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Effects of n-acetylcysteine amide in preventing/treating cataracts

Sri Krishna Yasaswi Maddirala

EFFECTS OF N-ACETYLCYSTEINE AMIDE IN PREVENTING/TREATING
CATARACTS

by

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ABSTRACT

Cataract, the opacification of an eye lens, is a common pathological abnormality of the lens accounting for approximately 50% of all blindness. The only effective treatment currently available for a cataract is the surgical removal of the affected lens and replacement with an artificial lens for the restoration of vision. Although, cataract surgery is considered to be a very successful procedure in terms of visual outcome, the cost of surgery, need for trained personnel and surgeons, and postsurgical complications, limit the worldwide availability and accessibility of this procedure. Hence, alternative preventive and treatment procedures are worthy of investigation. The lens depends on a balanced redox state for maintaining its transparency, and a high content of glutathione (GSH) in the lens is believed to play a key role in doing so. Several studies have reported that oxidative stress plays an important role in the etiology of cataract development and, therefore, the present study has sought to evaluate the efficacy of a thiol antioxidant, (R) - N-acetylcysteine amide (NACA), in preventing/reversing cataracts. To investigate NACA's ability to provide therapeutic benefits for cataracts, three different experimental models were utilized. The first was an *ex-vivo* cataract model, where culturing the rat lenses in dexamethasone resulted in posterior cataracts. The second was an *in vivo* mouse model, where injection of acetaminophen caused cataracts. The third model was a rat *in vivo* model where injection of sodium selenite generated nuclear cataracts. Treatment with NACA in each model helped to decrease the severity of cataracts. In summary, the results from this study suggest that NACA can potentially be developed into a promising therapeutic option for prevention and reversal of cataract formation.

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

LIST OF ABBREVIATIONS

Symbol	Description
4-HNE	4-hydroxynonenal
6PG	6-phosphogluconate
γ-GCS	γ-glutamylcysteine synthetase
APAP	N-acetyl-p-aminophenol (Acetaminophen)
$CaCO_3$	Calcium carbonate
CuZn-SOD	Copper-zinc superoxide dismutase
CYP	Cytochrome P450
Dex	Dexamethasone
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC-SOD	Extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FDA	Food and drug administration
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Glucocorticoid
GPx	Glutathione peroxidase

Symbol	Description
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
IL-1, IL-6	Interleukin-1, Interleukin-6
LOOH	Lipid hydroperoxides
LPO	Lipid peroxidation
LPOs	Lipid peroxides
MCE	2-mercaptoethanol
MDA	Malondialdehyde
Mn-SOD	Manganese superoxide dismutase
Na_2SeO_3	Sodium selenite
Na_3VO_4	Sodium orthovanadate
NAC	N-acetylcysteine
NACA	(R)-N-acetylcysteine amide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPQI	N-acetyl-p-benzoquinone imine
NEI	National Eye Institute
$NO^{\bullet}$	Nitric oxide radical
NPM	N-(1-pyrenyl)maleimide
$OH^{\bullet}$	Hydroxyl radical

Symbol	Description
$O_2^{\bullet-}$	Superoxide radical
PD	Postpartum day
PSC	Posterior subcapsular cataract
PSSG	Protein mixed disulfide
PSH	Protein thiol
PSSP	Protein thiol disulfide
PUFA	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SBB	Sodium borate buffer
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
Trx	Thioredoxin
Ttase	Thioltransferase
UV	Ultraviolet

1. INTRODUCTION

A cataract causes a progressive loss of transparency of the lens that affects vision. It is the most common cause of curable blindness, which accounts for approximately 50% of all blindness. Thus more than 17 million people are blind because of cataracts, and worldwide, 28 000 new cases are reported daily. Most cataracts are age related and, according to the National Eye Institute (NEI), by age 80, more than half of all Americans either have a cataract or have had cataract surgery. In the U.S. over 1.2 million cataract operations are performed per year; the costs are over 3.4 billion dollars [1]. The NEI and Prevent Blindness America estimate that more than 22 million people in the U.S. live with cataracts and, by 2020, this may exceed 30 million (NEI, 2008) [2]. Although, a cataract is not associated with signs such as pain or redness in the eye, it is still characterized by symptoms such as blurry vision, double vision, glare of light, and yellowing of colors. The only effective treatment currently available for a cataract is the surgical removal of the affected lens and replacement with an artificial lens for the restoration of vision. However, the cost of surgery and requirements for highly trained personnel to perform ocular surgery, limit the restoration of vision in certain parts of the world. Unfortunately, a majority of people with vision impaired by cataracts live in developing countries where access to surgery is limited [3]. Hence, there is an immediate necessity for prevention and non-invasive treatment of cataracts.

