased cell viability with glutathione concentration above 1 mM ($p < .001$). Hydrogen peroxide did not affect cell viability at or below 0.1 mM ($p > .9$) and significantly decreased cell viability at 0.6 mM ($p < .001$) for both lens and retinal cells (Figure 1(c)). Intermediate concentrations of hydrogen peroxide (0.2 mM and 0.4 mM) significantly decreased the viability of lens cells (25% – 35% of the control, $p < .001$) but had no effect on the retinal cells (87% – ~100% of the control, $p = .921$).

The lens and retinal epithelial cells were cultured with a concentration of vitamin C (1 mM) that demonstrated reduced cell viability in the presence or absence of a low (0.1 mM), medium (2 mM), or high (10 mM) concentration of glutathione (Figure 2). The viability of cells cultured in low concentration of glutathione (1 mM VC + 0.1 mM GSH) was significantly reduced to a level similar to that of cells cultured with just vitamin C without glutathione (1 mM VC, 60% of the control, $p < .001$). Medium and high concentrations of glutathione were effective at maintaining the viability of cells treated with 1 mM vitamin C (90–100% of the control, $p > .9$) and significantly different from the group with no or low concentration of glutathione (1 mM VC or 1 mM VC + 0.1 mM GSH, $p < .001$).

Different concentrations of vitamin C (0.1 mM and 1 mM), glutathione (1 mM, 2 mM, and 10 mM), and a combination of the two (1 mM VC + 1 mM GSH) were tested to determine the best formulations for reducing ROS activity induced by hydrogen peroxide (Figure 3). A limitation of this study was oxidation of vitamin C was explicitly measured as a way to indirectly measure antioxidant ability. Initial experiments demonstrated rapid oxidation of vitamin C, thereby limiting its ability to be delivered as an antioxidant with long-term efficacy. Therefore, the goal of this experiment was to find the concentrations and/or combinations of vitamin C and glutathione that could maintain the physiological level of ROS activity of the hydrogen-peroxide-treated lens and retinal cells. This information was then used as a guideline for the following experiments that focused on the oxidation of vitamin C and how to prevent or

