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Thiol antioxidants protect human lens epithelial (HLE B-3) cells against *tert*-butyl hydroperoxide-induced oxidative damage and cytotoxicity

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ABSTRACT

Oxidative damage to lens epithelial cells plays an important role in the development of age-related cataract, and the health of the lens has important implications for overall ocular health. As a result, there is a need for effective therapeutic agents that prevent oxidative damage to the lens. Thiol antioxidants such as tiopronin or *N*-(2-mercaptopropionyl)glycine (MPG), *N*-acetylcysteine amide (NACA), *N*-acetylcysteine (NAC), and exogenous glutathione (GSH) may be promising candidates for this purpose, but their ability to protect lens epithelial cells is not well understood. The effectiveness of these compounds was compared by exposing human lens epithelial cells (HLE B-3) to the chemical oxidant *tert*-butyl hydroperoxide (*t*BHP) and treating the cells with each of the antioxidant compounds. MTT cell viability, apoptosis, reactive oxygen species (ROS), and levels of intracellular GSH, the most important antioxidant in the lens, were measured after treatment. All four compounds provided some degree of protection against *t*BHP-induced oxidative stress and cytotoxicity. Cells treated with NACA exhibited the highest viability after exposure to *t*BHP, as well as decreased ROS and increased intracellular GSH. Exogenous GSH also preserved viability and increased intracellular GSH levels. MPG scavenged significant amounts of ROS, and NAC increased intracellular GSH levels. Our results suggest that both scavenging ROS and increasing GSH may be necessary for effective protection of lens epithelial cells. Further, the compounds tested may be useful for the development of therapeutic strategies that aim to prevent oxidative damage to the lens.

1. Introduction

Currently, surgery is the only treatment for cataracts, the most common cause of blindness worldwide [1]. However, this procedure may increase the risk of developing other ocular diseases such as glaucoma [2] and age-related macular degeneration [3], as the lens contributes substantially to redox homeostasis in ocular tissues [4–6]. Glutathione (GSH) is the most abundant non-protein thiol in the body and the most critical antioxidant in the lens [7]. In young, healthy lenses, epithelial cells synthesize and export high levels of GSH, which maintain the native structure of the transparent refractive crystallin proteins in the fiber cells and protect other ocular tissues from oxidative damage [4–6]. Several studies have demonstrated that oxidative damage to lens epithelial cells and loss of GSH precede crystallin aggregation and opacification [8,9]. After a cataract forms, a synthetic intraocular lens implant can restore vision, but it cannot replace the antioxidative functions of the natural lens, leaving surrounding ocular tissues more vulnerable to oxidative insult. However, if cataract formation could be slowed or prevented, millions could retain their natural lenses [10]. Loss of GSH and lens epithelial cell death represent critical steps in the progression towards cataract [7,9]. Thus, agents that preserve lens epithelial redox status and viability may be key to developing effective cataract preventive strategies.

Although GSH is the most important antioxidant in the lens, it is uncertain whether exogenous GSH can protect lens epithelial cells. High doses may be required to reach therapeutically relevant concentrations

Abbreviations: 7-AAD, 7-aminoactinomycin D; ATCC, American Type Culture Collection; Carboxy-H_2DCFDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; FDA, United States Food and Drug Administration; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; HLE B-3, human (eye) lens epithelial cell line B-3; MPG, *N*-(2-mercaptopropionyl)glycine; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); NAC, *N*-acetylcysteine; NACA, *N*-acetylcysteine amide; $OH^{\bullet}$, hydroxyl radical; PBS, phosphate-buffered saline; ROS, reactive oxygen species; *t*BHP, *tert*-butyl hydroperoxide.

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due to unfavorable biochemical and pharmacokinetic properties when GSH is administered systemically. GSH has a short half-life in human plasma (<3 min) and difficulty crossing cell membranes lacking specific transporters [11,12]. Instead, thiol antioxidants such as *N*-acetylcysteine amide (NACA), *N*-acetylcysteine (NAC), and *N*-(2-mercaptopropionyl)glycine (MPG) have been applied to circumvent the challenges associated with the administration of exogenous GSH. Like GSH, these compounds scavenge ROS and undergo thiol-disulfide exchange, restoring endogenous oxidized thiols to their functional, reduced state [13]. Therefore, it has been hypothesized that these compounds may protect lenticular protein thiols and existing GSH levels. Additionally, thiol compounds such as NAC and its amide derivative, NACA, provide cysteine for the rate-limiting step of GSH synthesis. NAC is used clinically to restore liver GSH levels and prevent hepatotoxicity after acetaminophen overdose, and it may be beneficial in other oxidative stress-related disorders [14,15]. NACA is not currently approved for clinical use, but its amide functionality remains uncharged at physiological pH, making it more lipophilic and bioavailable than NAC [16]. MPG is currently FDA-approved for the treatment of cystinuria [17], but it may also protect/spare endogenous GSH and protein thiols from oxidative processes that disrupt redox homeostasis in the lens [18,19].

While previous studies suggest that MPG, NAC, NACA, and exogenous GSH may be effective antioxidants, their ability to protect lens epithelium against oxidative insult has not been determined, nor have they been compared in relevant models, to the best of our knowledge. Therefore, we investigated the ability of MPG, NAC, NACA, and exogenous GSH to protect human lens epithelial cells against oxidative insult and death *in vitro*. We hypothesized that viability would be highest in groups treated with compounds able to scavenge ROS *and* support intracellular GSH levels. To induce oxidative stress, we used *t*BHP, a lipid peroxide prototype widely employed in lens epithelial cell models of oxidative damage [20–23]. We selected an immortalized human lens epithelial cell line that expresses β- and γ-crystallins [24], HLE B-3, as an *in vitro* model of lens epithelium. This cell line is often used for studies involving oxidative stress in the lens [23,25–28]. We then measured the effects of MPG, NAC, NACA, and exogenous GSH on cell viability, apoptosis, and intracellular GSH and ROS levels following *t*BHP-induced oxidative insult. The results of this study shed light on the limitations and advantages of these compounds and provide an impetus for optimizing their administration *in vivo*.

