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Research article

N-Acetylcysteine amide (NACA) and diNACA inhibit H_2O_2-induced cataract formation ex vivo in pig and rat lenses

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A B S T R A C T

Oxidative stress plays a central role in cataract formation suggesting that antioxidants might slow cataract progression. The anticataract activity of N-acetylcysteine amide (NACA) and (2 R, 2 R')-3,3'-disulfanediyl bis(2-acetamidopropanamide) (diNACA) and/or N-acetylcysteine (NAC), were evaluated in porcine and rat lens models. Cataractogenesis via oxidation was induced with H_2O_2 and/or glucose oxidase (GO). Porcine lenses were incubated in 0.1 mM, 1 mM, or 10 mM NAC, NACA or diNACA for 24 h. Lenses were then transferred to media containing 0.75 mM H_2O_2 and 4.63U of GO in order to maintain a constant H_2O_2 level for an additional 8 h. At the end of incubation, lenses were imaged under darkfield microscopy. Separately, rat lenses were extracted from 3-week-old Wistar rats and incubated with either 10 mM NACA or 10 mM diNACA for 24 h prior to treatment with 0.2U GO to generate a steady source of ~0.6 mM H_2O_2. Rat lenses were analyzed by LC-MS/MS to quantify changes in cysteine, cystine, glutathione (GSH) or oxidised glutathione (GSSG) levels in the lens epithelium, cortex or core. Pre-treatment with NACA or diNACA followed by oxidation with H_2O_2 and/or GO to stimulate cataract formation afforded rapid assessment in *ex vivo* porcine (32 h) and rat (48 h) lens models. Pre-treatment of isolated porcine lenses with 0.1 mM, 1 mM or 10 mM of either NAC, NACA or diNACA followed by H_2O_2/GO treatment resulted in reduced lens opacity relative to the lenses exposed to H_2O_2/GO, with NACA and diNACA reducing opacities to a greater extent than NAC. Rat lenses incubated with 10 mM NACA or 10 mM diNACA without exposure to H_2O_2 showed no signs of opacities. Pre-treatment of rat lenses with 10 mM NACA or 10 mM diNACA, followed by GO cataract induction resulted in reduced opacities compared to control (GO alone). LC-MS/MS analyses revealed that NACA, but not diNACA, increased cysteine, cystine and GSH levels in rat lens epithelium and cortex regions. Taken together, both NACA and diNACA inhibited cataract formation to a greater extent than NAC (all at 1–10 mM) in an *ex vivo* porcine lens model. Both NACA and diNACA (both at 10 mM) reduced cataract formation in rat lenses. Based on LC-MS/MS analyses, NACA-induced reduction in opacity observed in rat lenses was attributed to enhanced cysteine and GSH levels while the diNACA-induced reduction in opacity induced did not consistently increase cysteine, cystine and GSH levels and, therefore, appears to involve a different antioxidant mechanism. These screening studies warrant further testing of NACA and diNACA as anticataract agents.

1. Introduction

Cataract or opacity of our normally clear crystalline lens that focuses light onto the retina is the leading cause of blindness worldwide and accounts for approximately half of all forms of vision loss (Bourne et al., 2013). The main risk factors associated with cataract are predominantly age and diabetes, but others include nutrition (malnutrition and obesity), exposure to sunlight or chemicals, genetics, gender, smoking and alcohol (Brian and Taylor, 2001; Chatterjee et al., 1982; Kelly et al., 2005; Olafsdottir et al., 2012; Pan and Lin, 2014; Truscott, 2005; Weintraub et al., 2002; Ye et al., 2012). Currently, there are no approved drugs for the treatment or prevention of any type of cataracts. The only treatment is surgical removal of the cataractous lens followed by the replacement with an intraocular lens (Kohnen et al., 2009). However, given the increased demand for cataract surgery, there exists a need for treatment of cataract as an alternative to or adjunct to surgery.

It is well known that oxidative stress plays a major role in cataract formation (reviewed by Truscott, 2005; Berthoud and Beyer., 2009). As a result, the lens possesses several mechanisms to protect itself from oxidative stress including the glutathione reducing system which ensures that glutathione levels are maintained at high concentrations (Giblin, 2000). Glutathione is an endogenous tri-peptide comprising of sequentially linked glutamic acid, cysteine, and glycine amino acids, with the sulphydryl (-SH) group on the cysteine residue acting as a potent reducing moiety. We have previously identified transporters involved in the uptake of amino acids required for GSH synthesis

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including members of the Alanine Serine Cysteine Transporter family (ASCT1 and 2) which are involved in the uptake of cysteine (Lim et al., 2006). However, while cysteine is inherently unstable in solution, the oxidised form of cysteine, cystine, is more stable and abundant in the aqueous humour compared to cysteine, suggesting that the uptake of cystine may be a more relevant mechanism for the accumulation of cysteine in the lens. Accordingly, we have shown that the rat and human lens expressed the cystine/glutamate exchanger System xc- (Lim et al., 2005, 2013), an amino acid transport system capable of mediating cystine uptake in exchange for glutamate export. We showed that xCT, the light chain subunit of System xc-was expressed in the lens cortex and core in young rat and human donor lenses (Lim et al., 2005, 2013), suggesting that in the cortex of the lens, xCT mediates the uptake of cystine, where it is rapidly reduced to cysteine for GSH synthesis. On the other hand, in the core of the lens, which lacks the ability to synthesise GSH, the expression of xCT suggests that cysteine itself could act as an antioxidant in this region to protect it from oxidative stress. In older human donor lenses, xCT was not detectable in the lens centre suggesting that with advancing age, xCT may be modified, potentially reducing its ability to uptake cystine and thus accumulate cysteine and in turn reduce antioxidant protection within the lens centre. Hence, bypassing this transport system to enhance cysteine (reduced form) or cystine (oxidised form) accumulation in the lens, may represent a strategy for enhancing endogenous antioxidants levels in the lens to afford protection from cataract formation.

We have been exploring the biological activities of N-acetylcysteine (NAC), N-acetylcysteine amide (NACA) and ((2 R, 2 R')-3,3'-disulfanediyl bis(2-acetamidopropanamide) diNACA; small molecules with potential anti-cataract properties (Fig. 1) (Sunitha et al., 2013).

NACA and diNACA are analogues of NAC, a drug approved around the world for the treatment of acetaminophen overdose and as a mucolytic (Akakpo et al., 2022). NAC (Sunitha et al., 2013) and NACA (Sunitha et al., 2013) have demonstrated antioxidant properties in various biological models. NAC exerts antioxidant action via donation of cysteine, one of the building blocks of GSH, thereby potentiating biosynthesis of GSH in tissues (Sunitha et al., 2013). NACA, an amide analogue of NAC, has increased lipophilicity compared to NAC, and the ability to better penetrate cell membranes as well as the blood-brain barrier (Bahat-Stroomza et al., 2005; Offen et al., 2004) and the blood-retinal barrier (Dong et al., 2014). The anti-cataract properties of NACA have been previously studied by various groups. NACA inhibited L-buthionine-(S,R) sulfoximine (BSO, a glutathione synthesis inhibitor)-induced cataract in Wistar rat pups. Pretreatment with NACA before the injection of BSO and further supplementation until the day of sacrifice (day 15) was shown to prevent cataract formation compared to the control group (BSO-injection without NACA) (Carey et al., 2011). NACA was also shown to inhibit dexamethasone-induced cataract formation by limiting lipid peroxidation and increasing the ratio of GSH/GSSG in rat lens (Tobwala et al., 2014). In addition, NACA prevented acetaminophen-induced cataractogenesis in the rat lens (Maddirala et al., 2015) and decreased the severity of selenite-induced cataract in rat pups (Maddirala et al., 2017). DiNACA is the immediate precursor in the synthesis of NACA (Wall et al., 2023), and until now has not been tested for its ability to elevate cysteine and/or GSH levels in the lens and slow cataract formation. In this study, we developed rapid anticataract screening models with isolated porcine and rat lenses, and conducted *ex vivo* screening to ascertain the potential of NACA, diNACA and/or NAC to inhibit cataract formation in these models.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Merck Ltd. (Darmstadt, Germany) unless otherwise stated. NACA and diNACA were sourced from Nacuity Pharmaceuticals, Inc., Fort Worth, TX, USA.

