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Regulation of time-dependent lens opacification by hydrogen sulfide-releasing compounds

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Abstract

Purpose: Although cataract is the leading cause of blindness worldwide, there are no pharmacological agents to prevent or reverse the disease. Since oxidative stress is implicated in the pathogenesis of cataracts, and hydrogen sulfide (H_2S) exhibits antioxidant and neuroprotective properties, the primary objective of this study was to investigate the ability of H_2S-releasing compounds to prevent time-dependent loss of transparency in the cultured bovine lens.

Methods: Freshly isolated bovine lenses were cultured in DMEM containing H_2S-producing compounds, diallyl trisulfide (DATS), GYY4137 or ascorbic acid (AA; used as positive control). Lens opacity was determined by measuring transmittance (using Synergy H1 hybrid reader) and by visual inspection of cultured lenses up to 120 h. Total glutathione (GSH) content and superoxide dismutase (SOD) activity were determined in lens homogenates using Cayman Chemical Assay kits.

Results: After 120 hours of incubation, DATS (10^{-7} and 10^{-6} M) significantly ($p<0.001$; n=6) attenuated the time-dependent decline in transmittance by 16.91 ± 0.01% and 28.54 ± 0.15%, while GYY4137 (10^{-7} and 10^{-6} M) also reduced transmittance by 22.05 ± 0.02% and 2.45 ± 0.32%, respectively. Similarly, DATS (10^{-7} M and 10^{-6} M) and GYY4137 (10^{-7} M and 10^{-6} M)-treated lenses exhibited relatively clear grids after 120 hours, compared to control and AA (10 mM)-treated lenses. DATS (10^{-6} M) and GYY4137 (10^{-7} M) significantly ($p<0.001$; n=5) reversed time-dependent decline in total GSH content by 69.69 ± 0.01% and 80.52 ± 0.17% and SOD activity by 3.23 ± 2.05% and 19.31 ± 0.89%, respectively.

Conclusion: H_2S-releasing compounds prevented time-dependent cultured lens opacification by an action that depends, at least in part, on the integrity of the antioxidant defense pathways, ex vivo.

Introduction

Hydrogen sulfide (H_2S) is a gaseous signaling molecule with neuroprotective effects due to its antioxidant, anti-inflammatory, and anti-apoptotic properties in some diseases associated with the central nervous system. H_2S is endogenously biosynthesized from four major pathways which involve the following enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfur transferase (3-MST) coupled with cysteine aminotransferase (CAT) utilizing L-cysteine as a substrate [1-3], and 3MST coupled with d-amino acid oxidase (DAO) utilizing d-cysteine as the substrate [4]. In addition to an endogenous pathway for the biosynthesis of H_2S from L-cysteine, polysulfides have also been shown to generate H_2S in *in vivo* systems [5]. Among the polysulfides in garlic, diallyl disulfide (DADS), diallyl trisulfide (DATS), and S-allylcysteine (SAC) can produce H_2S in biological systems with DATS and DADS exhibiting a superior ability to generate H_2S [6]. In addition to DATS, GYY4137 (morpholin-4-ium-4-methoxyphenyl (morpholino) phosphinodithioate), is an inorganic, water soluble prodrug for the *in situ* production of H_2S. The prodrug GYY4137 could release H_2S at a constant rate and maintain steady concentrations, compared to the sulfide salt, sodium hydrosulfide (NaHS) [6].