2. Materials and methods

2.1. Materials

Tiopronin or *N*-(2-mercaptopropionyl)glycine (MPG), *tert*-butyl hydroperoxide solution (*t*BHP), *N*-acetylcysteine (NAC), glutathione (GSH), Tris-HCl, L-serine, boric acid, diethylenetriaminepentaacetic acid, and *N*-(1-pyrenyl)maleimide were purchased from MilliporeSigma (St. Louis, MO). *N*-acetylcysteine amide (NACA) was provided by Dr. Glenn Goldstein (David Pharmaceuticals, New York, NY, USA). Antioxidant stock solutions were generated in sterile Type 1 water prepared in-house with a Millipore Simplicity 185 System. All other reagents were purchased from Fisher Scientific (Fair Lawn, NJ) unless otherwise indicated.

2.2. Cell culture

B-3 human eye lens epithelial cells (ATCC CRL-11421) were purchased from ATCC (American Type Culture Collection, Manassas, VA). Cells were grown in ATCC-formulated Eagle's Minimum Essential Medium (# 30–2003, ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B (Thermo Fisher Scientific) in a humidified incubator with 5% CO_2/95% air at 37 °C. All experiments were performed in phenol red-free media, using tissue culture dishes coated with 20 μg/mL collagen type IV from human placenta (Advanced BioMatrix, San Diego, CA). The cells were passed twice per week at a subcultivation ratio of 1:3. All experiments were performed using cells between passages 2 and 38. The immortalized human cell line HLE B-3 was derived from human infant lens tissue and transformed with an adenovirus 12-SV40 hybrid [24].

2.3. Experimental design

Preliminary dose-response studies (Supplementary Information) indicated that exposure to 0.5 mM *t*BHP for 4 h followed by a "resting period" of incubation in *t*BHP-free media resulted in an approximately 60% decrease in cell viability, which was deemed appropriate for subsequent experiments. Four thiol-containing antioxidants were selected based on their antioxidant properties as reported in the literature: MPG [29–31], NACA [32–35], NAC [36], and GSH [11]. The chemical structures of each are shown in Table 1. A concentration of 1 mM of MPG, NACA, NAC, and GSH was selected based on reports by our group [37–39] and others [11,32]. Our overall goal for the investigation of thiol antioxidants described here was to prevent oxidative damage-induced lens epithelial cell death; therefore, cell viability was selected as the primary outcome for preliminary dose-response studies.

For all subsequent experiments, HLE B-3 cells were incubated in complete medium overnight prior to the experimental treatment. The cells were divided into 10 treatment groups: (1) control, (2) MPG-only, (3) NACA-only, (4) NAC-only, (5) GSH-only, (6) *t*BHP-only, (7) MPG + *t*BHP, (8) NACA + *t*BHP, (9) NAC + *t*BHP, (10) GSH + *t*BHP. The antioxidant and *t*BHP solutions were freshly prepared from concentrated stock solutions immediately prior to each experiment. After overnight incubation, the medium was removed and replaced with the corresponding treatment media (Table 2). The cells were incubated in the treatment media for 4 h. After the 4-h incubation time, the treatment media in the control and *t*BHP-only groups were replaced with plain medium, while the treatment media for the remaining groups were replaced with media containing 1 mM of the corresponding antioxidant for a "resting period" of 1 or 20 h. For convenience, the 4-h *t*BHP exposure followed by a 1-h resting period is referred to as the "short-term" treatment, and the 4-h exposure followed by a 20-h resting period is referred to as the "long-term" treatment.

*t*BHP increases intracellular ROS levels, leading to lipid peroxidation, protein and DNA oxidation, toxicity, and eventually cell death [40–43]. From the dose/exposure time optimization experiments (Supplementary Info), it was apparent that cell death continued after *t*BHP was removed. Therefore, we utilized the long-term treatment to examine the effects of the antioxidants on downstream consequences of oxidative insult, such as loss of viability and apoptosis. Because GSH depletion occurs relatively quickly after lens epithelial cells are exposed to *t*BHP [20], analyses of GSH and ROS levels were performed after a shortened resting period (short-term treatment). Apoptosis was assessed after this time point as well to determine whether antioxidants exerted any

Table 1
Chemical structures of antioxidants investigated in the present study.

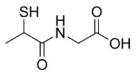
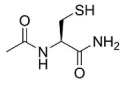
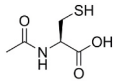
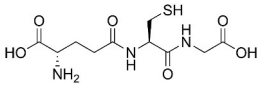
Name	Structure
N-(2-mercaptopropionyl)glycine (MPG)	
N-acetylcysteine amide (NACA)	
N-acetylcysteine (NAC)	
glutathione (GSH)	

Table 2
Groups and treatment conditions.

	Group	Treatment media	
		4 h	Resting period
1	Control	medium only	medium only
2	MPG only	1 mM MPG	1 mM MPG
3	NACA only	1 mM NACA	1 mM NACA
4	NAC only	1 mM NAC	1 mM NAC
5	GSH only	1 mM GSH	1 mM GSH
6	*t*BHP only	0.5 mM *t*BHP[a]	medium only
7	MPG + *t*BHP	1 mM MPG +0.5 mM *t*BHP[a]	1 mM MPG
8	NACA + *t*BHP	1 mM NACA +0.5 mM *t*BHP[a]	1 mM NACA
9	NAC + *t*BHP	1 mM NAC +0.5 mM *t*BHP[a]	1 mM NAC
10	GSH + *t*BHP	1 mM GSH +0.5 mM *t*BHP[a]	1 mM GSH

[a] For GSH analysis, the concentration of *t*BHP was increased from 0.5 mM to 1 mM.

protective effect against cell death shortly after *t*BHP was removed. Results from the antioxidant-only treatment groups (Groups 2–5) were omitted from figures when they were not significantly different from the controls ($p > 0.05$). The low toxicity of these compounds at physiologically relevant concentrations has been noted in our previous studies and by others [33,44–46].