2.2. Animal protocols

2.2.1. Porcine lens culture system for pre-treatment with NAC, NACA or diNACA and H_2O_2 and/or GO exposure

Fresh eyes from pigs were obtained from Sierra for Medical Science (Whittier, CA). The lenses were extracted and cultured in modified TC-199 media following a previously published method (Wang et al., 1997). Briefly, lenses were first incubated in 5 mL TC-199 medium and 1% porcine serum in a 12-well plate for 2 h. At the end of the incubation, lenses were imaged under darkfield microscopy and only clear, transparent lenses were used for further experimentation. Lenses were then pre-incubated with NAC (0.1 mM, 1 mM or 10 mM), NACA (0.1 mM, 1 mM or 10 mM) or diNACA (0.1 mM, 1 mM or 10 mM) for 24 h (n = 3 for each experimental group). After pretreatment, lenses were transferred to media containing 0.75 mM H_2O_2 and 4.63U of GO for an additional 8 h. Glucose oxidase (GO) is an oxidoreductase enzyme that converts glucose to H_2O_2 and D-glucono-δ-lactone and has been widely in lens studies to maintain a constant H_2O_2 level. The levels of H_2O_2 are within the mM range but have been shown to be required for cataract formation in the larger porcine lens (Persa et al., 2004). H_2O_2 levels were measured using the Hydrogen Peroxide Assay kit (Abcam, Cambridge, UK). At the end of the incubation, lens morphological changes were recorded under a dissecting microscope (Nikon, Melville, NY, USA) using darkfield illumination. Lenses incubated with media alone served as the control.

2.2.2. Rat lens culture system for pre-treatment with NACA or diNACA and exposure to H_2O_2 via GO

Rats were treated according to the Association for Research in Vision and Ophthalmology guidance and study was approved by the University of Auckland Animal Ethics Committee. Lenses were extracted from 3-week-old male Wistar rats euthanized by CO_2 asphyxiation followed by cervical dislocation. Whole eyes were removed immediately and placed in warm phosphate buffered saline (10 mM, pH 7.2). Lenses were then dissected from the globe and incubated in Medium 199, no phenol red with glutamine (Cat#11043-023, Thermofisher Scientific, Waltham, Massachusetts, USA), with 1% penicillin, streptomycin, neomycin

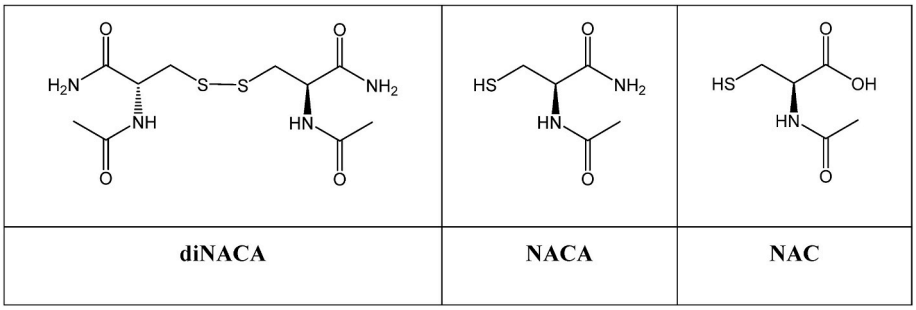

Fig. 1. Chemical structures of diNACA, NACA and NAC.

mixture (PSN; BioReagent P4083, Sigma-Aldrich, St. Louis, MO, USA) for 6 h. At the end of the 6-h incubation, lenses were imaged under darkfield and brightfield microscopy and only clear, transparent lenses were used for further experimentation.

Clear lenses were categorized into one of six groups: *1.* Control group with no NACA or diNACA, and no H_2O_2 (n = 5 lenses), *2.* GO only group (n = 8 lenses), *3.* NACA group with 10 mM NACA (n = 6 lenses), *4.* diNACA group with 10 mM diNACA (n = 6 lenses), *5.*10 mM NACA pre-treatment group with GO (n = 9 lenses), *6.*10 mM diNACA pretreatment group with GO (n = 7 lenses). The levels of H_20_2 are within the mM range consistent with that used previously in the literature (Lou et al., 1995; Zhang et al., 2022), and sufficient for inducing cataract formation within 24 h allowing for rapid screening of changes to lens opacities following pre-treatment with NACA or diNACA. Concentrations of H_2O_2 levels were measured using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (ThermoFisher, Massachuetts, USA). *Group 1:* Following pre-incubation, control lenses were incubated in M199 for 18 h at which time, darkfield and brightfield images were taken. Lenses were placed in fresh media and then incubated for another 24 h in M199 with 1% PSN. Images were then re-taken to assess lens transparency. *Group 2:* In the GO only group, lenses were also further incubated in M199 for 18 h at which time, images were taken. Lenses were placed in fresh media with 0.21U of glucose oxidase (GO) and 1% PSN (Lou et al., 1995), and incubated for a further 24 h, after which images were taken to assess lens transparency. *Groups 3 and 4:* For the NACA (Group 3) or diNACA (Group 4) groups, lenses were cultured in M199 with 10 mM NACA or diNACA and 1% PSN for 18 h at which time, darkfield and brightfield images were taken. *Groups 5 and 6:* For the 10 mM NACA + GO (Group 5) or 10 mM diNACA + GO (Group 6) groups, lenses were pre-treated in either 10 mM NACA or 10 mM diNACA for 18 h, images were taken, and then lenses placed in media containing 1% PSN and 0.21U of GO. Lenses were then incubated for a further 24 h, after which images were taken to assess lens transparency.

Following culturing, all lenses were snap frozen and stored at −80 °C. Each lens was then thawed and dissected on ice into epithelial, cortical, and core fractions. This was achieved by gently removing the capsule with attached epithelium. The lens cortex was then peeled away until only the stiff core remained. The fractions were homogenised in 50 μL for epithelial fractions and 100 μL for cortex and core fractions of 50 mM EDTA, spun down at 15,000 relative centrifugal force for 20 min at 4 °C (Eppendorf 5415 R Refrigerated Centrifuge, Hamburg, Germany) and the supernatant collected. The supernatant was then prepared for quantification of cysteine/cystine (CSH/CSSC) and glutathione/oxidised glutathione (GSH/GSSG) by liquid chromatography tandem mass spectrometry (LC-MS/MS). The data is presented in mM which represent the concentrations present in the supernatant following homogenisation and then centrifugation of either the epithelium, cortex or core fractions in homogenising solution.

2.3. Quantification of lens opacity using ImageJ

All darkfield and brightfield images were taken at the same magnification. Illumination intensity of the light source was kept constant. All images were captured using standardised parameters. Darkfield images were analyzed using the circle tool in ImageJ (National Institutes of Health, Maryland, USA) to quantify the mean pixel value of the entire lens. Background pixel values were measured, averaged and subtracted from the mean pixel value to generate a final mean pixel value.