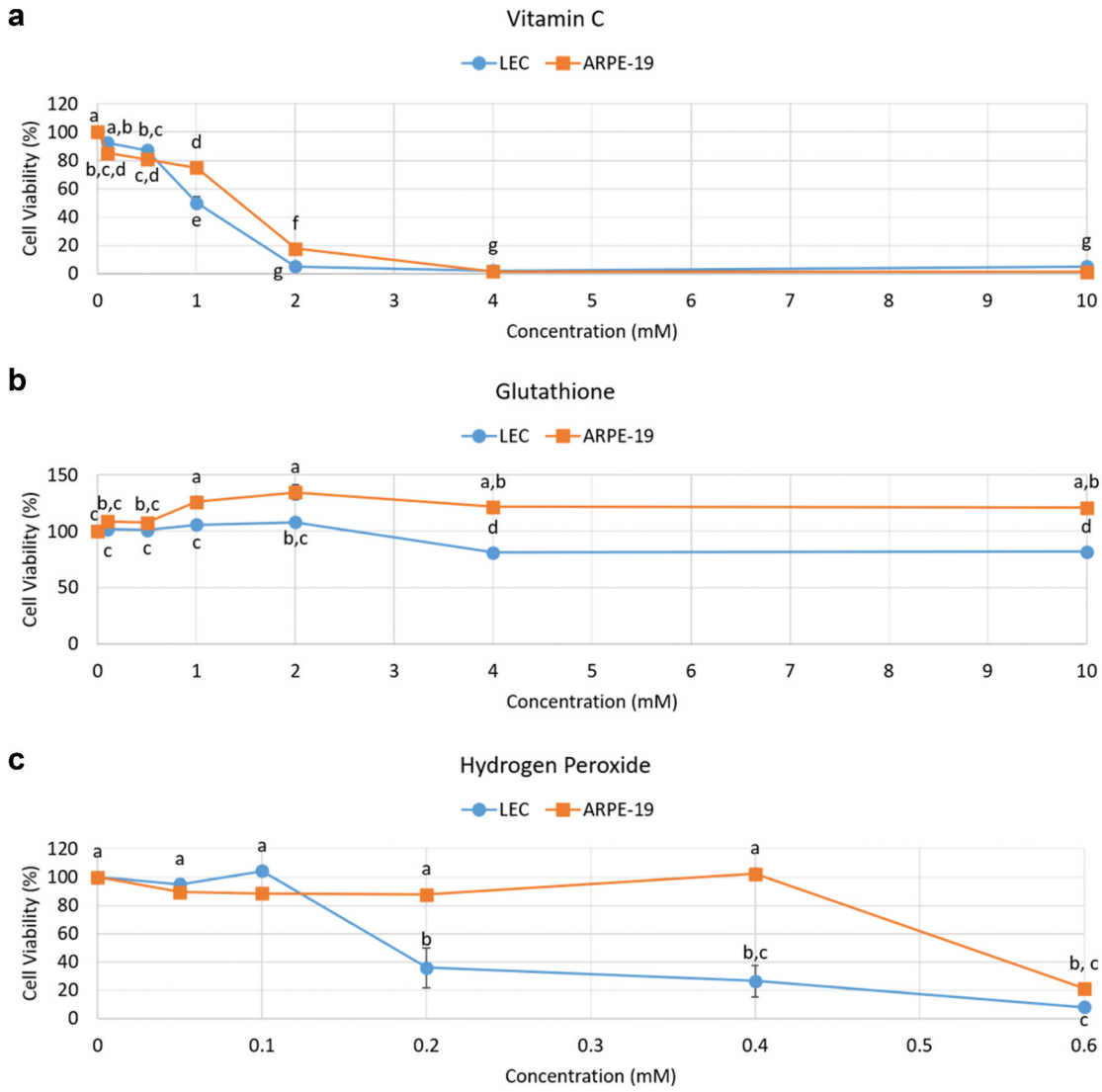

Figure 1. Vitamin C reduced cell viability at concentrations above 1 mM but glutathione did not reduce cell viability below 70%, even at 10 mM. Vitamin C reduced lens and retinal cell viability *in vitro* at physiological concentrations (1–2 mM) found in the vitreous (A). Glutathione did not significantly reduce the viability of lens and retinal cells, even at a high concentration of 10 mM (B). Hydrogen peroxide at concentrations 0.2–0.4 mM was toxic to lens cells but not to retinal cells (C). The letters indicate statistical differences, where means that do not share a letter are significantly different ($p < .001$, n = 8).

delay this oxidation, to evaluate feasibility of using it as a potential antioxidant that could be delivered in the eye. While 1 mM vitamin C significantly reduced the ROS activity of lens cells treated with hydrogen peroxide to 68% of the control ($p < .001$), low concentration of vitamin C (0.1 mM) was not effective at reducing ROS activity ($p = .938$). Vitamin C at both concentrations (0.1 mM and 1 mM) did not reduce the ROS activity for retinal cells ($p = .999$). Glutathione reduced the ROS activity of lens cells (60–65%) at all concentrations tested ($p < .001$). However, glutathione was not as effective at reducing the ROS activity of retinal cells ($p > .158$). A mixture of 1 mM vitamin C and 1 mM glutathione significantly reduced ROS activity for both lens and retinal cells (62% and 80%, respectively, $p < .010$).

Vitamin C and/or glutathione were mixed with a hydrogel vitreous substitute (PEGDA-co-PEGMA) to determine the potential efficacy of an antioxidant-loaded hydrogel at reducing ROS activity (Figure 4). The hydrogel vitreous substitute without antioxidants did not significantly reduce the ROS activity for both lens and retinal cells (92% and 86%, $p = .932$ and $p = .307$, respectively). The antioxidants (vitamin C and/or glutathione) were still effective at reducing ROS activity of lens cells when used with the hydrogel vitreous substitute (56–63%, $p < .001$). Vitamin C or glutathione loaded with the hydrogel did not significantly change the ROS activity of retinal cells (85–100%, $p > .202$), as previously determined (Figure 3).

Vitamin C (1 mM VC), glutathione (1 mM GSH), and a combination of the two (1 mM VC + 1 mM GSH) were mixed with DMEM and incubated at 37°C for 3 days. Media with fresh vitamin C and/or glutathione significantly reduced the ROS activity of lens cells compared to the control (Figure 5, 61–68%, $p < .001$). However, media with vitamin C or glutathione incubated for 3 days did not significantly reduce the ROS activity compared to the control (80% and 88%, $p = .062$ and $p = .763$, respectively). Only the combination of vitamin C and glutathione (1 mM VC + 1 mM GSH) was effective at reducing ROS activity after 3-day incubation (66%, $p < .001$). As previously determined (Figure 3), media with fresh vitamin