The lens is a transparent, biconvex, avascular structure that is responsible for focusing the images onto the retina [4]. The lens depends on a balanced redox state to maintain its transparency. Lens tissue is subjected to significant amounts of oxidative

stress requiring elevated levels of cellular antioxidants to neutralize reactive oxygen species (ROS). The endogenous high level of glutathione (GSH) is responsible for the first line of defense against oxidative stress and keeps lens proteins in a reduced state, thereby, maintaining lens transparency [5]. During the development stage of the lens, it has been determined that it contains a high concentration of antioxidant enzymes, as well as GSH. However, with aging, the *de novo* synthesis and the recycling system for GSH become less efficient [6], causing diminished levels of GSH and other antioxidant defenses. This leads to increased incidences of oxidative damage, which eventually results in the formation of cataracts.

Cataractogenesis is considered to be a multifactorial disease involving various pathogenic mechanisms that are not clearly understood, with oxidative stress being the foremost cause in initiating cataract formation [7-13]. Oxidative stress implies an imbalance between the rate of oxidant production and the rate of detoxification, with the rate of production being significantly higher when compared to that of detoxification by antioxidants [14]. An organism counteracts this condition by its natural antioxidant defense systems, but with age, oxidants accumulate while antioxidant defenses gradually diminish. This could probably be the most important mechanism in age-related cataract formation. Accumulation of oxidants results in aggregation and precipitation of crystallin proteins, which are responsible for maintaining transparency of the lens [15]. Since crystallins make up more than 90% of the total dry mass of the lens, aggregation and precipitation of these proteins leads to insolubility and fragmentation, eventually resulting in the formation of cataracts [16].

Since oxidative stress is implicated in cataract formation, a logical approach to counteract cataractogenesis would be the use of an effective lens permeating antioxidant that would neutralize and alleviate cumulative oxidative damage. Several compounds with antioxidant properties have been reported to prevent cataracts, with few of them in the clinical phase. One such powerful antioxidant, which was recently developed into an eye drop formulation, is N-acetylcarnosine. The topical administration of N-acetylcarnosine (as a bio-activating antioxidant for vision) in an eye drop formulation delivered pure L-carnosine, which was found to be present in appreciable levels in transparent human lenses and depleted in the lenses of mature cataracts [17]. This allowed an increased intraocular absorption of L-carnosine resulting in an increased anti-cataract drug efficacy [18]. Although recently published studies have reported that N-acetylcarnosine may ameliorate the risk for cataracts [18], a larger trial was needed to justify the long-term benefit of N-acetylcarnosine.

Use of antioxidants to treat/prevent oxidative stress-related disorders is becoming more popular, with thiol antioxidants (such as GSH, cysteine, and N-acetylcysteine (NAC)) providing some protection [19-21]. However, NAC is negatively charged at physiological pH, which limits its ability to permeate cell membranes and, therefore, requires higher doses and longer times of treatment. In contrast, N-acetylcysteine amide (NACA), an analog of NAC, has been shown to be more effective than NAC, owing to its neutral amide group, making it more lipophilic and, therefore, increasing its ability to penetrate cell membranes. Previous studies have also shown that NACA is lipophilic and can cross membranes, chelate Cu^{2+}, scavenge free-radicals, and protect against oxidative stress [22-29]. Our own studies have demonstrated that NACA is better than NAC at

alleviating oxidative stress [26, 30]. Furthermore, NACA is effective at lower concentrations (compared with NAC), which helps to prevent the various adverse effects that are associated with administration of higher doses of NAC [19, 24, 31-33].

The development of an antioxidant eye drop formulation that could prevent and reverse the cataract grade would significantly reduce the number of patients becoming blind worldwide because of the unavailability of surgical procedural treatments and also because of the financial burden involved in undergoing cataract surgery. By slowing the onset of cataract formation, or arresting the progression of the disease in its early stages, surgery could be completely avoided in many cases. Therefore, the present study has sought to evaluate the efficacy of the thiol antioxidant, NACA, in preventing/reversing cataracts in rodent models. A successful result of this research would provide impetus for further study of antioxidant-based approaches in treating cataracts, with the emphasis shifted towards a mechanistic approach rather than surgical intervention, which would be better for patients. This could help to achieve the goal of preventing unnecessary blindness worldwide.