2.4. Cell viability

Cells were seeded in 96-well plates precoated with collagen type IV at a density of 2×10^4 cells/well and allowed to adhere overnight. Then, cells were divided into groups and subjected to the long-term treatment as described previously under "Experimental Design." After the treatment, the treatment media were replaced with fresh EMEM medium, and cell viability was examined using the Vybrant MTT Cell Proliferation Assay kit (Invitrogen, Carlsbad, CA) as described by the manufacturer. The cell viability of each group was expressed as a percentage relative to the untreated control group.

2.5. Intracellular ROS measurement

Intracellular ROS content was measured using the fluorescein derivative, carboxy-H_2DCFDA (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, Molecular Probes, Invitrogen) [47]. HLE B-3 cells were seeded at a density of 2×10^4 cells/well in 96-well special optics black plates (Corning) precoated with collagen type IV and allowed to adhere overnight. Cells were subjected to the short-term treatment as described previously. Fluorescence measurements were performed at several time points during the treatment and immediately after. Prior to measurement, cells were washed with PBS and then incubated with 10 μM carboxy-H_2DCFDA in PBS for 30 min at 37 °C. The fluorescence in each well (λ_{ex} = 485 nm and λ_{em} = 520 nm) was measured using a microplate reader (Fluor Star Optima, BMG Labtech, Durham, NC, USA).

2.6. Apoptotic cell measurement

The effect of treatment on apoptosis of HLE B-3 cells was examined by flow cytometry (BD Accuri C6, BD Biosciences, Ann Arbor, MI) after the long-term and short-term treatment conditions. Cells were seeded on 24-well plates (Corning) precoated with collagen IV at a density of 2×10^5 cells/well, allowed to adhere overnight, and divided into groups. Trypsinized cells were washed using annexin-V binding buffer and stained with 7-AAD (7-aminoactinomycin D, # 559925, BD Pharmingen) and annexin V-Alexa Fluor 647 conjugate (Invitrogen) for 15 min at room temperature in the dark. The FL-3 channel (λ_{ex} = 533 nm and λ_{em} = 670 nm) was used for 7-AAD. Annexin V-Alexa Fluor 647 fluorescence was measured using the FL-4 channel (λ_{ex} = 640 nm and λ_{em} = 675 nm). Debris were excluded by forward vs. side scatter gating. Data was analyzed using Accuri C6 software, and results are reported as a percent of the total cell population. Percentages of late-apoptotic and necrotic cells were combined for comparison between treatment groups.

2.7. GSH quantification

HLE B-3 cells were seeded at a density of 7.5×10^5 cells/well in 6-well plates precoated with collagen IV and allowed to adhere overnight before the experimental treatment. Cells were subjected to the short-term treatment as described previously, except that the concentration of *t*BHP was increased from 0.5 mM to 1 mM based on the results of preliminary *t*BHP dose-response studies [48]. These experiments found no significant decrease in GSH levels after exposure to 0.5 mM *t*BHP (data not shown). Intracellular GSH levels were determined as described by Beltz *et al.* [44] using HPLC with fluorescence detection after pre-column derivatization by *N*-(1-pyrenyl)maleimide according to a method developed in our laboratory [49]. GSH levels were normalized to the soluble protein concentration in each sample determined using the Bradford dye-binding method [50].

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). All values reported represent the mean ± standard deviation of at least three separate experiments. Statistical significance was determined by two-way analysis of variance (ANOVA) for flow cytometry experiments and effect of *t*BHP exposure time on ROS levels. One-way ANOVA was used for all other experiments. Tukey's or Dunnett's multiple comparison tests were applied after ANOVA. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Effect of antioxidants on tBHP-induced cytotoxicity

To determine whether the selected antioxidants could prevent *t*BHP-induced loss of viability, HLE B-3 cells were subjected to the long-term treatment conditions described previously, and MTT cell viability was measured immediately afterward. Exposure to *t*BHP alone decreased viability to approximately 35% of the control (Fig. 1). However, cell viability in the NACA + *t*BHP and GSH + *t*BHP groups was significantly increased (55% and 45% of the control, respectively) compared to the *t*BHP-only group. In contrast, treatment with MPG and NAC did not provide any significant protection of cell viability. Apoptosis and ROS results (see below) suggest that MPG and NAC protect cells for a short time after oxidative insult but fail to protect cells against *t*BHP-induced cytotoxicity during the long-term treatment. Treatment of HLE B-3 cells with 1 mM MPG, NACA, NAC, or GSH alone did not markedly decrease cell viability compared to the control (data not shown).