Oxidative stress has been implicated in the pathogenesis of cataract [7-9]. Destabilized state of crystallins and disrupted ionic environment resulting from depletion UV-filters, α-crystallin, glutathione (GSH) levels, and other antioxidant systems have been reported to result in gradual opacification of lenses [7,10-15]. Moreover, the neuroprotective effects of H_2S have been described in ocular tissues by several investigators [16-20]. Deficiency of CBS due to CBS gene mutation has been linked to with ocular disorders such as ectopia lentis, myopia, and cataracts [21]. Furthermore, inhibition, proteolytic degradation, and downregulation of CSE resulted in lenticular GSH depletion and increased risk of cataract formation, suggesting a significant physiological role of H_2S in the eye [22-23]. In cultured bovine lenses, the substrate for endogenous production of H_2S, L-cysteine attenuated time-dependent cataract formation with a corresponding decline in GSH content and SOD enzyme activity [24]. Accordingly, we designed experiments in the present study using lenses that were cultured *ex vivo* to observe the time-dependent loss of lens transparency. The aim of the present study was, therefore, to determine whether H_2S-releasing compounds (DATS and GYY4137) can prevent the time-dependent loss of transparency in the cultured bovine lenses. Since ascorbic acid (AA) is an endogenous antioxidant that is present in aqueous humor

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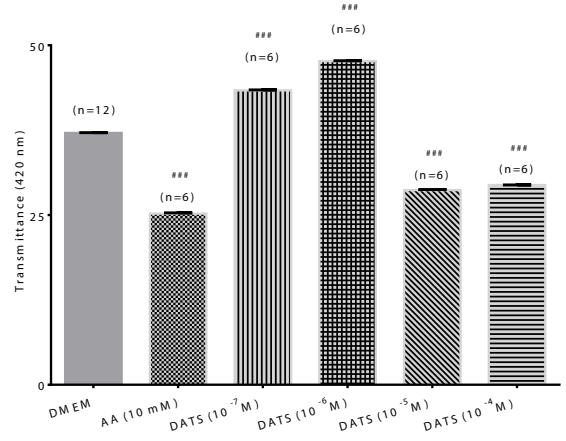

Figure 1(A)

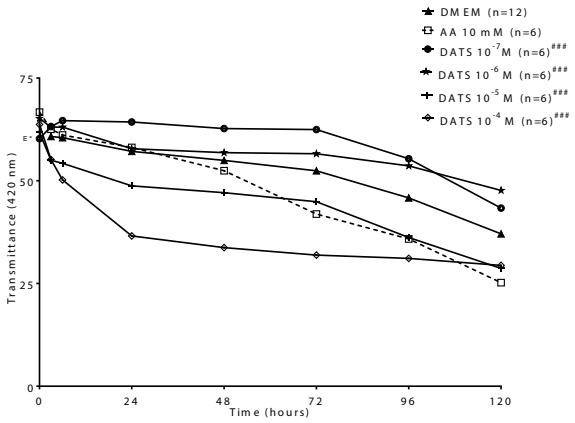

Figure 1(B)

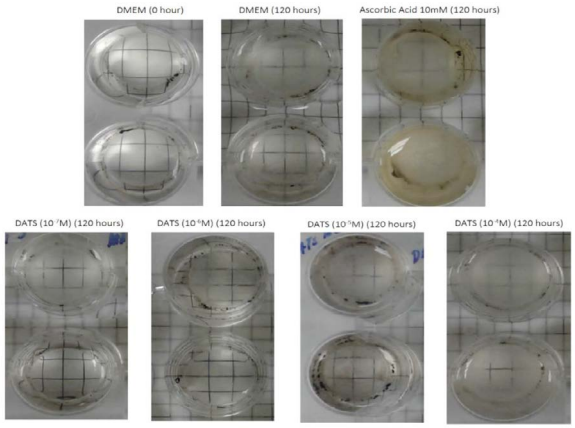

Figure 1(C)

Figure 1. Preventive effect of DATS on time-dependent loss of transparency in cultured bovine lenses. **Figure 1A:** Effect of DATS on transmittance (402 nm) after 120 hours. Results expressed as mean ± SEM. One-way ANOVA $^{###}p<0.001$, significantly different from lenses cultured in DMEM after 120 hours**. (**n) Denotes number of observations. **Figure 1B.** Effect of DATS on transmittance (420 nm) during cataractogenesis up to 120 hours. Two-way ANOVA $^{###}p<0.001$, significantly different from lenses cultured in DMEM up to 120 hours. **(**n) Denotes number of observations. **Figure 1C.** Optical clarity of lenses treated with different concentrations of DATS after 120 hours; Top panel: controls, bottom panel: lenses cultured in DATS (10^{-7} M to 10^{-4} M).

and lens in high concentrations [25-26] and its deficiency is correlated to cataract severity [27-29], it was used as a positive control for these studies. Parts of this manuscript have been presented in abstract format [30].