2. REVIEW OF LITERATURE

2.1. CATARACT

A cataract is an opacification of the lens of the eye affecting the vision. The lens is a transparent, biconvex, elliptical, semi-solid, avascular structure that is located between the iris and the vitreous (Figure 2.1). The lens, along with the cornea, focuses light onto the retina, the light-sensitive tissue at the back of the eye. To do this, the lens must be clear, have a relatively high refractive index, and be able to accommodate. The cloudiness in the lens scatters the light before arriving at the retina, hence the image produced appears blurred.
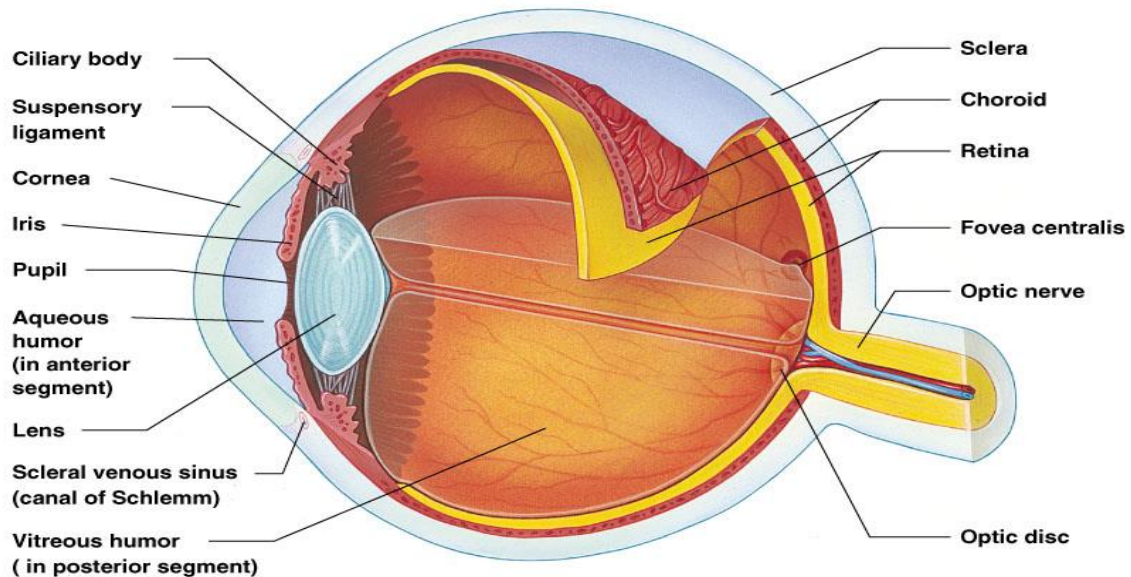
Figure 2.1. Eye. Diagrammatic representation of adult human eye showing the location of the lens in the eye.

In the embryonic stage, the lens appears as a hollow ball of cells surrounded by a capsule, with each cell containing a nucleus. The outside facing surface of the eye is designated as the anterior side of the lens and the inside facing surface is designated as the posterior side. As the lens develop, the posterior cells elongate into the cavity towards the anterior cell layer forming the primary lens fiber which forms the core of the lens nucleus [34, 35]. During this process, the cells also make a large quantity of proteins called crystallins which becomes the primary structural components of the lens fiber. The primary lens fibers soon lose their nuclei and other cellular organelles and become inert structures. However, the anterior cells remain in the lens as a single layer of living cells along the anterior surface and will continue to divide along the edges to form secondary lens fibers which form the core of the lens cortex. As the secondary fibers grow, they also make large quantities of crystallins; however, at maturation, they lose their nuclei and other cellular organelles resulting in the loss of ability to synthesize crystallins [36, 37]. New secondary lens fibers continue to form throughout the life of a person (Figure 2.2).

The primary structural proteins of the lens fiber cells that are responsible for the lens transparency and uniform refractive index, and for maintaining minimal light scattering are crystallins. The crystallins constitute more than 90% of the total dry mass of the lens. Crystallins are water soluble lens proteins which cannot be replaced and, thus, have to last the lifetime of an organism. Most of the vertebrate lenses contain three classes of crystallins- the α-, β-, and γ-crystallins. In the lens under 15 years of age, nearly 50% of the nuclear soluble protein is α-crystallin. By their mid-20's, this drops to 25% and by the mid-40's, α-crystallins represent less than 5% of water-soluble lens proteins, while the percentage of aggregated crystallins increases with age [38].
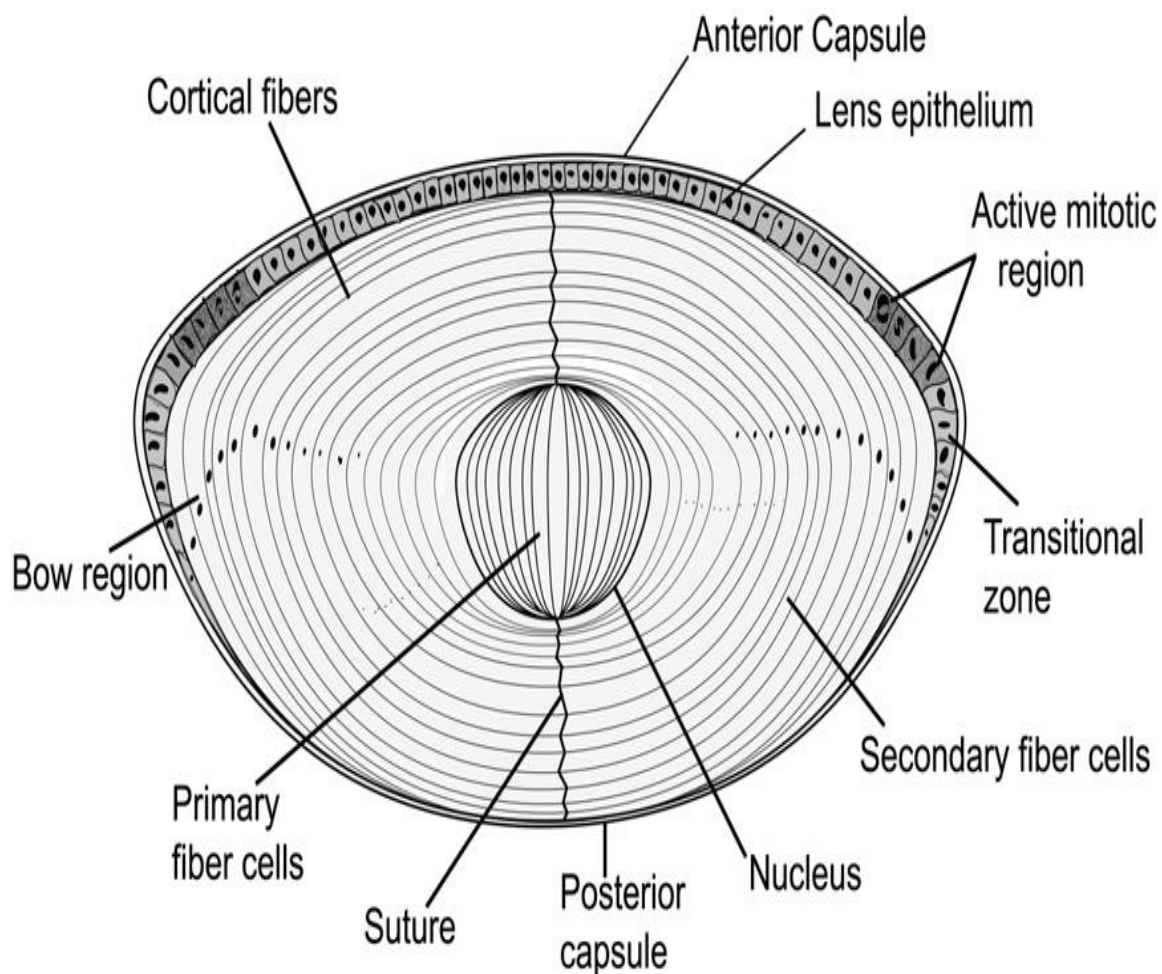
Figure 2.2. Lens Structure. This figure shows a cross section of the entire structure of a lens that has been divided into various regions. The anterior region of the lens capsule is surrounded by epithelial cells. The bow region is where cells differentiate, elongate, lose their organelles, and mature into fiber cells.
(Adapted from Sharma KK et al. [39].)