3.2. Effect of antioxidants on tBHP-induced apoptosis

MTT cell viability is based on the reduction of MTT to a colored formazan product by metabolically active cells in the culture [51,52]. Hence, cells in the early stages of apoptosis with residual metabolic activity may still be counted as viable. Further, MTT does not distinguish between necrotic and apoptotic cells or between stages of apoptosis. Therefore, flow cytometry was used to examine the effects of antioxidants on early apoptotic and late apoptotic/necrotic subpopulations after exposure to *t*BHP. For this analysis, cells were labeled with a double staining method using annexin V-Alexa Fluor 647 and 7-AAD. Annexin V binds to phosphatidylserine located on the outer leaflet of the plasma membrane during early apoptosis. 7-AAD binds to DNA and is unable to penetrate intact membranes [53,54]. Early apoptotic cells only bind annexin V-Alexa Fluor 647, whereas late apoptotic cells bind both annexin V-Alexa Fluor 647 and 7-AAD. Necrotic cells bind 7-AAD only, but not annexin V-Alexa Fluor 647 due to membrane

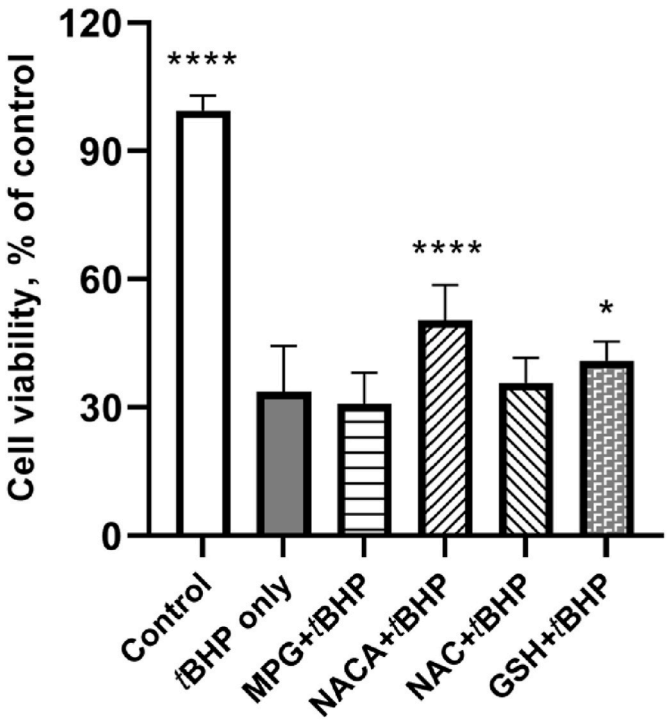


Fig. 1. Cell viability of HLE B-3 cells after long-term treatment (4-h exposure to 0.5 mM *t*BHP + 1 mM antioxidant, followed by 20-h treatment with 1 mM antioxidant). Cell viability was determined by MTT assay and is expressed as a percentage of absorbance at 570 nm compared to that of the control group. The height of the columns represents the mean of at least fourteen replicates, and the error bars indicate the standard deviation. *$p \leq 0.05$ and ****$p \leq 0.0001$ compared to the *t*BHP-only group. Cell viability results from the antioxidant-only groups (groups 2–5 in Table 2) are not shown because they were very similar to that of the control group.

disintegration and subsequent loss of phosphatidylserine.

Representative 2D-plots from the flow cytometry analysis are provided in Fig. 2. The effects of the antioxidants on the distribution of early apoptotic and late apoptotic/necrotic cells after exposure to *t*BHP are summarized in Fig. 3A and B. After the short-term treatment, there were indeed significantly more late apoptotic and necrotic cells present in the *t*BHP-only group (9.7%, $p \leq 0.01$), but the *t*BHP + antioxidant groups were not significantly different from the control. After the long-term treatment, exposure to *t*BHP alone significantly increased both early apoptotic and late apoptotic/necrotic populations (8.6 ± 4.6% and 27.9 ± 14.5%, respectively). However, all of the antioxidants significantly reduced the percent of late apoptotic/necrotic cells ($p \leq 0.0001$ compared to *t*BHP-only) after the long-term treatment. Among the antioxidants, NACA appeared to be the most effective, with early apoptotic and late apoptotic/necrotic populations (5.2 ± 1.7% and 7.2 ± 2.5%, respectively) that were not significantly different from the control. The percentage of late apoptotic/necrotic cells in the *t*BHP + GSH group was also not significantly different from the control, but the percentage of early apoptotic cells was significantly higher than the control. Our results suggest that all of the selected antioxidants prevented apoptosis after the short-term exposure, but only NACA and GSH prevented a significant increase in late apoptotic/necrotic cells up to 20 h after removal of *t*BHP. The relative effectiveness of each antioxidant correlates well with results from the cell viability experiments and suggests that NACA may be the most effective antioxidant at reducing the cytotoxic effects of *t*BHP.

3.3. Effect of antioxidants on intracellular ROS levels

Previous studies suggest that MPG, NAC, NACA, and GSH may prevent oxidative stress-induced cytotoxicity by scavenging ROS before they damage cellular components [11,16,44]. *t*BHP produces *t*-butoxyl and *t*-butyl peroxyl radicals, which give rise to other reactive species in the cells [55,56] through a variety of processes, including mitochondrial dysfunction and lipid peroxidation [40–43]. To determine whether the selected antioxidants could decrease ROS produced by *t*BHP, carboxy-H_2DCFDA was used to monitor intracellular ROS levels during and after the short-term treatment. Carboxy-H_2DCFDA is localized to the interior of the cell via cleavage of its acetate groups by intracellular esterases [47]. The short-term conditions were selected because ROS, by definition, are highly reactive and unstable, and their detection is thus time-sensitive. ROS levels were monitored at several time points (0.5, 1, 1.5, and 2 h) during incubation with *t*BHP and 1 mM antioxidant as well as after the short-term treatment. From 0.5 to 2 h, a significant time-dependent increase in ROS was observed in the *t*BHP-only group (Fig. 4A, $p \leq 0.0001$ compared to control), whereas ROS levels in the *t*BHP + antioxidant cells were not significantly different from the controls from 0.5 to 2 h, except for NAC + *t*BHP cells at 2 h ($p \leq 0.05$). By the end of the short-term treatment (Fig. 4B), ROS levels in all *t*BHP-exposed groups had decreased; this may be attributed to the absence of *t*BHP during the 1-h "resting period." Levels in the *t*BHP-only group were still significantly higher ($p \leq 0.0001$) than those in the control group, and levels in the antioxidant-treated groups were significantly lower ($p \leq 0.0001$) than those in *t*BHP-only cells but somewhat higher than those in the controls. Cells treated with MPG or NACA had the lowest ROS levels of the *t*BHP-exposed cells ($p \leq 0.01$ compared to controls). Overall, these results suggest that the protective effect of thiol antioxidants is partially mediated by their ability to scavenge *t*BHP-derived ROS. For NACA-treated cells, the ROS levels correlate well with the cell viability and apoptosis data, but for MPG-treated cells, the decrease in ROS does not correspond to improved viability. Taken together, these data indicate that ROS scavenging may be necessary but not sufficient for protecting HLE B-3 cells against oxidative insult from *t*BHP.