2.4. Quantification of antioxidant metabolites using liquid chromatography tandem mass spectrometry

LC-MS/MS was used to measure CSH, CSSC, GSH and GSSG levels in rat lenses from all six groups. A calibration curve was first prepared with known concentrations of CSH, CSSC, GSH and GSSG. Known concentrations or calibration curve samples and lens supernatants were immediately treated with 5 mM monobromobimane (MBrB) to derivatise and stabilise thiol groups. Similarly, stable isotopically-labelled standards GSH (13C 15 N) and CSH/CSSC (13C 15 N) were derivatised with MBrB. Then, the internal standard mixed solution of 5 mM CSH/CSSC(13C 15 N)-MBrB, GSH(13C 15 N)-MBrB or GSSG(13C 15 N)-MBrB (Cambridge Isotopes Laboratories, Inc., Massachusetts, USA) was added to the calibration curve and lens samples as an additional quality control. All known and unknown samples with internal mixed standard were then added to Strata-X-C solid phase extraction cartridges (30mg/1 mL) that had been previously conditioned with methanol and 0.1% formic acid, before eluting with 5% NH_4OH in Methanol. Calibration curve and lens samples were then vacuum-concentrated and reconstituted with 5% acetonitrile/0.1% heptafluorobutyric anhydride in H_2O. Separations were then performed by injecting a 10 μL sample into the LC equipped with a Phenomenex Synergi Hydro-RP C18 4 μm 150 × 2mm column (Phenomenex, Torrance, California, USA) and a 0.2 μm in-line filter (Phenomenex, Torrance, California, USA) in gradient mode, at a flow rate of 0.4 μL/min. The column effluent was then directed into an Agilent 6460 A Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, California, USA) with parameters set in Table 1. LC-MS/MS data was analyzed using the Agilent MassHunter Software (Agilent Technologies, Santa Clara, California, USA). CSH, CSSC, GSH and GSSG were quantified using the calibration curve with known concentrations of CSH (range 0–100 μM), CSSC (range 0–50 μM), GSH (range 0–400 μM) and GSSG (range 0–50 μM). Metabolite concentrations were expressed as μM and not normalised to lens wet weight as individual fractions, particularly of the epithelium, were too difficult to accurately measure.

2.5. Statistics

Statistical analyses were carried out with GraphPad Prism® Version 7. All numerical values and graphs are displayed as mean ± standard error of the mean (SEM). Statistical significance between each treatment group including control was carried out using the non-parametric Kruskal-Wallis test with a Dunn's multiple comparison post-hoc analysis. In all instances, P values of <0.05 were considered statistically significant. These are displayed as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, or ****$p < 0.0000.1$.

3. Results

3.1. Pre-treatment with NACA or diNACA reduced H_2O_2-induced opacities in porcine lenses greater than NAC

The ability of NAC, NACA and diNACA to reduce lens opacities

Table 1
MS/MS ions and parameters.

Analyte	Mass transition	Fragmentation Voltage	Collision Energy
CSH-mBrB	312.1 > 225.1	180 V	17 V
	312.1 > 192.1	180 V	24 V
CSH-mBrB (13C 15 N)	316.1 > 225.1	180 V	17 V
	316.1 > 192.1	180 V	24 V
CSSC	241.1 > 152	120 V	10 V
	241.1 > 120	120 V	18 V
CSSC (13C 15 N)	249.1 > 156	120 V	10 V
	249.1 > 124	120 V	18 V
GSH-mBrB	498.2 > 435.1	185 V	21 V
	498.2 > 192.1	185 V	45 V
GSH-mBrB (13C 15 N)	501.2 > 438.1	185 V	21 V
	501.2 > 192.1	185 V	45 V
GSSG	613 > 355	190 V	22 V
	613 > 484	190 V	15 V
GSSG (13C 15 N)	619 > 361	190 V	22 V
	619 > 490	190 V	15 V

caused by H_2O_2 exposure in porcine lenses was evaluated (Fig. 2). Exposure of lenses to 0.75 mM H_2O_2 plus GO resulted in lens opacification. A substantial region of superficial haziness was observed and in some areas, the opacification extended more deeply into the lens cortex. (Fig. 2B). Pretreatment with 0.1 mM, 1 mM or 10 mM NAC, NACA or diNACA appeared to dose-dependently reduce the degree of opacity induced by H_2O_2 plus GO. Quantification of the opacities using Image J revealed that pretreatment with 10 mM NAC resulted in significantly reduced mean pixel values ($p < 0.001$) compared to lenses exposed to H_2O_2 + GO alone (Fig. 2L). Pretreatment with 1 mM NACA or 10 mM NACA significantly reduced mean pixel values ($p < 0.0001$) compared to lenses exposed to H_2O_2 + GO alone (Fig. 2L), while pretreatment with diNACA at all concentrations resulted in significantly reduced mean pixel values ($p < 0.0001$) compared to lenses exposed to H_2O_2 + GO alone (Fig. 2L). This suggests that NACA and diNACA exhibited a stronger anti-cataract effect than NAC.

3.2. NACA and diNACA preserved lens transparency and differentially affected thiol constituents in the rat lens under physiological conditions

To further test the ability of NACA and diNACA to reduce lens opacification and to delineate the molecular mechanisms involved, we used rat lenses which offer advantages over porcine lenses in terms of freshness post-mortem that improves experiment reproducibility and repeatability. We first examined in the absence of H_2O_2, the effects of NACA and diNACA on rat lens transparency (Fig. 3) and whether supplementation with NACA and diNACA improved the bioavailability of CSH/CSSC and GSH/GSSG in the different regions of the lens (Fig. 4).

Rat lenses were cultured for 6 h in M199 media and clear transparent lenses were further cultured for 18 h in either vehicle (H_20) control (n = 6 lenses), 10 mM NACA (n = 6 lenses) or 10 mM diNACA (n = 6 lenses) (Fig. 3). It can be see that all lenses remained clear and transparent with the underlying grid visible during the 6 h pre-incubation period (Fig. 3A–C) and that addition of NACA or diNACA for a further 18 h did not result in lens opacification (Fig. 3B'-C').

In order to determine the effects of NACA and diNACA on CSH/CSSC and GSH/GSSG levels, cultured lenses from each treatment group were dissected into three fractions; epithelium, cortex and core, and LC-MS/MS was used to quantify analytes in each region of the lens. Lenses treated with 10 mM NACA exhibited significantly increased levels of CSH (Fig. 4A–C) in the epithelium (4-fold increase, $p = 0.0074$), cortex (10-fold increase, $p = 0.0004$), and core (8-fold increase, $p = 0.009$) compared to control lenses. Similarly, CSSC was also significantly increased (Fig. 4D–F) in the epithelium (4-fold increase, $p = 0.04$), cortex (32-fold increase, $p = 0.02$), and core (49-fold increase, $p = 0.002$) compared to control lenses. On the other hand, 10 mM diNACA did not alter CSH (Fig. 4A–C) or CSSC (Fig. 4D–F) in any regions of the lens, compared to control lenses.

Lenses treated with NACA did not exhibit significant changes in GSH levels in the epithelium (Fig. 4G), cortex (Fig. 4H), and core (Fig. 4I) when compared to control lenses. Similarly, lenses treated with diNACA exhibited no differences in GSH levels in the epithelium (Fig. 4G) and core (Fig. 4I) compared to control lenses. The diNACA treated lens cortex exhibited a slight increase in GSH (Fig. 4H), which was not significant compared to control lenses, but was significant ($p = 0.002$) when compared to lenses treated with NACA, suggesting that diNACA may be better than NACA at potentiating GSH levels in the cortex. GSSG levels did not significantly differ in the epithelium (Fig. 4J) and core (Fig. 4L) in lenses treated with NACA when compared to control lenses. However, lenses treated with NACA had a slight but significant ($p = 0.02$) reduction in cortex GSSG when compared to control (Fig. 4K). No differences in GSSG levels were seen between controls and lenses treated with diNACA in the epithelium or core (Fig. 4J &L). GSSG in the cortex was significantly increased ($p = 0.02$) in diNACA-treated lenses compared to NACA-treated lenses, but these levels were in the range of control lenses. Taken together, these results show that in rat lens (a) NACA and diNACA do not adversely affect lens transparency, (b) NACA statistically increases CSH levels in the epithelium, cortex and core, and