In addition, with time, these crystallins undergo several irreversible changes because of proteolysis, oxidation, deamidation, maillard reactions, and cleavage resulting in protein insolubilization and precipitation [40-45]. With aging, the antioxidant defense systems in the lens diminish causing decreased GSH levels, and reduced enzyme

efficiencies associated with GSH, resulting in the accumulation of crystallin proteins. The cumulative effects of the above ultimately leads to a cataract.

While the etiology of cataracts is still unclear, oxidation is considered as the major cause of cataract formation [5, 46-48]. Crystallin proteins contain amino acids (such as cysteine and methionine) which are prone to oxidation. While α-crystallin has only two cysteine residues, β-crystallin has several cysteine residues that are susceptible to oxidation, making intra-disulfide linkages more probable, resulting in high molecular weight aggregates. Tryptophan and histidine are susceptible to oxidation, but their oxidation in aging lenses is minimal, as compared to that of cysteine, although an evidence has been shown that these amino acids are oxidized in advanced cataract development [5]. Free radicals, such as hydroxyl radical, superoxide, and singlet oxygen, are the likely oxidizing species *in vivo* [49-52]. The α-crystallin binds to early unfolding intermediates of other crystallins, preventing their aggregation and insolubilization and hence, preventing light scattering [40]. Investigators have suggested that once all of the α-crystallin has been utilized, the concentration of the irreversibly denatured proteins would rise to a level at which aggregation occurs, resulting in a cataract [40]. Crystallin proteins cannot be replaced and, with aging, these proteins accumulate at the center of the lens. Since these proteins cannot be repaired nor catabolized, having a protein that has chaperon-like characteristics would be beneficial to the eye lens. The chaperon-like activity of α-crystallins may be the reason why these proteins were recruited by the eye [15]. Investigators have shown that non enzymatic glycation, truncation and oxidation of α-crystallins results in the loss of chaperon activity [53, 54]. There are two α-crystallin genes, αA and αB, that encode for proteins that share approximately 60% sequence