3.4. Effect of antioxidants on intracellular GSH levels

Loss of free GSH is among the earliest indicators of oxidative stress in lens epithelial cells [20]. GSH levels were therefore measured in the cells after the short-term treatment. We increased the *t*BHP dose to 1 mM for these experiments in order to observe a substantial and statistically significant decrease in GSH. Exposure to *t*BHP alone resulted in significantly decreased GSH levels, as compared to the control (Fig. 5). When cells were exposed to *t*BHP in the presence of NACA, NAC, and GSH, however, GSH levels increased significantly ($p \leq 0.0001$ compared to *t*BHP-only). The MPG + *t*BHP treatment group had increased GSH levels compared to the *t*BHP-only group, but the difference was not significant. Treatment of cells with antioxidants alone did not increase GSH levels compared to the control group (data not shown). Although the NACA + *t*BHP group had slightly lower GSH levels compared to the NAC + *t*BHP and GSH + *t*BHP groups, the difference was not statistically significant. As with the ROS results, effective GSH preservation by NACA correlates well with its effects on cell viability and apoptosis. GSH and NAC both increased GSH levels, but they did not protect against increases in ROS and cell death as well as NACA did. Therefore, GSH preservation may be an important component of protection against *t*BHP, but ROS scavenging may also be necessary for maximum efficacy.

4. Discussion

Taken together, the results support our hypothesis that viability would be highest in groups treated with compounds able to both scavenge ROS *and* support intracellular GSH levels. Cells treated with NACA or MPG exhibited the lowest ROS levels. Cells treated with NAC, NACA, or exogenous GSH exhibited significantly higher GSH levels than cells exposed to *t*BHP alone. However, NACA was more successful than the

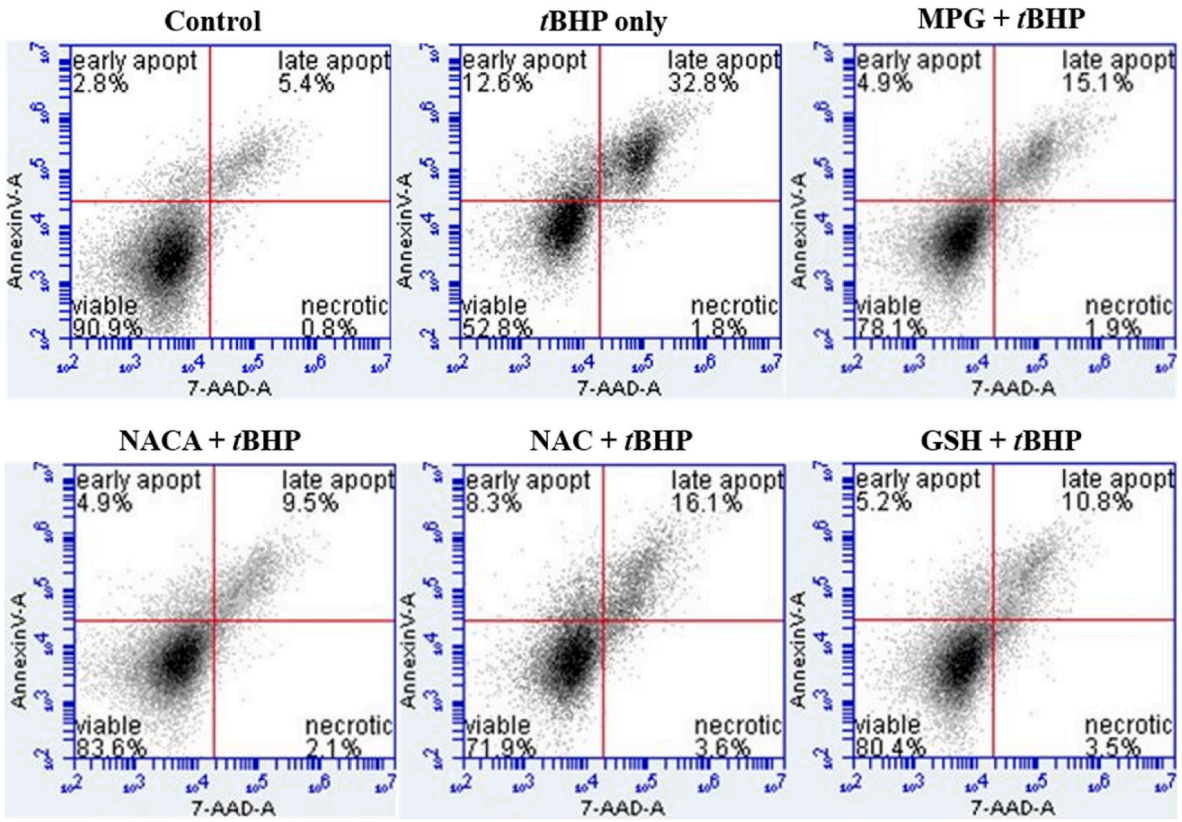


Fig. 2. Representative 2D plots from flow cytometry analysis of apoptotic cells after long-term treatment (4-h exposure to 0.5 mM *t*BHP + 1 mM antioxidant, followed by 20-h treatment with 1 mM antioxidant). The cells were gated excluding debris. The analysis was performed using 7-AAD (FL-3) and annexin V-Alexa Fluor 647 (FL-4) double staining.