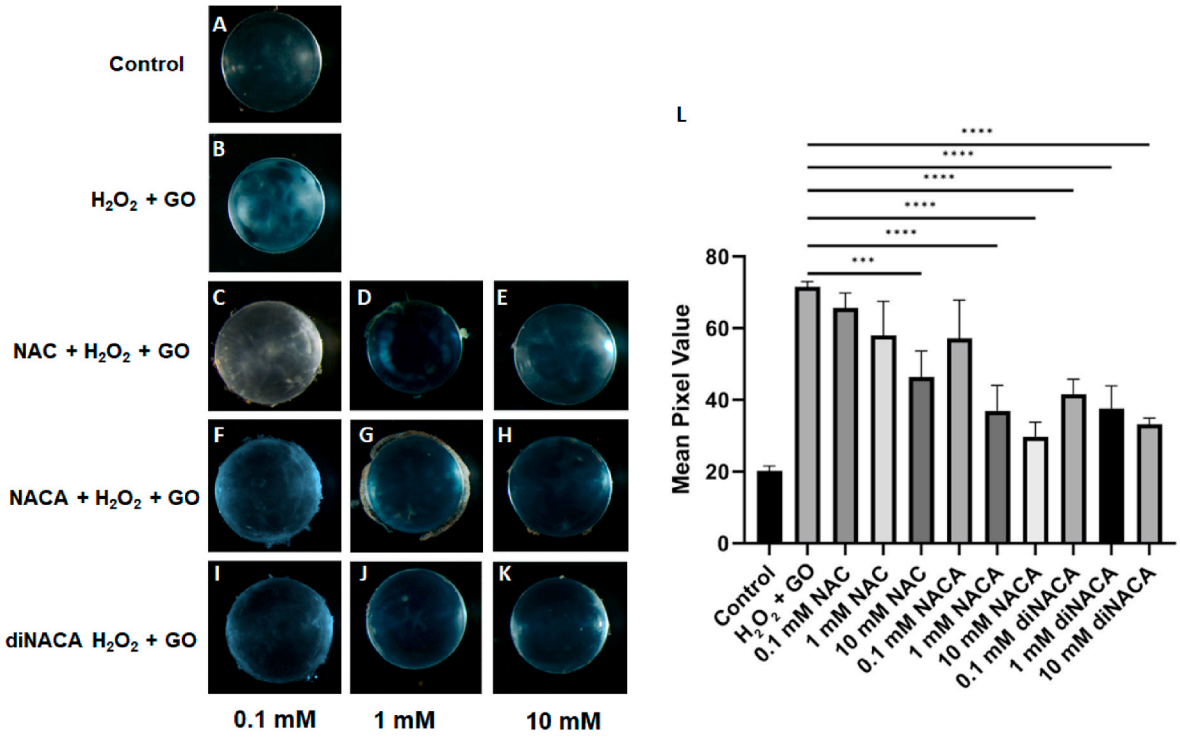

Fig. 2. NACA and diNACA pretreatment inhibited H_2O_2-induced cataract in isolated porcine lenses. Representative darkfield images of **(A)** control lenses, **(B)** lenses incubated with 0.75 mM H_2O_2 and 4.63U GO **(C-K)** Lenses pre-treated with **(C-E)** 0.1 mM, 1 mM, or 10 mM NAC, **(F-H)** 0.1 mM, 1 mM, or 10 mM NACA, or **(I-K)** 0.1 mM, 1 mM, or 10 mM diNACA followed by incubation with 0.75 mM H_2O_2 and 4.63U GO. n = 3 lenses/group. Lenses are cultured and imaged anterior side up. **(L)** Darkfield images were quantified using ImageJ to generate a measurement of mean pixel value ± SD. ***$p < 0.001$, ****$p < 0.0001$.).

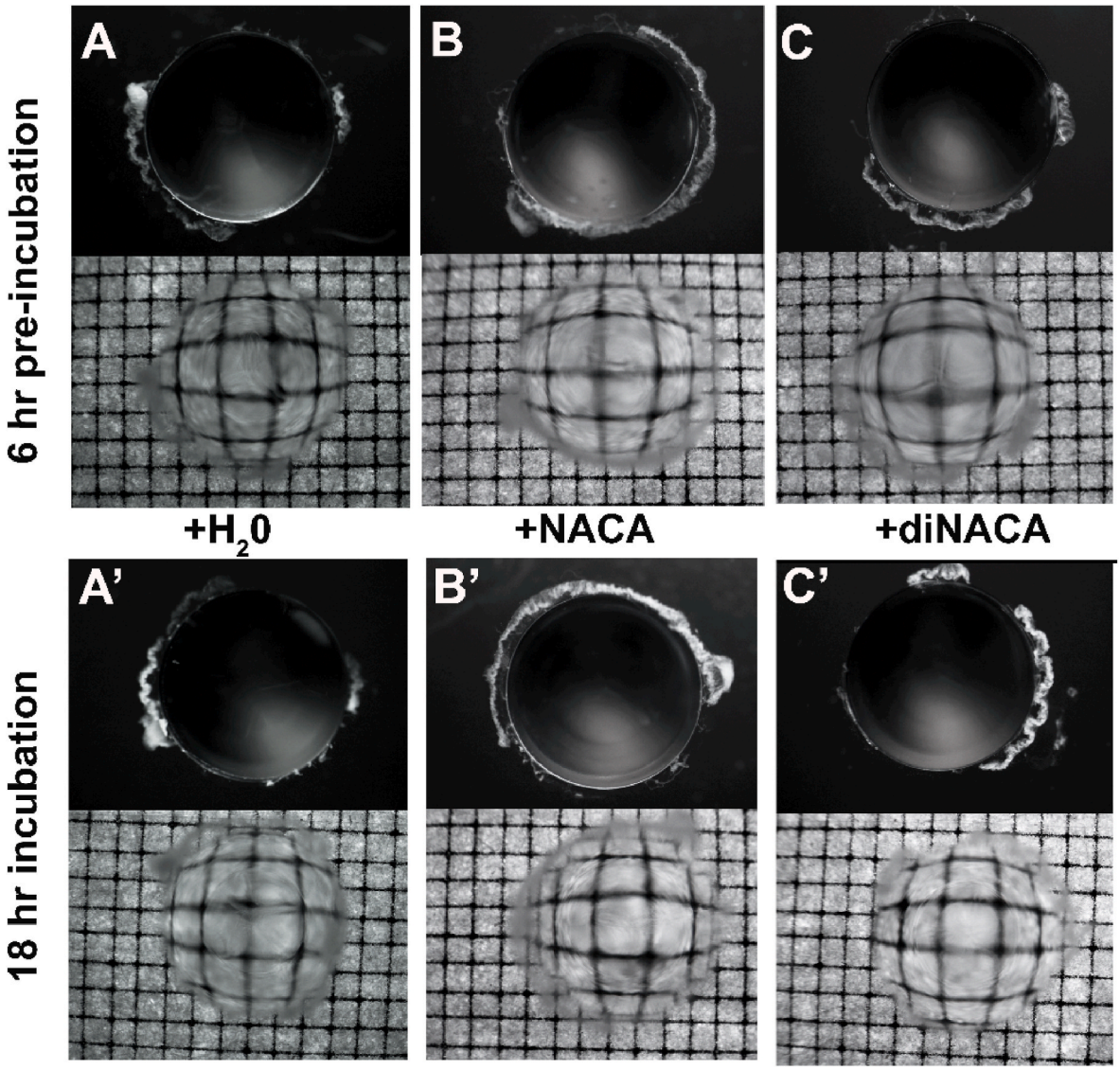

Fig. 3. Effects of NACA or diNACA on rat lens transparency. (A-C) Representative darkfield (upper panel) and brightfield (bottom panel) images of lenses cultured for 6 h in M199 media and (A'-C') representative darkfield (upper panel) and brightfield (bottom panel) images of lenses from A-C cultured for a further 18 h in either (A') vehicle (H_2O), (B′) 10 mM NACA, or (C') 10 mM diNACA. Note: In darkfield images, a reflection is detected in all lenses in the lower right hand quadrant which is due to an artifact of the imaging system.