identity [55]. In the mammalian lens, the molar ratio of αA to αB is generally three to one [40]. The αA crystallin is mainly expressed in lens, with trace amounts found in other tissues [56-58]. The αB crystallin is considered ubiquitous and is expressed abundantly in the brain, heart, and muscle [59]. Since crystallins have to last the lifetime of an organism, they are susceptible to the accumulation of various post-translational modifications that are thought to damage their structure and alter the function of crystallins [60-62] . The crystallin proteins of the lens have several phosphorylation sites that have been shown to regulate the chaperone activity of α-crystallin proteins [63-65]. Phosphorylation at serine residues 19, 45, and 59 have been identified as a major modification for α-crystallins [66]. Despite phosphorylation being a common feature of α-crystallin, both inside and outside the lens [67], the *in vivo* significance of these modifications and their effects on chaperone-like activity remain unclear [55]. Many researchers have reported that phosphorylation has no effect on chaperone activity [68, 69], but one study demonstrated that a sub-unit of α-crystallin protein from a cataract lens was not phosphorylated [70]. The significance of this absence of phosphorylated α-crystallin is yet to be determined. S-Methylation, that is specific for Cys 26 and Cys 24, is one of the most prominent post-translational modifications of human lens crystallins. In contrast to most post-translational modifications, which continue to increase with lens age, methylation levels reach to 40% at about 19 years of age, increase further to 50%, and then decrease slightly as the lens gets older. Methylation of these exposed cysteine residues prevents their participation in the intermolecular disulfide bonding that leads to protein cross linking, aggregation and, eventually, cataracts. Hence, methylation could be a defense mechanism against cataracts [71-73].

2.2. OXIDATIVE STRESS

The lens depends on a balanced redox state for maintaining its transparency, and a high content of GSH in the lens is believed to play a key role in doing so. Alterations in the GSH metabolism within the lens result in an increased oxidative stress that has been implicated in the pathogenesis of a cataract [10, 48]. As the lens age, the oxidants (ROS) accumulate as a result of oxidative stress, causing damage to the eye. The lens defends this condition by employing antioxidant enzymes and proteins which help in protecting important biochemical functions within the eye. ROS, such as superoxide radical, hydrogen peroxide, and hydroxyl radical, attacks the proteins and polyunsaturated fatty acids (PUFAs) of lipid membranes causing damage to the eye. UV light also generates oxidative stress and the eye is more susceptible to UV damage as the lens age. The levels of UV filters in the lens decrease linearly with age, at a rate of 12% per decade [74].

2.2.1. Reactive Oxygen Species (ROS). Highly reactive biochemical compounds containing oxygen are collectively known as ROS. Most ROS are generated as by-products during mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation [75-82]. It was originally thought that only phagocytic cells produce ROS as a part of their host cell defense mechanism. Recent studies have demonstrated that ROS have a role in cell signaling, including apoptosis, gene expression, and the activation of cell signaling cascades [83]. However, during times of environmental stress, such as UV or heat exposure, ROS levels, that can increase dramatically [84], have the potential to cause a number of deleterious events. There are numerous types of ROS; however, those which are also free radicals are the most reactive and damaging species. The three most important free radicals include

superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2).

Although H_2O_2 is not a free radical by itself, via Fenton reaction it generates $OH^{\bullet}$ radical

which is the most reactive free radical. Because of the presence of unpaired valence

electrons, these species are highly reactive and can damage cellular structures.

2.2.2. Lipid Peroxidation (LPO). This is a process under which free radicals

attack lipids containing carbon-carbon double bonds, especially PUFAs that involve

abstraction of hydrogen from a carbon, with an oxygen insertion leading to lipid peroxyl

radicals and hydroperoxides[85]. Glycolipids, phospholipids, and cholesterol are the most

susceptible lipid structures that promote free radical attack. LPO produces a wide variety

of oxidation products. Lipid hydroperoxides (LOOH) are the primary products of LPO.

Among different aldehydes that are produced as secondary products, malondialdehyde

(MDA), hexanal, and 4-hydroxynonenal (4-HNE) have been extensively studied [86-97].

These lipid by-products are formed via a free radical chain reaction that occurs within the

cellular environment. MDA is considered to the most mutagenic by-products of LPO,

whereas 4-HNE is the most toxic. The mechanism of LPO is shown in Figure 2.3.

Initiation of LPO begins with the abstraction of hydrogen by a hydroxyl radical

from the methylene group, adjacent to a double bond of PUFAs, leading to an unstable

lipid radical. This leaves an unpaired electron on the carbon, forming a carbon-centered

radical, which is stabilized by a molecular rearrangement of the double bonds to form a

conjugated diene, which then readily reacts with oxygen to form a peroxyl radical. The

lipid radical or the peroxyl radicals can abstract hydrogen from the neighboring fatty acyl

chain of PUFAs, thereby leading to the propagation of the peroxidation process and a

chain reaction that causes damage to the membrane proteins [98]. The chain reaction

terminates with the formation of LOOH. The LOOH is the first, comparatively stable

product of lipid peroxidation [99]. Lipid peroxidation causes injury to cells and

intracellular membranes which can lead to cell destruction and, subsequently, cell death

[100]. The LOOH reacts with transition metal ions, leading to the generation of radicals

that are capable of re-initiating the lipid peroxidation by redox-cycling of the metal ions