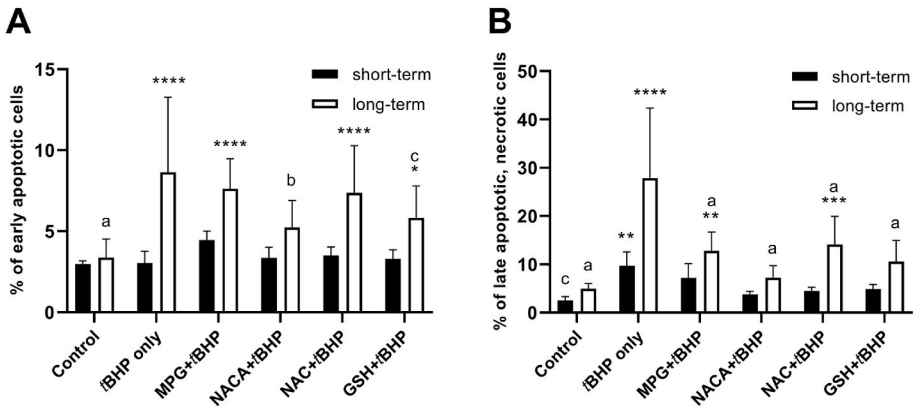


Fig. 3. Quantitative results of flow cytometry on early apoptotic cells **(A)** and late apoptotic and necrotic cells (**B**). The measurements were taken after short-term (4-h exposure to 0.5 mM *t*BHP + 1 mM antioxidant, followed by 1-h treatment with 1 mM antioxidant) and long-term (4-h exposure to 0.5 mM *t*BHP + 1 mM antioxidant, followed by 20-h treatment with 1 mM antioxidant) treatments. The height of the columns indicates the mean of at least six replicates, and the error bars represent the standard deviation. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$ compared to the control group. **a:** $p \leq 0.0001$, **b:** $p \leq 0.001$, **c:** $p \leq 0.01$ compared to the *t*BHP-only group. The antioxidant-only groups (groups 2–5 in Table 2) are not shown because the percentages of early and late apoptotic/necrotic cells in these groups were not significantly different from those in the control group ($p > 0.05$).

other agents at preventing loss of viability, apoptosis, and necrosis.

The substantial reduction in viability observed after exposure to *t*BHP is in good agreement with previous studies using similar concentrations and exposure times in lens epithelial cell lines [20–22,57,58]. Mitochondrial disruption may play an important role in *t*BHP's toxicity in these cells. Preliminary studies in our lab indicate that *t*BHP significantly increases mitochondrial superoxide in B-3 cells (Supplementary Info), and a study by Cai *et al.* suggests that exposure to *t*BHP results in significant loss of the mitochondrial membrane potential and apoptosis [58]. Additionally, an enzyme localized to mitochondria in lens epithelial cells, methionine sulfoxide reductase, has been shown to confer resistance to oxidative insult [57]. Recently, an LC-MS/MS metabolomic approach was employed to investigate the effects of *t*BHP-induced oxidative stress on the nonprotein thiol metabolites in several cell lines, including B-3. This study indicates that cell lines vary widely in their response to *t*BHP exposure, with B-3 cells exhibiting a robust antioxidant defense mediated by upregulation of γ-glutamyl-cysteine, a precursor for GSH [59].

Treatment with NACA significantly increased the viability of cells exposed to *t*BHP. Protection by NACA against *t*BHP-induced cell death has been reported in several other studies [33,60], although it was not investigated in lens epithelial cells. The substantial reduction in viability observed after exposure to *t*BHP alone is in good agreement with previous studies using similar concentrations and exposure times in lens epithelial cell lines [21,57,58]. Although millimolar *t*BHP concentrations appear high in relation to physiological ROS concentrations in

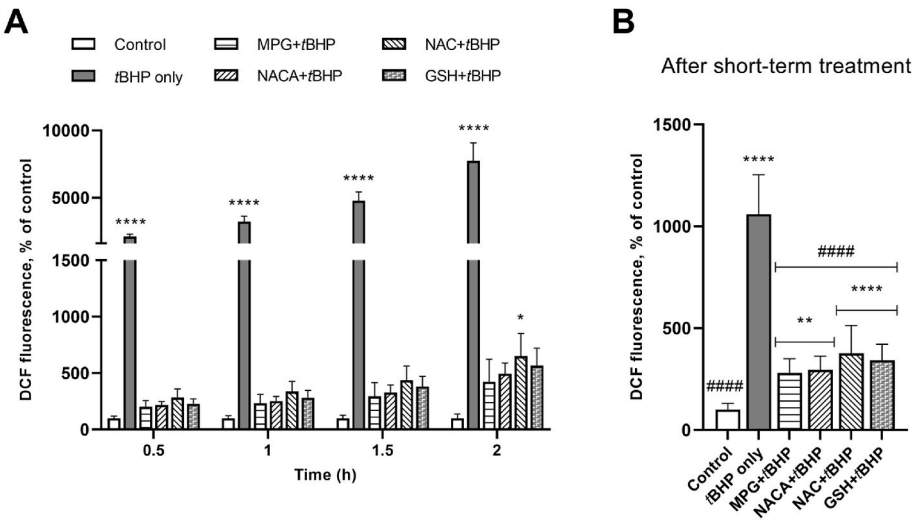


Fig. 4. Time-response for *t*BHP-induced generation of ROS during the short-term treatment (4-h exposure to 0.5 mM *t*BHP + 1 mM antioxidant, followed by 1-h treatment with 1 mM antioxidant). The ROS levels of the antioxidant-only groups (groups 2–5 in Table 2) are not shown because they were not significantly different from those of the control group ($p > 0.05$). **A**. Time-response for *t*BHP-induced generation of ROS *during* treatment with 0.5 mM *t*BHP and 1 mM antioxidants. The height of the columns represents the mean of at least three replicates, and the error bars indicate the standard deviation. *$p \leq 0.05$, and ****$p \leq 0.0001$ compared to the control group. **B**. ROS levels *after* the short-term treatment. The height of the columns represents the mean of at least seven replicates, and the error bars represent the standard deviation. **$p \leq 0.01$ and ****$p \leq 0.0001$ compared to the control group. ####$p \leq 0.0001$ compared to *t*BHP-only group.