Materials and methods

Lens culture

Bovine eyeballs from J. F. O'Neal Packing Co, Omaha, Nebraska were transported to the laboratory in an ice pack within one hour of enucleation. These eyeballs were dissected, and lenses were extracted and transferred into Kreb's buffer solution. Dulbecco's Modified Eagle's medium (DMEM) that contains antibiotic (10,000 IU/mL penicillin and 10,000 µg/mL streptomycin) (5 mL/500 mL DMEM) was used as the culturing medium. Twelve-well plates (Thermo Fisher Scientific) were filled with 3 mL of either DMEM with AA (positive control), or DMEM and test compounds (DATS or GYY4137). The lenses were then transferred from Kreb's buffer solution into each well. The cultures were incubated in a CO_2 incubator (Napco e series CO_2 incubator model 5100) at 5% CO_2 and 35^{0}C; where plates were replenished with respective media enriched with the treatment compound every 24 hours.

Assessment of opacity

Transmittance was measured at 420 nm using a plate reader (Synergy H1 hybrid reader; Bio Tek Instruments, Inc) at zero, three, and six hours after culture on the first day followed by 24 hours measurements up to 120 hours. There is evidence that maximum decrease in transmittance is achieved at the 420 nm wavelength, rendering it as an acceptable indicator for the development of opacification in cultured bovine lenses [24]. No transmittance change from initial measurement (0 hour) or an increase in transmittance compared to untreated lenses at 120 hours was taken as prevention of time-dependent loss of lens transparency. Lenses were also evaluated for cataract formation visually and by taking picture against a black grid. Visually assessing lenses for opacity is a classical method that has been used to assess cataract [31]. Visual assessments were conducted at the same time interval along with transmittance measurement.

Biochemical studies

Total glutathione (GSH) content and total superoxide dismutase (SOD) activity of homogenized lenses were evaluated.

Total glutathione content: After 120 hours of culture, each lens was halved (1 g size) and homogenized, deprotonated and supernatant was collected and stored at -20^{0}C. The supernatant containing GSH (reduced) and GSSG (oxidized GSH) was assessed as per the protocol (Cayman Chemical, Item no. 703002).

Total superoxide dismutase activity: Similarly, after 120 hours of culture, each lens was halved (1 g size) and homogenized, and supernatant was collected and stored at -80^{0}C. The supernatant was assessed for total SOD activity (both cytosolic and mitochondrial) as per the protocol for SOD assay kit (Cayman Chemical, Item no. 706002).

Data analysis

Results are described as arithmetic mean ± standard error of the mean (SEM). Significance of differences between groups was evaluated using two-way analysis of variance (ANOVA) and one-way ANOVA

followed by Dunnett's test (Graph Pad Prism 6), where p values of less than 0.05 were considered statistically significant.

Results

Prevention of time-dependent loss of lens transparency

DATS: The polysulfides, DADS, DATS, and SAC have been shown to release H_2S in both *in vivo* and *in vitro* systems [5-6,32]. Among these polysulfides, DATS was reported to exhibit a superior ability to releasing H_2S in biological media [6]. In the present study, we examined the pharmacological actions of different concentrations of DATS in protecting lenses from time-dependent loss of lens transparency. The lower concentrations of DATS (10^{-7} M and 10^{-6} M) attenuated time-dependent decrease in transmittance up to 120 hours. Both concentrations of DATS significantly ($p<0.001$; n=6) increased transmittance by 16.91 ± 0.01% and 28.54 ±0.15% respectively at 120 hours (Figures 1A). In contrast, the higher concentrations of DATS tested (10^{-5} M and 10^{-4} M) did not protect lenses from the time-dependent loss in transparency. The protective action of low concentrations of DATS (10^{-7} M and 10^{-6} M) was superior to that of the positive control, AA (10 mM), which was only effective up to 24 hours (Figures 1B). These observations were corroborated by optical clarity of lenses treated with DATS (10^{-7} M and 10^{-6} M) up to120 hours (Figure 1C).