[101, 102].
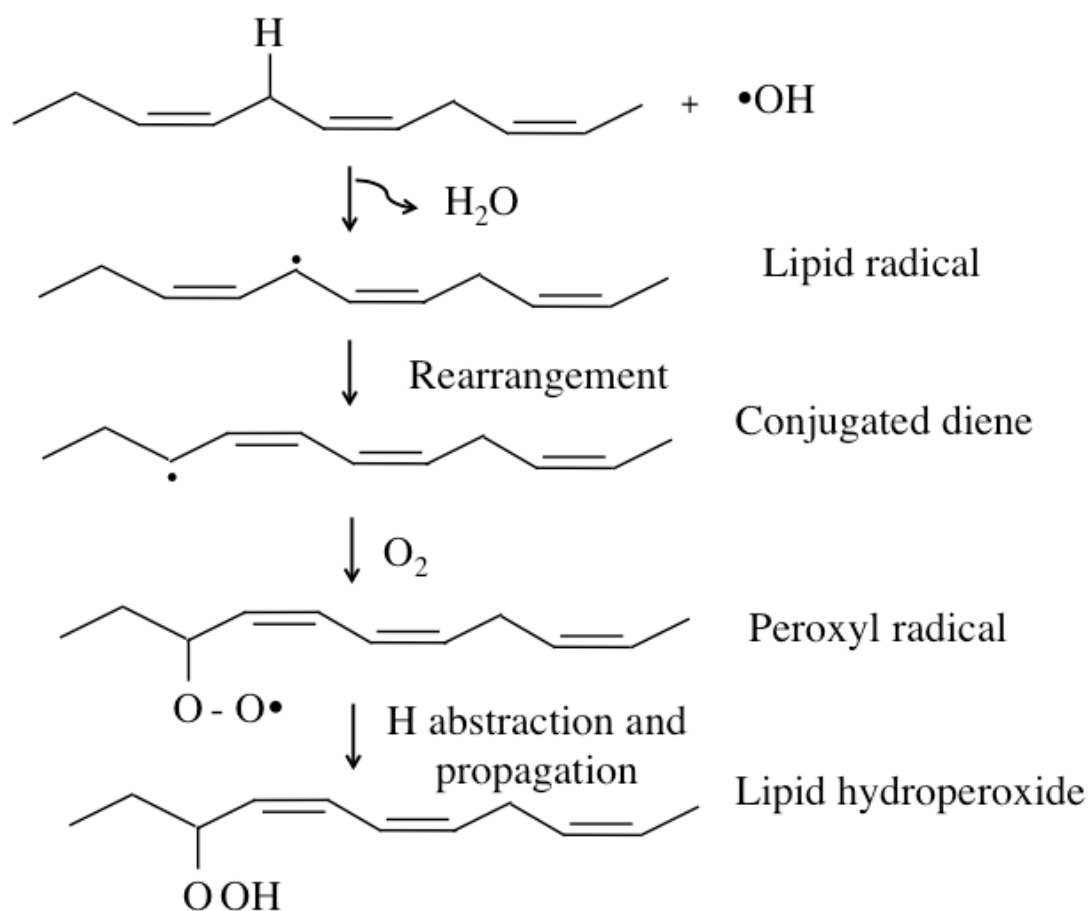
Figure 2.3. Lipid Peroxidation. Chain of events in lipid peroxidation, starting with
initiation, propagation and termination stages.
(Illustration: Vikram Jairam, Koji Uchida, and Vasanthy Narayanaswami.
Pathophysiology of Lipoprotein Oxidation. October 2012)

2.2.3. Protein Oxidation. Protein oxidation involves a number of modifications including hydroxylation of amino acids, such as tyrosine, phenylalanine, oxidation of methionine to methionine sulfoxide, and oxidation of cysteine residues to disulfides. The thiols in the cysteine residues of proteins may undergo different reversible and irreversible redox modifications in reaction with ROS and reactive nitrogen species (RNS) that are considered to be important for protein regulation. However, most of these modifications are readily reversible through mechanisms involving GSH. ROS and RNS can oxidize proteins in different pathways. RNS can cause S-Nitrosylation of the thiol residues of proteins, whereas oxidants (like H_2O_2) oxidize thiols to sulfenic acid, sulfinic acid, and sulfonic acid. Although sulfenic acid is readily reversible by GSH, because of its instability it undergoes further oxidation to sulfinic and sulfonic acids, which cannot be reversed by GSH. In the presence of ROS or RNS, a cysteine residue forms a thiol radical which then reacts with another thiol radical of the vicinal or neighboring protein resulting in protein disulfides. The mechanism of protein oxidation is shown in Figure 2.4.

2.3. ANTIOXIDANT DEFENSE

The human body has employed different antioxidants to counterbalance the oxidative stress caused by various oxidants. Sies [103] stated antioxidant as 'any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate'. Antioxidants acts as a first line of defense against ROS formation. These can be classified into two categories: enzymatic and non- enzymatic. The important ones are listed in Table 2.3.