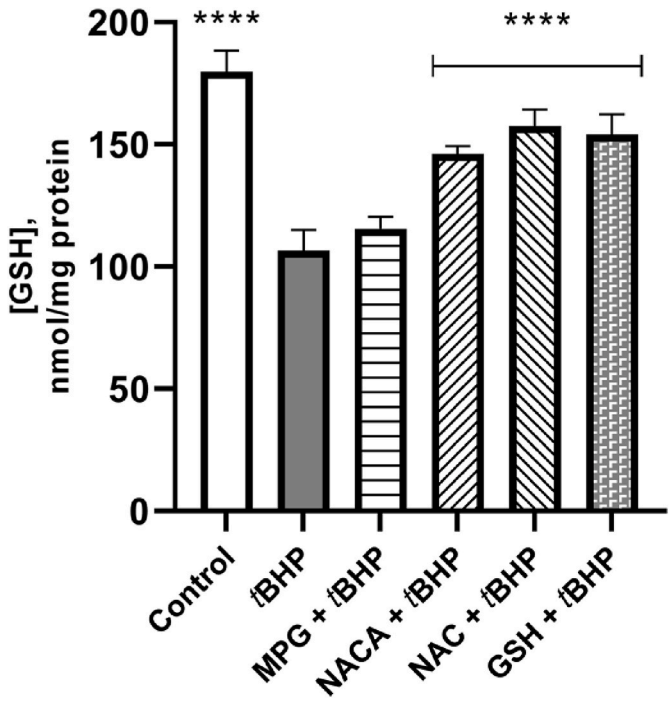


Fig. 5. Intracellular GSH levels in HLE B-3 cells after short-term treatment (4-h exposure to 0.5 mM *t*BHP + 1 mM antioxidant, followed by 1-h treatment with 1 mM antioxidant). The height of the columns represents the mean of three replicates, and the error bars indicate the standard deviation. ****$p \leq 0.0001$ compared to the *t*BHP-only group. The GSH levels of the antioxidant-only groups (groups 2–5 in Table 2) are not shown because they were not significantly different from those of the control group ($p > 0.05$).

some tissues, lens hydrogen peroxide levels in cataract patients have been reported to reach 0.6 mM [61]. In addition, the robust antioxidant defenses of the lens epithelial cells may necessitate the use of high oxidant concentrations to decrease cell viability and GSH levels enough to study the effects of exogenous antioxidants [48,62].

Results from apoptosis analysis after the short-term treatment indicate that all four antioxidants prevented the increases in necrotic/late apoptotic populations observed in the *t*BHP-only group. After the long-term treatment, NACA and GSH were the only compounds to prevent significant increases in the late apoptotic/necrotic populations, which supports the MTT cell viability results. However, the percentages of apoptotic and necrotic cells determined via flow cytometry were lower than expected from the MTT viability results. Several factors may have contributed to this discrepancy: First, MTT may still be converted to the formazan compound by early apoptotic cells, resulting in populations undergoing apoptosis being counted as viable. Also, because MTT is only reduced by metabolically active cells, it does not distinguish between apoptotic and necrotic populations. Additionally, labeling cells with fluorescent probes for flow cytometry involves several additional staining and washing steps, during which most fragile, damaged cells may be lost. As a result, necrotic cells with disintegrated membranes may have been excluded as cellular debris prior to quantification. Ultimately, cell viability and apoptosis results indicate that all four antioxidants may provide some short-term protection, but NACA appears to be the most effective at preventing apoptotic processes downstream of *t*BHP-induced oxidative stress. Examination of the ROS and intracellular GSH results may shed some light on why NACA conferred greater protection against *t*BHP-induced cell death.

MPG and NACA were the most effective at eliminating ROS in HLE B-3 cells. ROS formed by exposure to UV radiation, environmental pollutants, and normal metabolic processes can damage the lens, retina, and other ocular components over time. One of the ways in which antioxidants can prevent oxidative damage is through reaction with ROS to form less harmful products [16,63–65]. Improved ROS scavenging by NACA has been demonstrated in the literature, where NACA exhibited greater reducing power and H_2O_2 and $OH^{\bullet}$ scavenging power than NAC [16]. While no similar comparison exists for MPG, peroxyl and alkoxyl radical scavenging by this compound may be facilitated by the proximity of its amide and thiol functional groups [29]. However, ROS scavenging does not appear to be sufficient for HLE B-3 cytoprotection, as the MPG-treated cells did not exhibit significantly higher viability than the *t*BHP-only cells. By the end of the short-term treatment, ROS levels in all *t*BHP + antioxidant groups were significantly higher than those of the controls but significantly lower than in cells exposed to *t*BHP alone. This implies that even the most effective ROS scavengers were not able to eliminate all ROS, leaving some to be removed by endogenous antioxidant defense systems. For example, glutathione peroxidase uses GSH reducing equivalents to eliminate *t*BHP [13]. Consequently, in addition to scavenging ROS, protection of HLE B-3 cells may require the support of endogenous antioxidant defenses, including GSH and its associated enzymes (glutathione reductase and peroxidases).