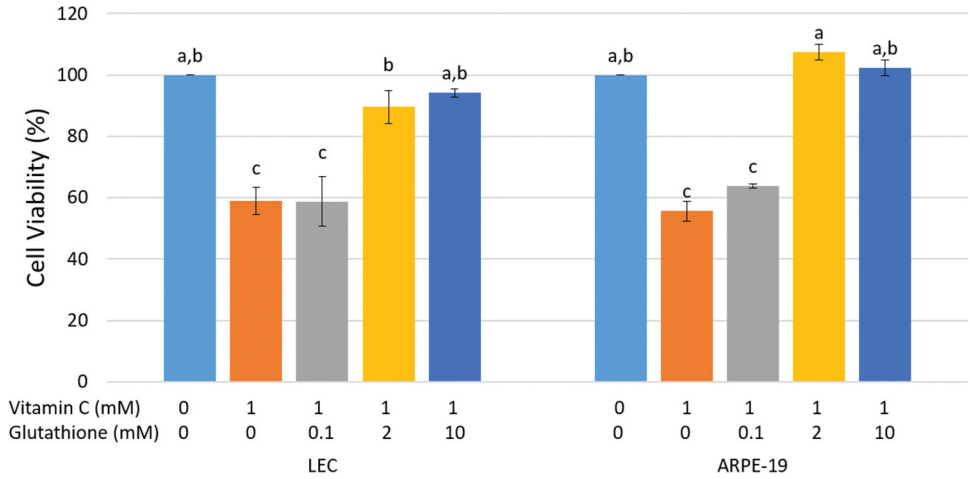

Figure 2. Glutathione (2–10 mM) negated the negative effect of vitamin C (1 mM) on cell viability. Medium and high concentrations of glutathione (2 mM and 10 mM) improved the viability of lens and retinal pigment epithelial cells treated with 1 mM vitamin C. Low concentrations of glutathione were not effective at protecting cells from the negative effects of vitamin C. The letters at the top of the bars indicate statistical differences, where means that do not share a letter are significantly different ($p < .001$, n = 8).

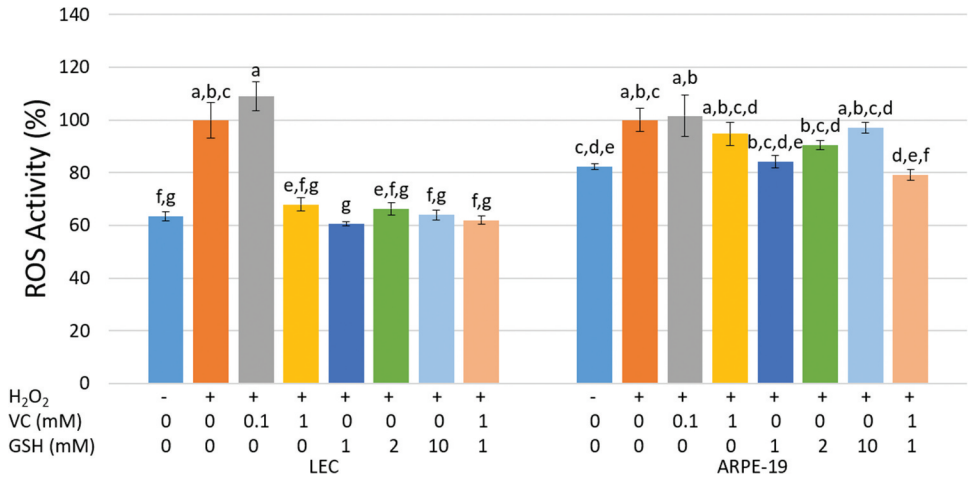

Figure 3. Vitamin C (1 mM) and glutathione reduced ROS activity for lens cells but not for retinal cells. Vitamin C reduced the ROS activity of lens cells when used at 1 mM and did not reduce the ROS activity of retinal cells at 0.1 mM or 1 mM. Glutathione reduced the ROS activity of lens cells at all concentrations tested (1 mM, 2 mM, or 10 mM) but did not significantly reduce the ROS activity of retinal cells. Mixture of 1 mM vitamin C and 1 mM glutathione significantly reduced ROS activity for both lens and retinal cells. The letters at the top of the bars indicate statistical differences, where means that do not share a letter are significantly different ($p < .001$, n = 8).

C or glutathione did not significantly reduce the ROS activity for retinal cells compared to the control (95% and 84%, respectively, $p > .344$). A combination of both antioxidants significantly reduced the ROS activity, but only when used fresh (79%, $p = .044$). Media containing glutathione (1 mM GSH) after 3-day incubation increased the ROS activity of retinal cells compared to control (126%, $p = .002$), suggesting that the oxidized products of glutathione might induce additional oxidative damage to the retinal cells.

To further investigate the long-term stability of vitamin C solution with/without glutathione or hydrogel vitreous substitute, the absorbance of vitamin C solutions (without cells) was measured using a microplate reader at 265 nm and compared to a standard curve with known vitamin C concentrations. The vitamin C remaining in solution decreased rapidly from 100% to 10% after 3 days (Figure 6). Incorporation of vitamin C in hydrogels slightly improved vitamin C retention, remaining at 20% on day 3. Mixing vitamin C with glutathione significantly extended the duration of detected vitamin C. The percent vitamin C remaining increased with the amount of glutathione used. More than half of the vitamin C remained past 14 days when combined with high concentrations of glutathione (4–10 mM). For direct comparison, the time for vitamin C to decrease to 80%, 50%, and 20% levels for each group were determined (Table 1). Glutathione clearly extended the duration of vitamin C stability in a concentration-dependent manner. While the hydrogel extended the duration of vitamin C release, this extension was not statistically significant ($p > .9$). It should be noted that the solutions were exposed to the atmospheric concentration of oxygen (20–21%) in an incubator.