GYY4137: GYY4137 is an inorganic, water soluble prodrug used for the *in situ* production of H_2S [6]. In the present study, we also examined the ability of GYY4137 to protect lenses from time-dependent change in transmittance (420 nm). We observed that GYY4137 (10^{-7} M and 10^{-6} M) attenuated the time-dependent decrease in transmittance up to 120 hours. Both lower concentrations of GYY4137 significantly ($p<0.001$; n=6) increased transmittance by 22.05 ± 0.20% and 2.45 ± 0.32%, respectively after 120 hours (Figures 2A). In comparison with DATS, the higher concentrations of GYY4137 (10^{-4} M and 10^{-3} M), also failed to reverse the time-dependent decrease in transmittance. As observed with DATS, the protective action of lower concentrations of GYY4137 (10^{-7} M and 10^{-6} M) was also superior to that of AA (10 mM) (Figures 2B). In support of the earlier observation on transmittance by GYY4137 (10^{-7} M and 10^{-6} M), lenses displayed a relatively higher optical clarity at 120 hours compared to untreated lenses and AA (10 mM)-treated (Figure 2C).

Role of antioxidants

Total glutathione content: To assess the contribution of oxidative stress in the pharmacological responses elicited by the H_2S-releasing compounds, a series of experiments were performed to measure GSH content and SOD activity in bovine lenses in the absence and presence of DATS and GYY4137. There was a significant ($p<0.001$; n=5), time-dependent decrease (46.08 ± 0.12%) in lenticular total GSH content of untreated lenses after 120 hours. This decline in total GSH content was significantly ($p<0.001$; p=5) reversed by DATS (10^{-6} M; 69.69 ± 0.01 %) and GYY4137 (10^{-7} M; 80.52 ± 0.17%) (Figure 3A and Table 1). Similarly, the lenticular total SOD activity significantly ($p<0.001$; n=5) decreased by 42.02 ± 8.87% over 120 hours, compared to untreated lens at 0 hour. This decline in SOD activity was also significantly ($p<0.001$) reversed by both DATS (10^{-6} M) and GYY4137 (10^{-7} M) by 3.23 ± 2.05% and 19.31 ± 0.89%, respectively (Figure 3B and Table 1).

Discussion

In the past few years, H_2S that is synthesized endogenously from L-cysteine by CBS, CSE and 3-MST, has been reported to act as a signaling molecule that regulates cellular redox homeostasis [1-3,33-

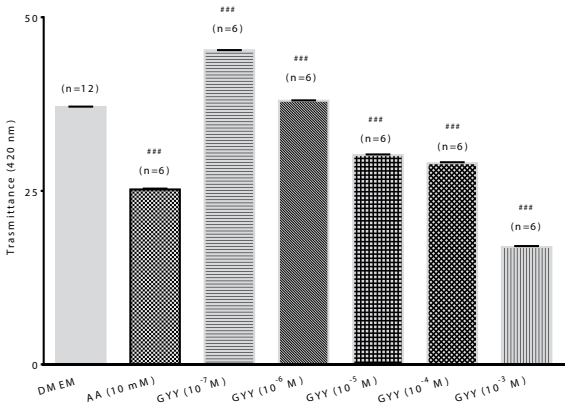

Figure 2 (A)

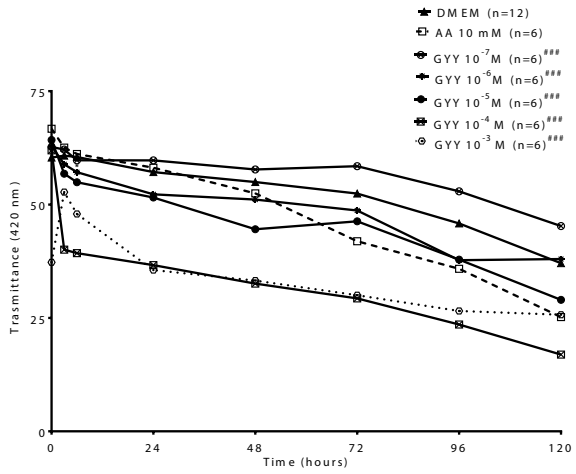

Figure 2 (B)