S-Nitrosylation

Protein-S-NO

S-Glutathionylation

Protein-S-S-G

Protein-SH

NO•

GSSG

ROS (H₂O₂)

S-Hydroxylation

ROS + P-SH

P-SOH $\xrightarrow{\text{ROS}}$ P-SO₂H $\xrightarrow{\text{ROS}}$ P-SO₃H

Sulfenic Acid   Sulfinic Acid   Sulfonic Acid

P-S-S-P

Disulfide Linkage

Irreversible modifications
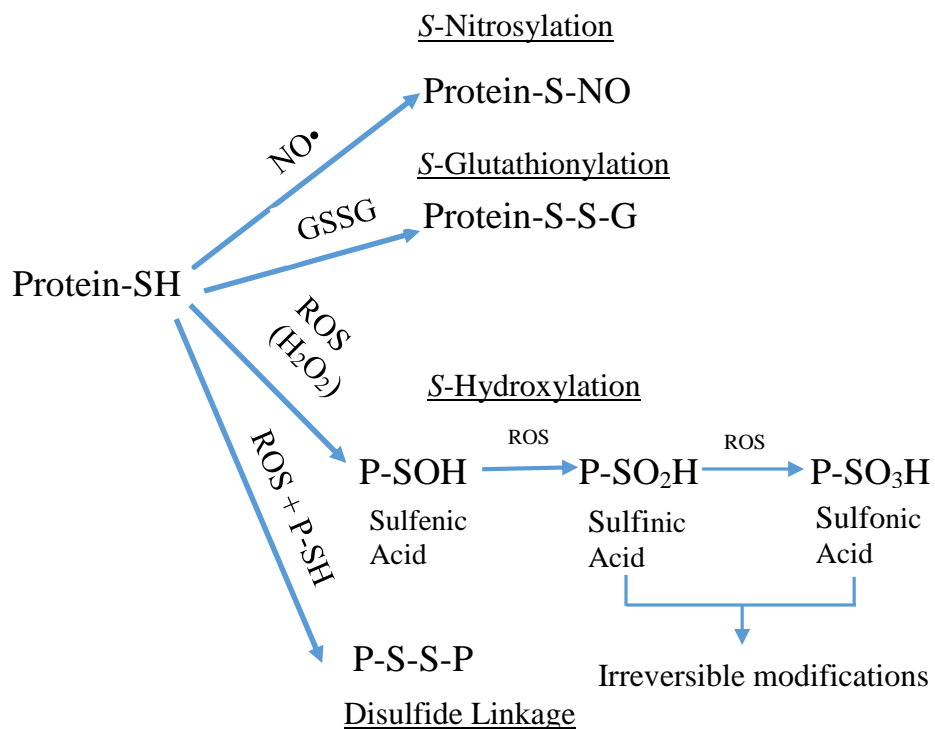
Figure 2.4. Modification of Protein Thiols. Protein thiols undergo different modifications after reactions with ROS and RNS. NO• reacts with the protein thiols causing S-Nitrosylation. Glutathione disulfide (GSSG) undergoes disulfide exchange with protein thiols resulting in S-Glutathionylation. Oxidation of thiols in the presence of ROS (such as H_2O_2) leads to sulfenic acid, which can then be oxidized to sulfinic acid and sulfonic acid. In the presence of ROS, a cysteine residue forms a thiol radical which then reacts with another thiol group to form a disulfide bond.

Table 2.3. Classification of Antioxidant Defense Network

Antioxidant Enzymes	Small antioxidants
Superoxide dismutase	Ascorbic acid
Catalase	α-Tocopherol
Glutathione peroxidase	GSH
Glutathione reductase	β- Carotene
Thioltransferase	
Thioredoxin	

2.3.1. Antioxidant Enzymes. The eye contains several antioxidant enzymes that play key role in protecting the eye against oxidative damage. The important enzymes include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, thioltransferase, and thioredoxin.

Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen [104].

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad \text{(Reaction 1)}$$

The antioxidant system of SOD is formed by three isoenzymes, with CuZn-SOD existing in cytosol [105], Mn-SOD in the mitochondrial matrix [106], and secretory EC-SOD in the tissue interstitium [107]. A large difference in the total amount of SOD activity and isoenzymes distribution in different compartments of the human eye have been reported [108], with the retina having the highest activity of SOD and the lens having the least SOD activity of any mammalian tissue examined so far [109]. CuZn-SOD isoenzyme activity per milligram of DNA was found to be highest in the retina, when compared to most other extraocular tissues. EC-SOD activity was reported to be highest in the sclera and cornea which accounts for more than half of the total SOD activity in these tissues [108].

The aqueous humor of many species (including human eye) has been shown to contain significant amounts of H_2O_2 [110-112], which may be formed from the light catalyzed reaction of ascorbic acid that is present in the aqueous humor [111, 112]. H_2O_2 is also formed from the dismutation of superoxide by SOD. H_2O_2 accumulation, if not counteracted, could lead to severe oxidative damage resulting in the loss of cell function [113-115]. However, the eye is endowed with defense systems, such as catalase and

glutathione peroxidase (GPx) enzymes, that can counteract the H_2O_2 that is generated in

the eye. Catalase catalyzes the degradation of hydrogen peroxide to water and oxygen

[116] (Reaction 2). However, GPx also detoxifies the H_2O_2 using GSH as a substrate