To better mimic the continuous presence of glutathione in the eye, fresh glutathione was added to vitamin C solution in two study groups with additions every 2 or 6 days. Glutathione could maintain the level of vitamin C (~40%) at which glutathione was added (starting on day 2) (Figure 7). However,

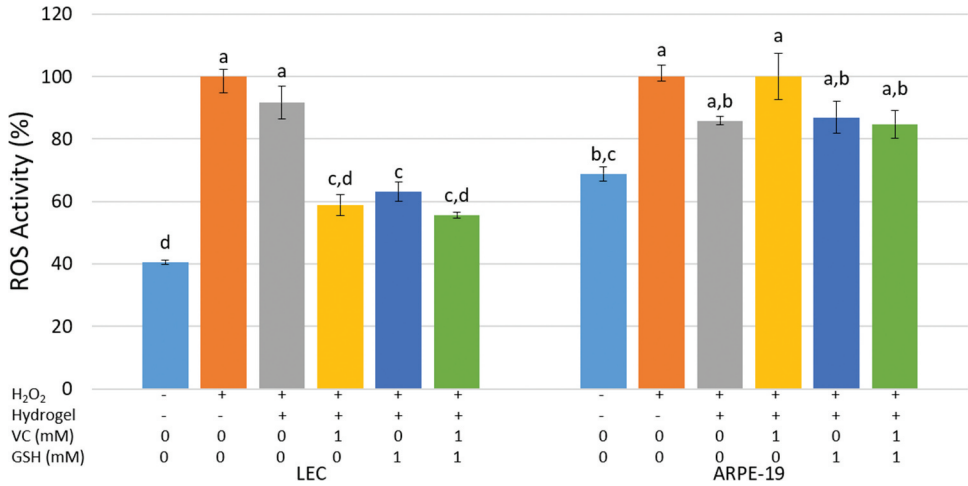

Figure 4. Vitamin C and glutathione loaded in a hydrogel vitreous substitute reduced ROS activity. The hydrogel vitreous substitute did not provide significant reduction to the ROS activity of lens cells compared to the control. However, the combinations of hydrogel and antioxidants were still effective at reducing ROS activity induced by hydrogen peroxide. For the retinal cells tested, the hydrogel and antioxidants did not provide significant reduction to the ROS activity compared to the control. The letters at the top of the bars indicate statistical differences, where means that do not share a letter are significantly different ($p < .001$, n = 8).

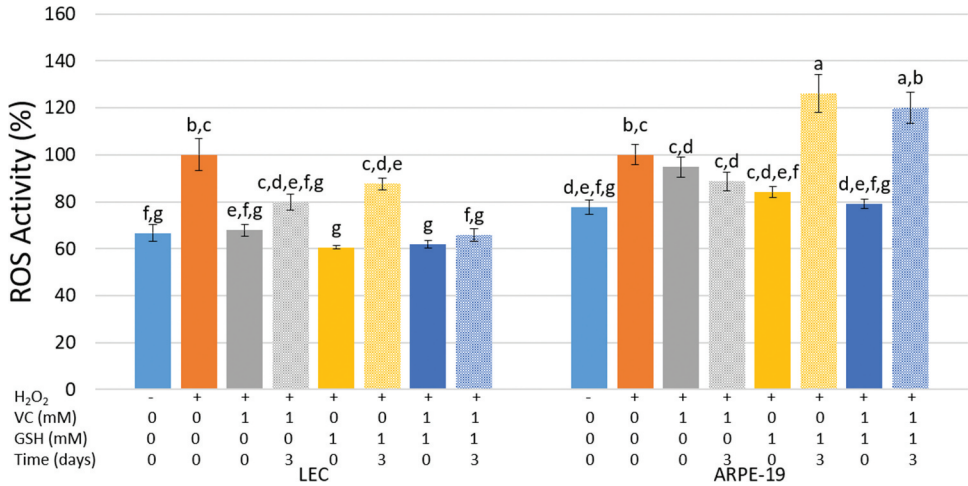

Figure 5. Glutathione prolonged the antioxidant activity of vitamin C up to 3 days after incubation. Media mixed with antioxidant(s) were used immediately after preparation (fresh) or after a period of incubation (3 days, without cells). For lens cells, fresh vitamin C or glutathione reduced the ROS activity induced by hydrogen peroxide. However, media with vitamin C or glutathione incubated for 3 days did not significantly reduce the ROS activity compared to the control. A combination of vitamin C and glutathione significantly reduced the ROS activity compared to the control, even after 3 days of incubation. For the retinal cells, medium with glutathione (1 mM GSH) incubated for 3 days significantly increased the ROS activity compared to the control, suggesting that the oxidized products of glutathione might induce ROS for retinal cells. The letters at the top of the bars indicate statistical differences, where means that do not share a letter are significantly different ($p < .001$, n = 8).

vitamin C could not be regenerated with the added glutathione, even when more glutathione was used (7.5 mg, equivalating to 6 mM in solution vs. 2.5 mg, equivalating to 2 mM in solution). It should be noted that no glutathione was added at the start of the experiment (day 0); therefore, there was an irreversible loss of vitamin C by day 2.