GSH is an integral part of the lens' robust antioxidant defense system [7,20,26,66], and substantial loss of free lenticular GSH is associated with cataract formation [48]. Therefore, agents that prevent GSH depletion may protect lens epithelial cells from oxidative damage and thereby prevent cataract formation *in vivo*. *t*BHP exposure depleted

intracellular GSH, but NAC, NACA, and exogenous GSH significantly increased GSH levels in cells exposed to *t*BHP. Increased GSH levels in NAC- and NACA-treated cells may be explained by these compounds' action as cysteine prodrugs. Upon entry into cells, NAC and NACA are converted to cysteine, which is necessary for the rate-limiting step in *de novo* GSH synthesis [67,68]. GSH levels were also increased in cells that were treated with exogenous GSH, which were washed carefully prior to analysis. While GSH generally does not diffuse across cell membranes [11,12], lens epithelial cells may express transporters for GSH [69]. MPG increased GSH levels, but not significantly. MPG is metabolized to thiolactic acid and glycine [17], neither of which contributes to GSH synthesis. However, its action in reducing cystine (Cys-S-S-Cys) kidney stones in the treatment of cystinuria suggests that it may also reduce GSSG to GSH and restore thiol redox balance, which has been demonstrated in other studies [45,70]. Since reduced GSH was measured, this slight increase could be the result of thiol-disulfide exchange with the oxidized form GSSG. Regardless, it appears that MPG does not significantly impact GSH levels under the conditions studied here.

A comparison of ROS, GSH, cell viability, and apoptosis results reveals the relative strengths and weaknesses of the antioxidants (Table 3): MPG decreased ROS but did not significantly increase viability or intracellular GSH. NAC increased intracellular GSH, but its impact on ROS was limited, and it did not improve viability. Exogenous GSH increased intracellular GSH, but its effects on cell viability and ROS were less substantial than those of NACA. Only NACA-treated cells exhibited the highest viability as well as ROS and GSH levels that were significantly improved compared to those of the *t*BHP-only group. These results support our hypothesis that the ability to both eliminate ROS and preserve GSH confers better protection against *t*BHP-induced cell death. One possible explanation may be that scavenging ROS limits potential damage, and providing GSH supports cellular detoxification and repair processes. By doing both effectively, NACA is able to better prevent *t*BHP-induced cell death.

The results reported here have important implications for the development of non-invasive cataract treatments. Although NACA appears to be the most promising, it is not an FDA-approved drug. MPG and NAC, on the other hand, are FDA-approved. It is interesting that MPG has been applied in various models of cataract before, with limited success [18,19]. Although MPG did not significantly improve cell viability under the conditions used in this study, other studies suggest that it may be an effective cytoprotectant, especially at higher concentrations [44]. Our results suggest that its capacity to scavenge ROS may be beneficial, but like NAC, it may suffer from inadequate uptake due to its negative charge at physiological pH. It is possible that using higher concentrations of MPG or increasing its uptake with drug delivery platforms may improve its effectiveness. Other nanoplatforms such as nanodiamonds, gold nanoparticles, fullerol, nanoceria, and nanolipids/nanoemulsions, have been investigated for delivery of antioxidants like quercetin and lutein in ocular and other tissues [71–78]. Additionally, the protective effects of exogenous GSH suggest that if it were able to penetrate the physiological barriers of the anterior eye to reach the lens epithelium, it may be able to provide some benefit with very low risk of toxicity.

A primary limitation of the present study is that *t*BHP-induced oxidative stress in immortalized lens epithelial cell cultures does not fully recapitulate the pathophysiology of age-related cataract *in vivo*. HLE B-3 cultures are not a substitute for whole organ culture or animal studies. Rather, they are a useful tool for determining whether agents such as those studied here may warrant further investigation in more complex systems. Additionally, cell death induced by *t*BHP is more rapid than that induced by UV radiation, one of the primary sources of oxidative damage in human lens [79]. Consequently, it remains to be seen whether MPG, NAC, NACA, or exogenous GSH affect UV-induced lens epithelial cell death. It is possible that the antioxidants primarily scavenged extracellular ROS in the media, eliminating most of them before they attacked the cells. However, it is also plausible that the agents investigated here may react with ROS in the extracellular environment *in vivo*. Finally, additional studies are needed to understand the role of drug uptake in the protective effects of the antioxidants observed here.

Table 3
Summary of protective effects of antioxidants in oxidatively challenged HLE B-3 cells.

Cytoprotective effect	Antioxidant			
	MPG	NACA	NAC	GSH
Scavenged ROS	+	+	*	*
Increased GSH	–	+	+	+
Prevented late apoptosis/necrosis	–	+	–	*
Increased cell viability	–	+	–	*

+ indicates greatest improvement compared to *t*BHP only group, * indicates some improvement compared to *t*BHP-only group, and – indicates that no significant improvement was observed compared to *t*BHP only group.

5. Conclusion

Oxidative damage to lens epithelial cells plays an important role in the development of age-related cataract, and the health of the lens has important implications for overall ocular health. As a result, there is a demand for effective therapeutic agents that prevent oxidative damage to the lens. These investigations provide much-needed insight into the protective effects of thiol antioxidants in oxidatively challenged lens epithelial cells and highlight the need for further studies in this important area to advance the development of non-invasive cataract treatments, which could benefit millions.

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Authors' contributions

Nuran Ercal: conceptualization, resources, project administration, supervision, funding acquisition, Writing-Review and Editing. **Anna Chernatynskaya, Annalise Pfaff, and Hannah Vineyard**: Methodology, Investigation, Formal analysis, Writing-Original Draft, Review, and Editing, Visualization.

Declaration of competing interests

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101213.

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