(c) diNACA statistically increases GSH levels in the cortex compared to NACA-treated lenses. This suggests that NACA and diNACA can enhance antioxidant availability in the lens either via increasing CSH levels or GSH levels in the lens.

3.3. Pre-treatment of rat lenses with NACA or diNACA reduced lens opacities in a H_2O_2 cataract model

Given that the rat lens is able to tolerate exposure to NACA and diNACA under normal physiological conditions, we next determined whether NACA and diNACA were protective of the lens using a model of H_2O_2−induced cataract. As our aim was to pretreat rat lenses with NACA or diNACA for 24 h before cataract induction, we optimized protocols to determine the most appropriate method of H_2O_2 insult. Optimization of this cataract model revealed that a single bolus of 0.5 mM H_2O_2 for 24 h resulted in lenses that remained completely clear and transparent at 48 h (Fig. 5A and A'; n = 7 lenses) and is consistent with the lens being able to effectively and rapidly detoxify H_2O_2. A combination of a bolus of 0.6 mM H_2O_2 and 1 μg/mL GO added at 24 h s, which provides a steady concentration of ~0.6 mM H_2O_2 over the culture period, induced a severe cataract with lenses turning completely opaque at 48 h (Fig. 5B and B'; n = 8). On the other hand, the culture of lenses in 1 μg/mL GO alone at 24 h produced a less dense cataract (Fig. 5C and C'; n = 8') at 48 h compared to a bolus of H_2O_2 plus GO, as some of the gridlines were visible from brightfield images (compare B′ with C'). This suggests that the use of GO alone was sufficient for cataract induction and would be a suitable model for testing the anticataract activity of NACA and diNACA within a brief period of time in isolated rat lenses.

Using the GO only-cataract model, we next tested the ability of NACA and diNACA to protect against opacities (Fig. 6). Control lenses (no oxidant treatment) remained clear throughout the incubation period (Fig. 6A–B & A'-B'). However, lenses exposed to GO developed a dense cataract (Fig. 6D & D'). Lenses that were pre-incubated in NACA and then GO developed an opacity (Fig. 6F & F′), but this opacity appeared reduced compared to lenses exposed to GO alone (Fig. 6D & D'). A similar finding was also observed in lenses pre-incubated in diNACA in which the opacities caused by GO were reduced (Fig. 6H & H') relative to lenses exposed to GO alone. Quantification of the opacities using Image J revealed that pretreatment with NACA and GO exposure (NACA + H_2O_2) or diNACA and GO exposure (diNACA + H_2O_2) resulted in

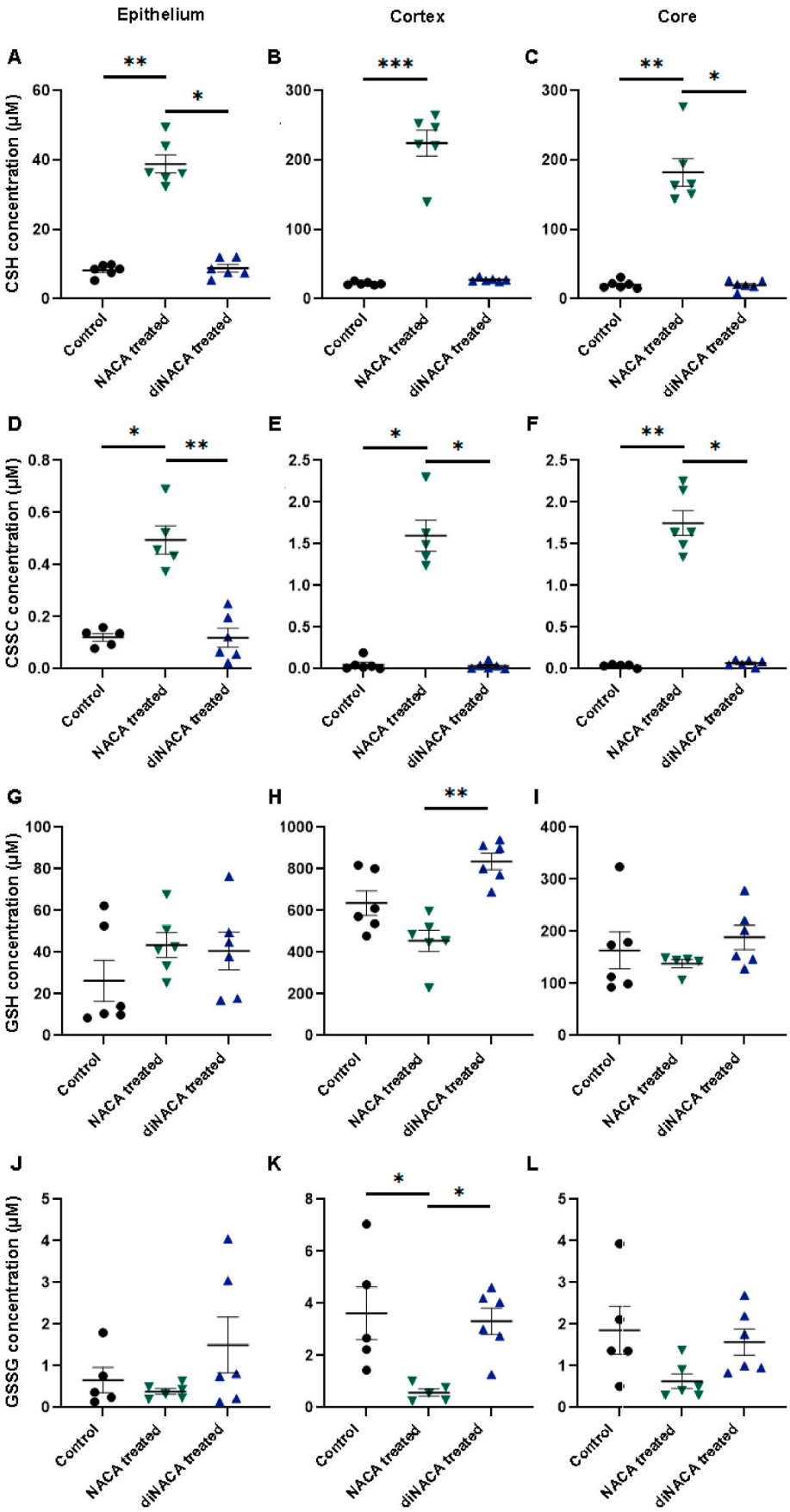

Fig. 4. Cysteine, cystine, glutathione, and oxidised glutathione levels in the rat lens following treatment with NACA or diNACA. Liquid chromatography tandem mass spectrometry quantification of cysteine (CSH), cystine (CSSC), glutathione (GSH), and oxidised glutathione (GSSG) levels in rat lenses treated with either H_2O (*black circles*), 10 mM NACA (*upside down triangles*) or 10 mM diNACA (*triangles*). CSH levels in the **(A)** epithelium, **(B)** cortex, and **(C)** core. CSSC levels in the **(D)** epithelium, **(E)** cortex, and **(F)** core. GSH levels in the **(G)** epithelium, **(H)** cortex, and **(I)** core after a 24 h incubation. GSSG levels in the **(J)** epithelium, **(K)** cortex, and **(L)** core after a 24 h incubation. Note: the concentrations represent the concentrations present in the solution following homogenisation of either the epithelium, cortex or core in homogenising solution. n = 5–6 lenses/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

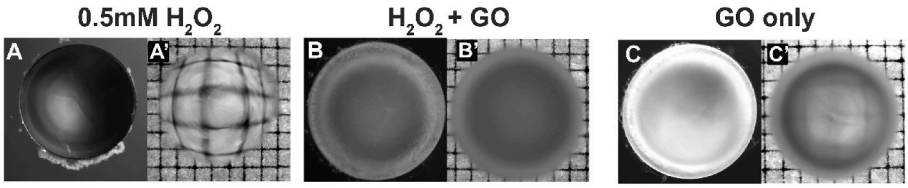

Fig. 5. Optimization of the H_2O_2 cataract model in rat lenses. To determine an appropriate model of H_2O_2 induced cataract, lenses were incubated in either **(A-A')** 0.5 mM H_2O_2, **(B-B')** H_2O_2 plus 0.21U of glucose oxidase (GO), or **(C-C')** 0.21U of GO only. Representative **(A-C)** darkfield and **(A'-C')** brightfield images were taken at 48 h after a 24 h incubation in H_2O_2. (n = 7–8 lenses).