Discussion

This is the first proof-of-concept study that investigated the feasibility of a vitamin C – glutathione loaded hydrogel vitreous substitute in preventing oxidative damage to ocular cells, particularly in the lens, post-vitrectomy. Tissues in the eye are particularly susceptible to oxidative damage from light exposure and have compensated for this by having high physiological levels of antioxidants. While vitamin C has been noted as a vital antioxidant in the vitreous humor, it was shown to be toxic to lens and retinal epithelial cells and cause lens protein crosslinking at physiological concentrations (1–2 mM) *in vitro*.[3,26,52-55] Vitamin C also degrades rapidly in solution, limiting its long-term effectiveness. We proposed to use glutathione in conjunction with vitamin C to prolong the antioxidant activity of vitamin C and curtail its cytotoxicity at high concentrations. This was systematically tested on both human lens and retinal epithelial cells *in vitro*.

Vitamin C reduced the viability of retinal and lens epithelial cells at physiological concentrations found in the vitreous (at or above 1 mM) (Figure 1(a)). Previous studies corroborate the presented data and showed that 0.1 mM was the optimal concentration at preventing oxidative damage.[54,55] Since the reported concentration of vitamin C in the vitreous is between 1 and 2 mM, there must be an explanation for this discrepancy.

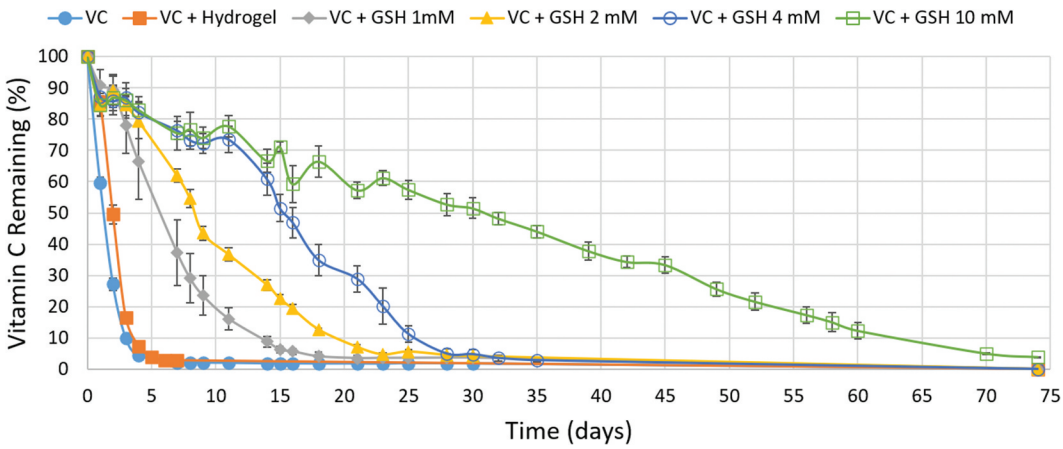

Figure 6. Glutathione improved the stability of vitamin C for more than 2 weeks *in vitro*. Vitamin C remaining decreased rapidly from 100% to 10% after 3 days. Loading in hydrogels slightly improved the stability of vitamin C. Glutathione effectively improved percent vitamin C remaining for at least 15 days in a concentration-dependent manner. Low concentration of glutathione (1 mM) extended the vitamin C remaining to 14 days, while the maximum concentration of glutathione tested (10 mM) extended the vitamin C remaining to more than 70 days (n = 4). It should be noted that the solutions were exposed to the atmospheric concentration of oxygen (20–21%).

Table 1. Stability of vitamin C in the presence of glutathione or hydrogel vitreous substitute.

Sample	80% Vitamin C Remaining (days)	50% Vitamin C Remaining (days)	20% Vitamin C Remaining (days)
Pure VC solution (2 mM)	0.50 ± 0.02^{g}	1.30 ± 0.04^{g}	2.40 ± 0.10^{g}
VC + Hydrogel	1.15 ± 0.04^{g}	2.00 ± 0.08^{g}	$2.89 \pm 0.04^{f,g}$
VC + GSH 1 mM	$3.03 \pm 0.73^{f,g}$	$5.85 \pm 0.94^{e,f,g}$	9.48 ± 1.44^{e}
VC + GSH 2 mM	$3.51 \pm 0.98^{f,g}$	$8.37 \pm 0.26^{e,f}$	15.65 ± 0.36^{d}
VC + GSH 4 mM	$4.87 \pm 1.19^{e,f,g}$	15.30 ± 0.88^{d}	22.73 ± 0.69^{c}
VC + GSH 10 mM	$4.62 \pm 2.13^{e,f,g}$	30.43 ± 1.90^{b}	54.10 ± 1.95^{a}

Means that do not share a superscript letter are significantly different ($p < .001$, n = 4).