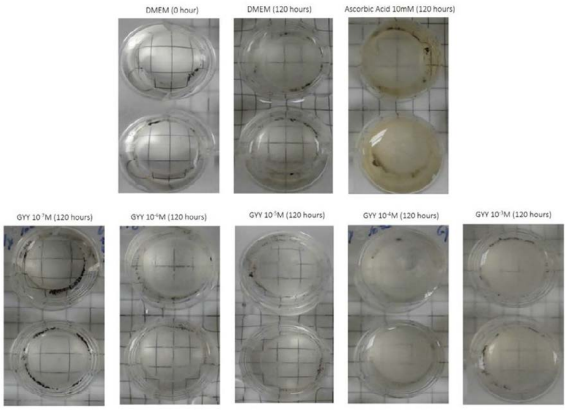

Figure 2 (C)

Figure 2. Preventive effect of GYY4137 (GYY) on time-dependent loss of transparency in cultured bovine lenses. **Figure 2A.** Effect of GYY on transmittance (420 nm) after 120 hours. Results expressed as mean ± SEM. One-way ANOVA ###p<0.001, significantly different from lenses cultured in DMEM after 120 hours**.** (n) Denotes number of observations. **Figure 2B.** Effect of GYY on change in transmittance (420 nm) during cataractogenesis up to 120 hours. Two-way ANOVA ###p<0.001, significantly different from lenses cultured in DMEM up to 120 hours. (n) Denotes number of observations. **Figure 2C:** Optical clarity of lenses treated with different concentrations of GYY after 120 hours. Top panel: controls, bottom panel: lenses cultured in GYY (10^{-7} M to 10^{-3} M).

Table 1. Effect of hydrogen sulfide producing compounds on total glutathione content and total superoxide dismutase enzyme activity in cultured bovine lens homogenates

Groups	Total GSH Concentration (µM/ g wet tissue)	Total SOD (U/ml/g wet tissue)
Controls		
DMEM (0 hour)	103.5245 ± 0.088	3.0345 ± 0.1747
DMEM (120 hours)	55.8213 ± 0.0624***	1.7595 ± 0.0544***
Prevention of time-dependent loss of lens transparency		
DATS (10^{-6} M; 120 hours)	94.7204 ± 0.0630###	1.8163 ± 0.0382###
GYY (10^{-7} M; 120 hours)	100.7663 ± 0.0199###	2.0993 ± 0.0613###

*Result expressed as mean ± SEM; one way ANOVA ***p<0.001 significantly different from untreated lenses at 0 hour, ###p<0.001 significantly different from untreated lenses at 120 hours. DMEM, Dulbecco's Modified Eagle's medium; GSH, glutathione; SOD, superoxide dismutase. One unit (U) is amount of superoxide dismutase (SOD) needed to induce 50% dismutation of superoxide radical.*

34]. In the mammalian eye, enzymes responsible for the biosynthesis of H_2S (CBS, CSE and 3-MST) have been identified in ocular tissues [35-39]. Defects in CBS and CSE have been linked to cataracts, adding more evidence to the physiological role of H_2S in the eye [21-22,40-41]. Taken together, evidence available supports a significant role for H_2S in the pathophysiology and pharmacology of ocular tissues.