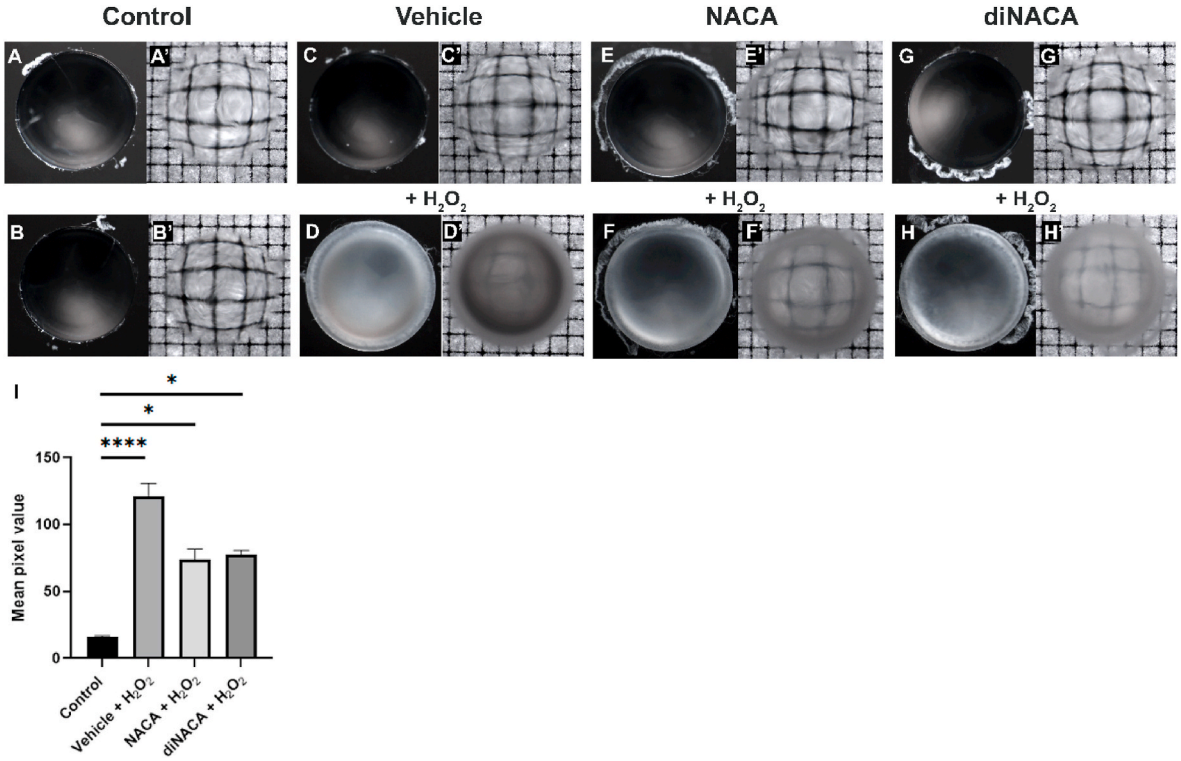

Fig. 6. Effects of NACA and diNACA on GO-mediated H_2O_2-induced rat lens cataract. Representative **(A-H)** darkfield and **(A'-H')** brightfield images of lenses were first incubated in M199 for 6 h to ensure transparency after dissection. Lenses were then further incubated for another 18 h in either **(A)** media **(C)** vehicle, **(E)** 10 mM NACA, **(G)** 10 mM diNACA before being imaged. **(D,F,H)** Lenses were then exposed to 0.21U of glucose oxidase (GO) for another 24 h. **(B)** Control lenses were not exposed to any GO. **(B,D,F,H)** Images were then taken at 48 h to determine the extent of the cataract. **(I)** Darkfield images were quantified at 48 h using ImageJ to generate a measurement of mean pixel value ± SEM. (n = 10 control, n = 7 vehicle + H_2O_2, n = 8 NACA + H_2O_2, n = 7 diNACA + H_2O_2; *$p < 0.05$, ***$p < 0.001$.)

reduced mean pixel values compared to lenses exposed to GO only (vehicle + H_2O_2), though not statistically significant (Fig. 6I).

3.4. NACA but not diNACA was able to increase CSH and CSSC levels in the rat lens epithelium and cortex but not the core

In order to understand the mechanisms involved in reducing lens opacities caused by H_2O_2, we examined the ability of NACA and diNACA to increase the bioavailability of CSH or CSSC to the different regions of the lens. Compared to the control group, CSH levels were significantly reduced in all three regions of the lens (epithelium, cortex, and core) when exposed to GO (Fig. 7A–C). On the other hand, CSSC levels were relatively similar in all regions of the lens between the control group and lenses that were exposed to GO (Fig. 7D–F).

Pre-treatment with NACA, followed by GO (Fig. 7A-C) caused a slight but significant increase (3.3-fold, $p = 0.01$) in CSH levels in the cortex region when compared to lenses exposed to GO but this was not sufficient to restore CSH back to control levels. NACA also significantly increased CSSC levels in the epithelium and cortex relative to lenses exposed to GO alone (Fig. 7D–E). However, the levels of CSSC were much lower than CSH levels indicating that NACA was able to maintain a reducing environment in the presence of H_2O_2. There were no changes to CSH or CSSC levels in the core with NACA.

Pre-treatment with diNACA followed by GO exposure revealed significantly reduced CSH levels when compared to control lenses in all three regions of the lens (Fig. 7A–C), while CSSC levels were similar to control lenses in all three regions. Moreover, pre-treatment with diNACA did not alter CSH or CSSC levels in all regions of the lens compared to lenses exposed to just GO (Fig. 7A–C & Fig. 7D–F).

3.5. NACA but not diNACA was able to increase GSH and GSSG levels in the lens epithelium and cortex but not the core

We also examined the levels of GSH and GSSG in the different regions of the lens after NACA or diNACA pretreatment. Compared to the control group, GSH levels were significantly reduced in all lens regions when lenses were exposed to GO (Fig. 8A–C). Pre-treatment with NACA, followed by GO exposure significantly increased GSH levels in the epithelium (2.3-fold increase, $p = 0.02$) and cortex (4.9-fold increase, $p = 0.002$) when compared to lenses exposed to GO (Fig. 8A–B). This was