One possible explanation is that the *in vitro* results reported in this study (Figure 1) and other studies do not completely reflect the *in vivo* conditions that cells in the lens and retina experience in the eye. The vitreous humor is known to possess several potent antioxidants and there might be other antioxidants that complement vitamin C.[56] The results from this study showed that glutathione can protect both the lens and the retinal cells from the negative effect of vitamin C on cell viability (Figure 2). The viability of cells treated with 1 mM vitamin C was around 50–60% compared to the control (no vitamin C). Treating the cells with vitamin C and glutathione at the physiological concentrations found in the lens (2–10 mM) brought cell viability to 90–100%, similar to the control with normal culturing medium, suggesting that glutathione might be able to neutralize the negative cytotoxic effects of vitamin C in physiological systems. Similarly, Mantha et al. (2020) recently showed that

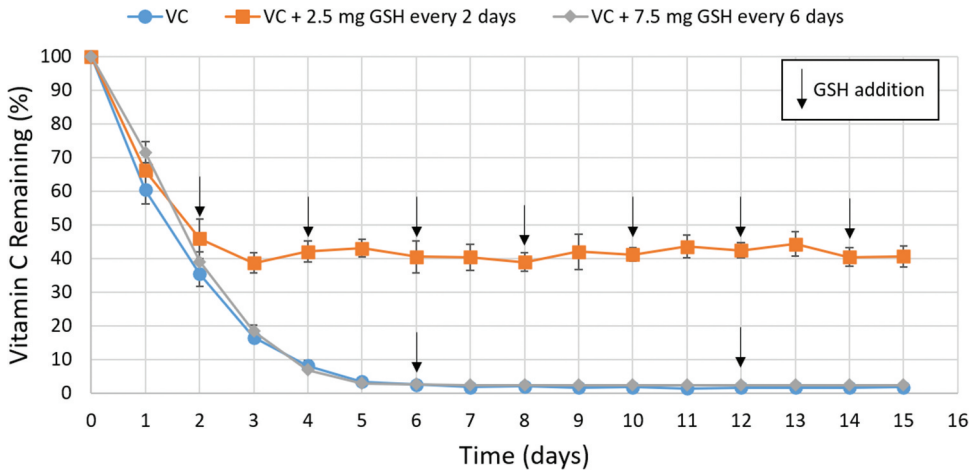

Figure 7. Glutathione maintained the level of vitamin C without regenerating vitamin C. The added glutathione maintained the level of vitamin C but could not regenerate new vitamin C once the vitamin C level decreased. It should be noted that no glutathione was added at the start of the experiment (day 0); therefore, there was an irreversible loss of vitamin C by day 2. Data were presented as mean ± standard deviation. Black arrows indicate when glutathione was added (n = 4).

vitamin C (1 mM) negatively affects the stability of intravitreal therapeutic proteins but glutathione (1 mM) protects these proteins from degradation to some extent.[57]

If vitamin C might be toxic to ocular cells, a logical follow-up question, then, is why there is a large concentration of vitamin C in the vitreous humor? Shui et al. (2009) previously showed that high concentration of vitamin C is needed in the vitreous humor to consume oxygen.[3] Vitamin C was shown to be one of the main factors that establishes the oxygen gradient in the vitreous humor, with a high concentration of oxygen near the retina and a low concentration of oxygen near the lens.[3,9,10] After reacting with oxygen, oxidized products of vitamin C are created. These products, particularly dehydroascorbic acid and L-threose, have been previously shown to be toxic to lens epithelial cells.[58,59] Glutathione, with its ability to recycle vitamin C, might provide some protection against the negative effects of the oxidized vitamin C products. The high concentration of glutathione in the lens would presumably consume any excess dehydroascorbic acid, eliminating its toxic effect and thereby protecting the lens from cataract formation. It should be noted that glutathione was not toxic to the cells, even when tested at high concentrations (4–10 mM, Figure 1(b)). Cells have *de novo* glutathione synthesis and a very well-regulated intracellular glutathione homeostasis.[25,34,60] Therefore, it is not surprising that the extracellular addition of glutathione did not have much effect on cell viability. Interestingly, vitamin C was also shown to function as an essential cellular antioxidant in endothelial cells under oxidative challenge, even in the presence of a vast molar excess of glutathione.[61] This highlights the complex interactions between vitamin C and glutathione in establishing and maintaining the intricate redox environment in biological systems, especially in ocular tissues, and the need to restore and maintain the oxygen gradient in the vitreous post-vitrectomy.