There is evidence that H_2S possesses neuroprotective, antioxidant, anti-inflammatory and anti-apoptotic properties in several CNS disease models [3,42-43] and ocular tissues [16,19,43]. For instance, H_2S-releasing compounds attenuated excitatory amino acid neurotransmission in bovine retina, *in vitro* [16] and attenuated RGC loss [19] and retinal apoptosis [43] in experimental rat glaucoma models. Since oxidative stress has been reported to play a major role in the pathogenesis of age-related cataract, several studies have been aimed at mitigating cataract formation by using antioxidants to target various antioxidant systems [44-50]. Mechanisms reported to account for the antioxidant activity of H_2S include free radical scavenging reaction [51], increase in GSH content [24,52] and SOD activity [24]. Therefore, in this study, we further examined the role of SOD and GSH on H_2S-mediated protection from time-dependent lens opacification in bovine lenses, *ex vivo.*

In the present study, we found that both H_2S-releasing compounds tested, DATS and GYY4137 protected lenses from time-dependent degradation in opacity. Comparing the effect elicited by these different H_2S-releasing compounds, the lower concentrations of DATS and GYY4137 were more effective at attenuating time-dependent decline in transmittance than those elicited by the higher concentrations after 120 hours. Indeed, at the highest concentrations tested, both H_2S-releasing compounds did not protect lenses from time-dependent loss in transparency. Interestingly, lower concentrations (10^{-6}M and 10^{-5}M) of L-cysteine, the substrate for endogenous biosynthesis of H_2S were more potent at preventing cataract formation in cultured bovine lenses than that exhibited by higher concentrations (10^{-3}M) of the amino acid [24]. The increased ability of low concentrations of DATS and GYY4137 to prevent time-dependent loss of lens opacity may be due to several factors such as the rate of H_2S release from these compounds, physical/chemical characteristics of the agents (e.g hydrophobicity and /or hydrophilicity), and the presence of transporters. To the best of our knowledge, this is the first report in literature of the protective action of DATS and GYY4137 against cataract formation in an *ex vivo* study.

AA is an endogenous antioxidant that is present in high concentrations in the aqueous humor and lens [25-26] and its deficiency has been reported to correlate to cataract severity [27-29]. AA plays a pivotal antioxidant role in the lens, where it mitigates oxidative stress by serving as free radical scavenger and a filter against ultraviolet light [53-54]. In experimental animals, AA has been reported to attenuate cataract formation in selenite-induced cataract formation in New Zealand albino rabbits [55] and Sprague–Dawley rat pups [50,56]; and galactose-induced cataract formation in guinea pigs [57], *in vivo*. It was interesting to note that the lower concentrations of both DATS and GYY4137 were more potent than that elicited by AA (used as a positive control), which exhibited a modest protection up to 24 hours only. It is conceivable that H_2S donors mitigate cataract formation by mechanisms that are distinct from that elicited by AA, thereby affirming the multifactorial etiology of cataract.

The lens has a sturdy defense system against oxidation [58-60]. Yet, depletion of the antioxidant defense systems has been associated with lens opacification [7,60-63]. In the present study, when compared to untreated lenses (at time 0 hour), lenticular total GSH content and total

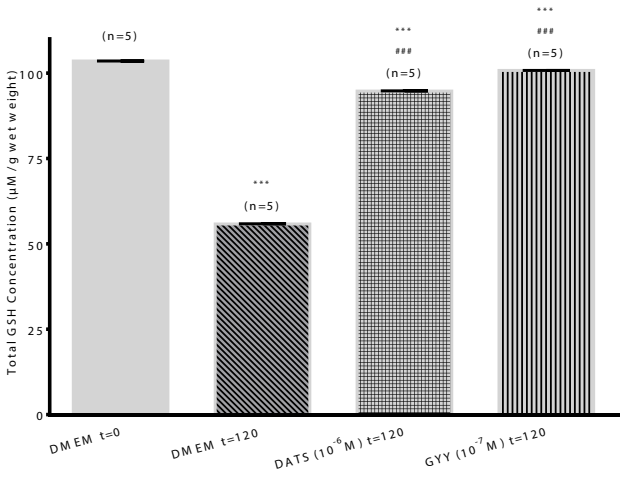

Figure 3 (A)