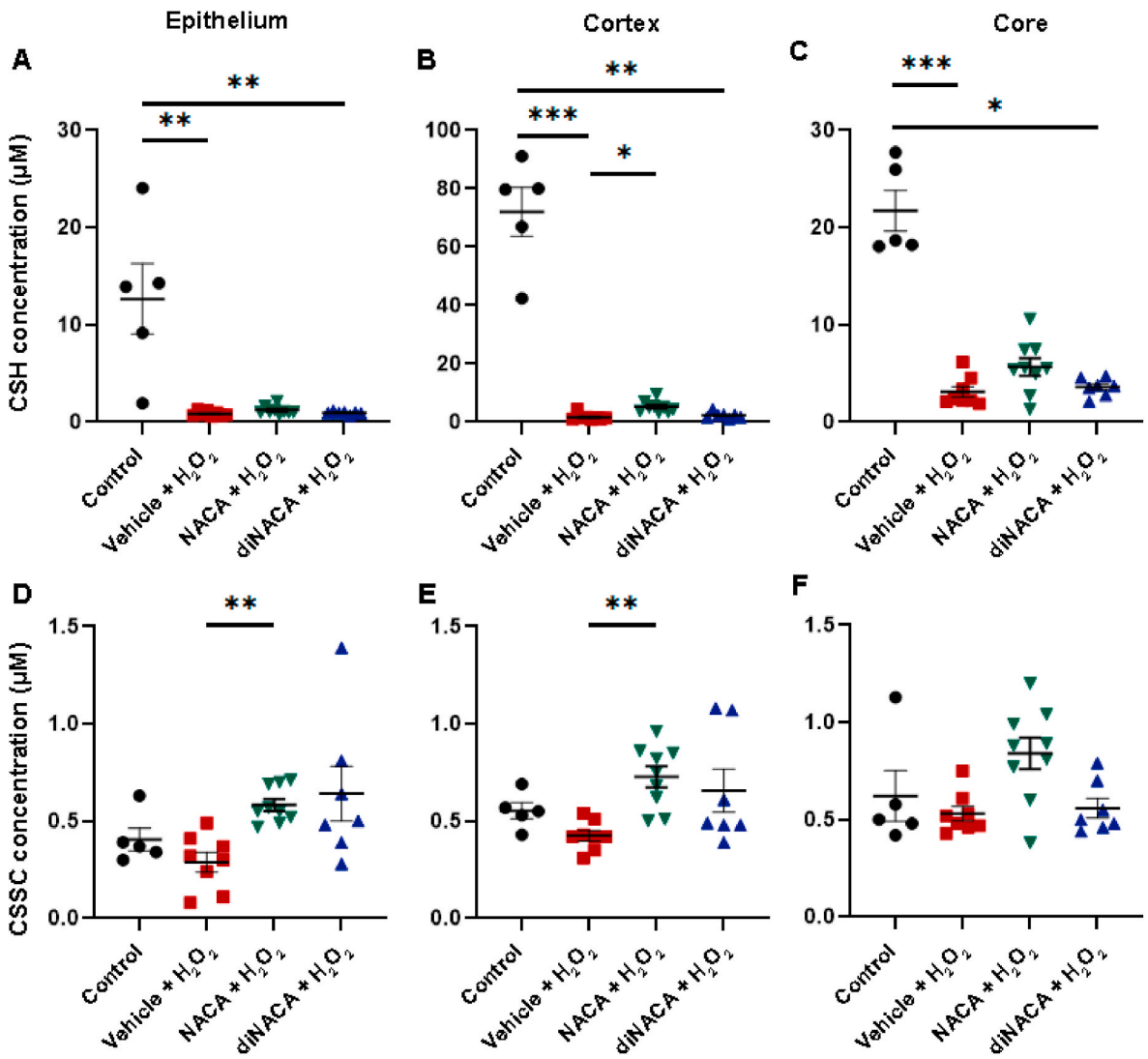

Fig. 7. CSH and CSSC levels following pre-treatment with NACA and diNACA followed by the addition of GO. Liquid chromatography tandem mass spectrometry was used to determine cysteine (CSH), cystine (CSSC), levels in rat lenses that were not exposed to GO or NACA or diNACA (control; *black circles*), lenses exposed to GO (vehicle (H_2O) + GO; *red squares*), lenses pre-treated with NACA and then GO (*green down triangles*), lenses treated with diNACA and then GO (*blue up triangles*). CSH levels in the **(A)** epithelium, **(B)** cortex, and **(C)** core after 48 h of culture. CSSC levels in the **(D)** epithelium, **(E)** cortex, and **(F)** core after 48 h of culture. Note: the concentrations represent the concentrations present in the solution following homogenisation of either the epithelium, cortex or core in homogenising solution. n = 5–9 lenses/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

not evident in the lens core (Fig. 8C). Pre-treatment with NACA, followed by GO, increased GSSG levels in the epithelium (65-fold, $p < 0.0001$) and cortex (93-fold, $p < 0.0001$) compared to lenses exposed to GO alone. However, these GSSG levels were substantially lower compared to GSH levels. This suggests that pretreatment with NACA enables lenses to increase overall GSH levels when exposed to oxidative insult, albeit less than control lenses. Interestingly, though pre-treatment with diNACA was just as effective as NACA (Fig. 6I) at preventing cataract, pre-treatment with diNACA, followed by GO exposure did not increase GSH or GSSG levels in any of the lens regions relative to lenses exposed to GO only (Fig. 8A–C & Fig. 8D–F).

4. Discussion

As we age, GSH levels are reduced in the lens, initiating the development of cataract (Giblin, 2000; Reddy, 1990). Glutathione is an endogenous tri-peptide comprised of glutamic acid, cysteine and glycine amino acid residues, with the sulphydryl (-SH) group on the cysteine residue acting as a potent reducing moiety. Supplementing in vitro cell preparations with cysteine or with its precursor, NAC, can theoretically ameliorate the effects of ROS via promotion of GSH biosynthesis (Forman et al., 2009; Lee et al., 2011). However, GSH, cysteine and NAC have restricted entry through cellular plasma membranes at physiological pH, theoretically limiting their therapeutic potential (Samuni et al., 2013). Other antioxidant moieties have been theorized to possibly compensate for low levels of GSH, e.g., uptake of cystine and its rapid reduction to cysteine (Lim et al., 2005, 2013), but such modalities have not made their way into antioxidant drug development programs.

Though potential anticataract agents have been reported (Lee and Afshari, 2023; Wang et al., 2022; Xu et al., 2020), there are currently no drugs approved to treat or slow progression of cataract. Numerous literature reports by various groups have demonstrated promising anticataract activity for NACA (Carey et al., 2011; Maddirala, 2015; Maddirala et al., 2017; Tobwala et al., 2014) Furthermore, NACA has a favorable lipophilic nature that renders it permeable to the blood-brain barrier (Bahat-Stroomza et al., 2005; Offen et al., 2004) as well as the blood retinal barrier (Dong et al., 2014), thereby offering alternate delivery strategies. We have explored this strategy by screening two