Both vitamin C and glutathione reduced the ROS activity induced by hydrogen peroxide in lens cells (Figure 3). However, it should be noted that the ROS reducing effect of vitamin C and glutathione was not additive, since the ROS reduction by the combination was not significantly lower than those reduced by each antioxidant individually (Figure 3, 4, and 5). The hydrogel vitreous substitute itself did not offer significant reduction to ROS activity (Figure 4). A low concentration of vitamin C (0.1 mM) was also not effective at reducing ROS activity for lens cells. Higher concentration of vitamin C (1 mM) can protect lens cells from increased ROS activity (Figure 3). Vitamin C (1 mM) was also previously shown to partially protect rat lenses from cytochalasin D cataractogenesis.[62] Paradoxically, high concentrations of vitamin C were shown to decrease cell viability (Figure 1(a)). Mixing glutathione with vitamin C not only negated the negative effect of vitamin C on cell viability (Figure 2) but also elongated its antioxidant effect (Figure 5). A combination of vitamin C and glutathione significantly reduced the ROS activity in lens cells, compared to the control, even after 3 days of incubation ($p < .001$). In contrast, media containing vitamin C or glutathione alone did not significantly reduce ROS activity, compared to the control, after 3 days of incubation ($p = .064$ and $p = .733$, respectively). It should be noted that the goal of these experiments was to maintain the physiological level of ROS activity for the hydrogen-peroxide-treated cells, rather than optimizing it. Therefore, a preliminary experimental design analyzing combinations at five levels was used based on approximate physiological concentrations, and this study did not further investigate what concentrations of vitamin C and/or glutathione would provide the best antioxidative functions. Future work will be needed to determine these concentrations, and the results presented in this study can serve as a starting point for an approximate concentration to incorporate in a drug delivery system to evaluate release and antioxidant efficacy of vitamin C and/or glutathione after delivery *in vivo*.

Glutathione also significantly increased the reading of vitamin C detected by UV-Vis spectroscopy over time, with the half-life (50% vitamin C remaining) increased to 30 days at the maximum concentration of glutathione used (10 mM) (Table 1). However, it should be noted that in these experiments, the solutions were exposed to the atmospheric concentration of oxygen (20–21%) in an incubator. Physiological concentration of oxygen in the vitreous is lower (1%)[63]; therefore, vitamin C will likely degrade slower *in vivo*. It should also be noted that the eye has a continual supply of glutathione in the vitreous *in vivo* that maintains the level of vitamin C.[25,34] Glutathione can reduce oxidized vitamin C products, particularly dehydroascorbic acid, through the glutathione-ascorbic acid cycle, thereby protecting vitamin C from oxidation.[26–29] Additionally, vitamin C is actively transported into the eye by the ciliary body,[13–15] where biotransport of vitamin C and oxidized vitamin C continually occur. In the eye, preservation of ascorbic acid additionally occurs by reduction of dehydro-L-ascorbic acid through enzymatic processes.[64] We hypothesize that, with both the intravitreal addition and *in vivo* transport of vitamin C and glutathione, the physiological level of vitamin C (2 mM) will be maintained in the vitreous post-vitrectomy. The *in vitro* experiments conducted in this study represent the worst-case scenario in which vitamin C is released and depleted rapidly. This is an interesting and important question that will be investigated in future studies.

The concentration of vitamin C has been shown to be reduced after vitrectomy.[16,17] Liquefied vitreous, commonly seen with advanced age, has also been correlated with a lowered vitamin C level.[3] The removal of the natural vitreous and replacement with current liquid or gaseous vitreous substitutes (saline, silicone oil, or sulfur hexafluoride) abolish the natural reserve of vitamin C in the vitreous humor and incapacitate its ability to consume oxygen. This puts more oxidative stress on the other ocular tissues, thereby depleting the other antioxidants such as glutathione. In fact, the level of reduced glutathione in the lens has been repeatedly shown to decrease with the development of cataract.[59] The concentration ratio of the redox couple dehydroascorbic acid/ascorbic acid was also shown to be higher in lenses with mature cataract.[65,66] The removal of the lens, which presumably removes the eye's reservoir of glutathione, has also been shown to correlate with increased incidence of open angle glaucoma.[10,67] However, the delivery of antioxidants (vitamin C or glutathione) either via topical eye drops or oral daily supplementation has not significantly reduced the risk of cataract formation or improved the visual outcome of patients.[68–73] The lack of evidence in these studies could be due to the indirect introduction of the

antioxidants to the lens (topically or systemically). Due to its proximity to the lens, a hydrogel vitreous substitute loaded with antioxidants allows ophthalmologists a unique opportunity to directly introduce antioxidants to the lens and the surrounding ocular tissues, potentially providing better outcomes for the treatment and prevention of cataract formation. Indeed, it may be important for current vitreous substitutes to replenish not only vitamin C but also glutathione to restore the homeostatic redox balance of the vitreal environment. Our PEGDA-co-PEGMA hydrogel mixed with vitamin C and glutathione demonstrated its ability to reduce the ROS activity of lens cells induced by hydrogen peroxide. Combinations of hydrogel vitreous substitutes, antioxidants, and other therapeutics need to be further investigated in future experiments with the goal of protecting the lens from cataract formation.