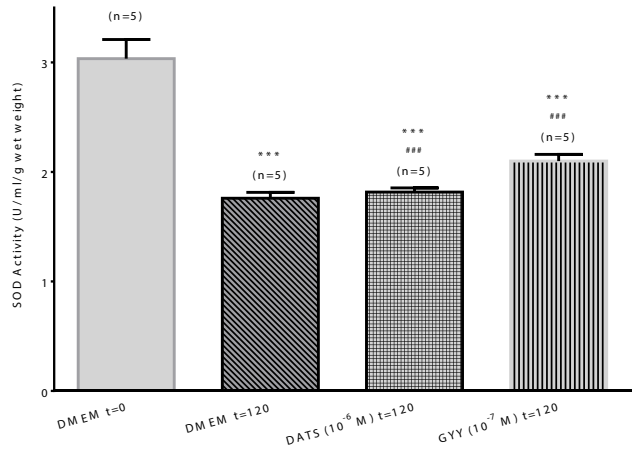

Figure 3 (B)

Figure 3. Preventive effect of hydrogen sulfide producing compounds on time-dependent loss in total glutathione (GSH) content **(Figure 3A)** and loss in superoxide dismutase (SOD) activity **(Figure 3B)** in cultured bovine lens homogenates. Results expressed as mean ± SEM. One way ANOVA ***p<0.001, significantly different from untreated lenses at 0 hour, ###p<0.001 significantly different from untreated lenses at 120 hours.

SOD activity were significantly ($p<0.001$) reduced by about 46% and 42% respectively, after 120 hours of incubation. In support of these observations, Heruye *et al* (2019) reported a similar time-dependent loss in GSH content and SOD activity in cultured bovine lenses, *ex vivo* [24]. Taken together, these observations support the view that the time-dependent opacification was accompanied with compromises in antioxidant defense system. It was interesting to note that both DATS and GYY4137 significantly ($p<0.001$) reversed time-dependent decline in total GSH content by 70% and 80%, respectively. Furthermore, these H_2S-releasing compounds, DATS and GYY4137, blocked time-dependent reduction in total SOD activity by 3% and 19% respectively. Similarly, lower concentrations of L-cysteine have been shown to attenuate time-dependent loss in SOD activity and GSH content in cultured bovine lenses, *ex vivo* [24]. These results indicate that the loss of total GSH content and reduction in SOD activity may have contributed, at least in part, to the pharmacological actions of these H_2S-releasing compounds on compounds on lens opacification.

The slow releasing H_2S-compound, GYY4137 was the most prominent in elevating lenticular non-enzymatic antioxidant (GSH) and enzymatic antioxidant activity (SOD) than DATS, while it was not as prominent in protecting lenses from time-dependent loss of transparency. A similar observation was made by other investigators in astrocytes where these H_2S-releasing compounds enhanced glutamate transporter-1 activity and elevated GSH production as with varying capabilities [63]. It appears that that the differences in responses elicited by the two H_2S -releasing compounds in the present study may be due to their mechanism/s of action on enzymatic and non-enzymatic pathways. It is feasible that multiple and distinctive pathways are involved in the time-dependent cataract formation and upregulation of total GSH content and total SOD activity.

Although cataract is a leading cause of blindness worldwide, there are no FDA-approved pharmacological treatments that can mitigate or reverse this ocular disease in humans. Surgery has been the treatment of choice for decades, presenting a major treatment barrier in developing nations with limited access to proper healthcare. The finding that H_2S-releasing compounds can mitigate cataract formation suggests a potential prophylactic role for these compounds in cataractogenesis. Interestingly, H_2S-releasing compounds such are DATS are abundant in nature, rendering them accessible to developing nations that lack easy access to cataract surgery. Therefore, data from the present study support the development of new treatment modalities that can mitigate or reverse cataracts.

We conclude that H_2S-releasing compounds can mitigate the time-dependent loss in opacity in cultured bovine lenses up to a period of 120 hours. The pharmacological actions produced by these compounds are due, at least in part, to their interaction with the antioxidant defense pathways in the cultured bovine lens.

Authorship and contributorship

All the authors made significant contribution to the study.

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Competing interest

Authors have no conflict of interest

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