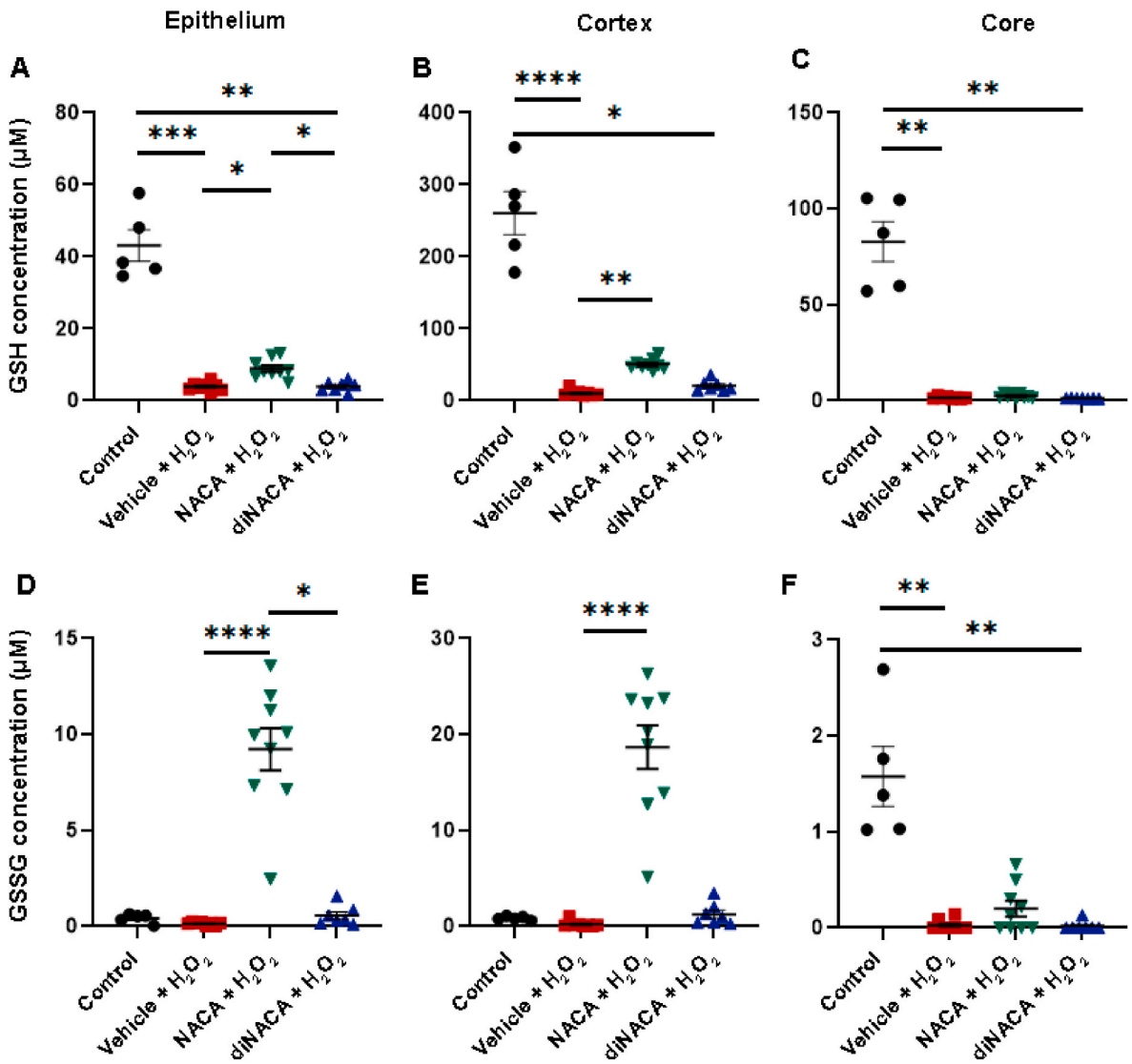

Fig. 8. GSH and GSSG levels following pre-treatment with NACA and diNACA followed by the addition of GO. Liquid chromatography tandem mass spectrometry was used to determine glutathione (GSH) and oxidised glutathione (GSSG), in rat lenses that were not exposed to GO or NACA or diNACA (control; *black circles*), lenses exposed to GO (vehicle (H_2O) + GO; *red squares*), lenses pre-treated with NACA and then GO (*green triangles*), lenses pre-treated with diNACA and then GO (*blue triangles*). GSH levels in the **(A)** epithelium, **(B)** cortex, and **(C)** core after 48 h of culture. GSSG levels in the **(D)** epithelium, **(E)** cortex, and **(F)** core after 48 h of culture. n = 5–9 lenses/group. Note: the concentrations represent the concentrations present in the solution following homogenisation of either the epithelium, cortex or core in homogenising solution. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

cysteine-based analogues, NACA and diNACA, which exhibit increased lipophilicity compared to NAC and can enter the lens without the need to be taken up by cysteine or cystine transporters to potentially enhance antioxidant protection in the lens.

We showed that rat lenses remained clear in the presence of up to 10 mM NACA or diNACA (Fig. 3). Biochemical analyses measuring CSH, CSSC, GSH and GSSG revealed that GSH levels in particular were lower than expected in control lenses (Fig. 4) (Whitson et al., 2016). It is unclear whether this was due to the effect of the freeze/thaw process prior to lens dissection which, although conducted carefully, may have ruptured cells resulting in a small amount of metabolite loss, or the culture process, where it is known that GSH can be exported from the lens (Umapathy et al., 2018). However, since NACA- and diNACA-treated lenses were cultured and processed in an identical manner to control lenses, any changes in metabolite levels observed with NACA or diNACA treatment are strongly suggestive of a treatment effect. Our results revealed that NACA and diNACA differentially affected CSH, CSSC, GSH and GSSG levels in the different regions of the rat lens. NACA increased CSH and CSSC levels in rat lenses, but this did not affect the GSH levels in the epithelium and cortex relative to control lenses (Fig. 4). This suggests that while NACA can increase the bioavailability of CSH in the absence of oxidative stress, rather than being used to synthesise GSH, this source of CSH may be used as an antioxidant itself. On the other hand, biochemical analyses of lenses pre-treated with 10 mM diNACA revealed no changes to CSH or CSSC levels relative to control lenses, and significantly decreased CSH and CSSC levels compared to lenses cultured with 10 mM NACA (Fig. 4). This was surprising as it was expected that diNACA would increase the bioavailability of CSH and CSSC in the lens. However, it may be that CSSC is readily reduced to CSH for GSH synthesis, resulting in lowered levels of CSH and CSSC. This idea is supported by the finding that in the cortex, the region of the lens capable of GSH synthesis, GSH levels were increased in diNACA-treated lenses compared to NACA-treated lenses (Fig. 4). Future work will be required to track the delivery of NACA and diNACA to the different regions of the lens to determine whether these compounds can reach the lens centre, but these results suggest that in

the absence of oxidative stress NACA and diNACA are protective in the lens cortex by increasing the bioavailability of CSH and GSH respectively.

We next tested the ability of NACA and diNACA to reduce H_2O_2 induced opacities in porcine and rat lenses. To do this, we used H_2O_2 and/or GO to produce a steady concentration of H_2O_2 (0.6 mM–0.75 mM) over the culture period. This was important as it known that a single bolus of H_2O_2 is often rapidly consumed and detoxified by the lens and is therefore insufficient to induce lens opacification. However, the steady production of H_2O_2 at the concentrations used in this study were sufficient to stimulate cataract formation. We showed that NACA and diNACA were more effective in reducing H_2O_2 induced opacities compared to NAC in porcine lenses (Fig. 2) and that NACA and diNACA appeared to reduce opacities in rat lenses, although quantitative analysis showed that this was not significant (Fig. 6). In the presence of oxidative stress, lenses pretreated with NACA increased CSH levels in the lens cortex (Fig. 7). Furthermore, NACA was able to increase GSH levels in the epithelium and cortex (Fig. 8), suggesting that under oxidative stress conditions, NACA can increase the bioavailability for CSH resulting in increased GSH synthesis and protection of the lens from H_2O_2-induced cataract. Pretreatment with diNACA did not increase the bioavailability of CSH, CSSC, GSH or GSSG levels in rat lens when exposed to GO, which was surprising given that diNACA was shown to reduce lens opacites (Fig. 6), suggesting that the diNACA mechanism of action was not via enhanced GSH synthesis. DiNACA theoretically should increase the bioavailability of CSSC which in turn can either be rapidly reduced to cysteine or form mixed disulfides with proteins (PSSCs) to buffer against oxidative stress. Thiolation of a protein or enzyme is an initial process for protecting thiol groups from irreversible damage by an oxidant until a repair enzyme system can dethiolate the PSSC and restore the original structure and function of the protein/enzyme (Lou, 2003). As such, PSSCs may protect the lens from further modifications such as protein disulphide formation and protein aggregation and may explain why lenses treated with diNACA were still able to reduce opacities without increasing GSH levels. Future work to measure PSSCs in the lens will help to clarify the mechanism by which diNACA is able to protect the lenses from H_2O_2-induced opacities.

Taken together, these preliminary screening results warrant further anticataract testing of NACA and diNACA to better understand the mechanisms involved and to further test the applicability of these compounds to other models of cataract, e.g., the hyperbaric oxygen model which mimics many of the biochemical features associated with age related nuclear cataracts (Lim et al., 2016). This work is of ongoing importance given the lack of any anticataract drug products in an ever growing, aging and diabetic population.

Author's contributions

Study of diNACA and further study of NACA for anticataract activity was envisioned by GMW. HW and GMW contributed to development of the pig study protocol. RMM, ACG, JCL, PJD and GMW contributed to development of the rat study protocol. All authors contributed to interpretation of the data, writing and reviewing the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

GMW is an employee and equity holder of Nacuity Pharmaceuticals, Inc. Other authors are independent academic investigators with no conflicts.

Data availability

No data was used for the research described in the article.

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