While the oxygen gradient is well-known in the vitreous humor, it is unclear whether vitamin C or glutathione also exist in a gradient similar to the oxygen gradient. Murali et al. (2016) reported a significant difference between ascorbate content in the mid-vitreous (0.26 ± 0.03 mM) and the posterior vitreous (0.38 ± 0.4 mM) in the porcine eye.[40] Similarly, Whitson et al. (2016) reported that glutathione was found most concentrated at the vitreous base near the lens (80 μM) compared to cortex or core (20 μM) in the human vitreous.[25] Umapathy et al. (2018) reported that glutathione is released from the rat lens into the surrounding environment, corroborating the hypothetical glutathione gradient.[42] Additionally, vitreous humor was shown to passively provide high levels of glutathione to the mouse lens.[25] These pieces of evidence from the literature suggest that the hypothetical vitamin C and glutathione gradients might indeed exist, with increasing concentration of vitamin C from the lens to the retina and increasing concentration of glutathione from the retina to the lens. If these gradient hypotheses are true, a natural follow-up question might be how the vitamin C and glutathione uniformly loaded in a hydrogel vitreous substitute can restore or mimic these gradients *in vivo*. The interplay between vitamin C, glutathione, and oxygen in the vitreous are likely to drive the initially uniform concentrations to a steady state where the vitamin C and glutathione concentrations change spatially due to the production, consumption, and recycling rates of vitamin C, glutathione and oxygen within specific locations of the vitreous humor. Taken all together, the dual vitamin C/glutathione gradient hypotheses complement each other, provide a compelling explanation to the complex redox environment of the vitreous humor, and should be investigated in future studies.

The cell culture conditions utilized in this study, while standard for *in vitro* cell culture experiments, do not completely mimic *in vivo* conditions. As a proof-of-concept study on the feasibility of developing a vitamin C – glutathione loaded hydrogel vitreous substitute, this study focused on quantitative measures of ROS activity. Details on the mechanistic roles of these antioxidants in protecting the ocular tissues remain to be evaluated in future studies. This study also used lens and retinal cell lines which may not directly translate to *in vivo* conditions. The cells were cultured on a nonbiological, two-dimensional well-plate surface rather than on a biological, pseudo three-dimensional surface. This difference in substrates has been previously shown to alter the morphology of lens cells, potentially affecting its cellular functions and behaviors.[74,75] The lens cells are also surrounded by a lens capsule *in vivo*, which might provide some additional protection from ROS. A recent paper showed the feasibility of culturing whole lenses *in vitro*, which might solve the problems of nonbiological substrate and the lack of lens capsule for lens cells.[76] Future studies will build on these findings by using whole lens culture models, quantifying intercellular levels of the antioxidants, evaluating the effects of these antioxidants on endogenous cellularly produced ROS levels, and quantifying the concentrations of the reduced and oxidized forms of vitamin C and glutathione in a hypoxic environment that better mimics *in vivo* conditions. A limitation of this study was measurement of ROS using a DCF assay rather than hydrogen peroxide directly. Finally, while glutathione could extend and maintain the stability of vitamin C, it could not regenerate new vitamin C once the vitamin C levels had decreased (Figure 7). Vitamin C and glutathione might, therefore, need to be encapsulated in nanoparticles embedded in a hydrogel vitreous substitute to further extend the stability and controlled release of these antioxidants. Regardless, the current *in vitro* experiments provided some insights into the responses of lens and retinal cells to ROS and antioxidants in a hydrogel vitreous substitute, which merits further investigation in the future.

Conclusions

This is the first study that shows a potential relationship between vitamin C and glutathione in the context of the vitreal environment. Vitamin C was found to reduce the viability of human lens and retinal epithelial cells at physiological vitreous concentrations (1–2 mM). Glutathione was found to protect the cells from the negative effect of vitamin C on cell viability and significantly improve the stability of vitamin C to more than 14 days. A combination of glutathione and vitamin C was found to provide the best antioxidant effect. The interplay between the vitamin C, glutathione, and oxygen in the vitreous might be responsible for the intricate redox balance in the eye where the oxygen, vitamin C, and glutathione concentrations change spatially due to the production, consumption, and recycle rates of these molecules within the vitreous humor. Glutathione should be further investigated in the future as an effective addition to vitamin C in intraocular implants and vitreous substitutes with the goal of minimizing oxidative stress after vitrectomy.

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Disclosure statement

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Data availability statement

The data that support the findings of this study are available from the corresponding author, Dr. Katelyn Swindle-Reilly, upon request.

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