(Reaction 3). Relative activities of catalase and GPx suggest that GPx is more significant

in detoxifying the peroxide at low concentrations[117, 118], whereas, at concentrations

exceeding the physiological limit, catalase becomes more important [119, 120]. Also, in

contrast to catalase, GPx can use substrates other than H_2O_2 such as hydroperoxides

which serve as substrates for the metal catalyzed Fenton reaction that generates highly

reactive hydroxyl radicals.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \qquad \text{(Reaction 2)}$$

$$2GSH + 2H_2O_2 \xrightarrow{\text{GPx}} GSSG + 2H_2O \qquad \text{(Reaction 3)}$$
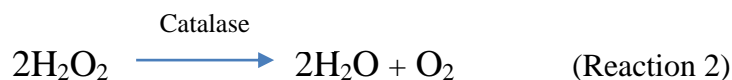
Glutathione reductase (GR) is an enzyme that catalyzes the reduction of GSSG to

GSH in the presence of NADPH as an electron source [121-123] (Reaction 4).

$$GSSG + NADPH \xrightarrow{\text{GR}} 2GSH + NADP^+ \qquad \text{(Reaction 4)}$$

Thioltransferase (Ttase), also called glutaredoxin, catalyzes the reversible thiol-

disulfide exchange reactions in the presence of GSH. The whole reaction system plays a

key role in maintaining the thiol/disulfide ratio in the cell [124-126] (Reaction 5-7). The

standard spectrophotometric analysis of Ttase depends on coupling the formation of

GSSG and its reduction by GR in the presence of NADPH.

$$PS\text{-}SP + GSH \underset{}{\overset{\text{Ttase}}{\rightleftharpoons}} P\text{-}SH + P\text{-}SSG \quad \text{(Reaction 5)}$$

$$P\text{-}SSG + GSH \underset{}{\overset{\text{Ttase}}{\rightleftharpoons}} P\text{-}SH + GSSG \quad \text{(Reaction 6)}$$

$$GSSG + NADPH \underset{}{\overset{\text{GR}}{\rightleftharpoons}} 2GSH + NADP^+ \quad \text{(Reaction 7)}$$
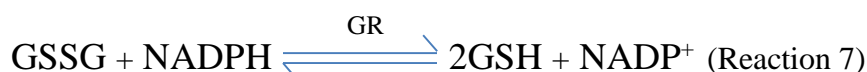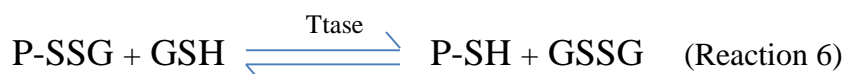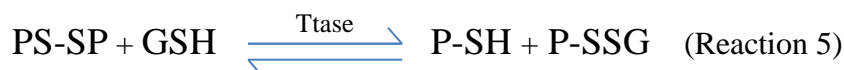
Thioredoxin (Trx) system is the major ubiquitous disulfide reductase system with two redox-active cysteine residues, -Cys-Gly-Pro-Cys-, in its active site [127]. It is responsible for maintaining proteins in their reduced state with the help of NADPH and Trx reductase [128]. The cysteine residues are used by Trxs to catalyze the breakage of protein thiol disulfide bonds. Upon completion of one cycle, these two cysteine residues are oxidized resulting in a disulfide bond. Thioredoxin reductase (Trx reductase) converts these oxidized cysteine residues to their native reduced form with the help of NADPH [129] (Reaction 8).

$$PS\text{-}SP + Trx\text{-}(SH)_2 \longrightarrow 2P\text{-}SH + Trx\text{-}S_2 \quad \text{(Reaction 8)}$$

Trx reductase

$$NADP^+ \qquad NADPH$$
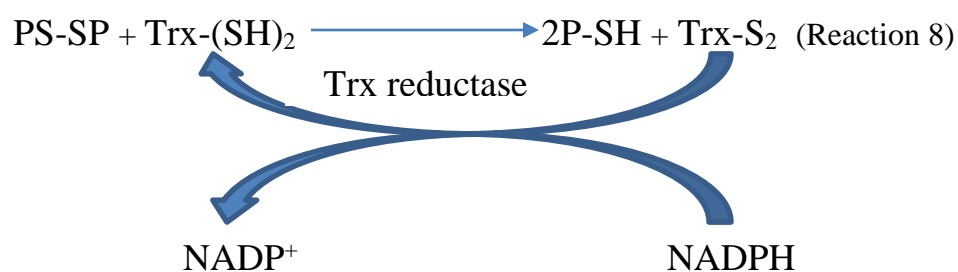
2.3.2. Small Antioxidants. Antioxidant proteins, along with antioxidant enzymes, form the first line of defense against oxidants that are generated during oxidative stress. They play an important role in maintaining cellular functions and have been implicated in processes associated with aging, cancer, diabetes, and several brain disorders. Among these small antioxidants that are mentioned in table 2.3, GSH is the most important one.

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is the major low molecular mass protein thiol that is found in cells of plants and animals [130]. It is a tripeptide containing the amino acids glutamate, cysteine, and glycine (Figure 2.5). The cysteine associated with the GSH is non-toxic [131], and the thiol group in the cysteine gives

antioxidant properties to the GSH which promote for its cellular defenses against oxidative stress [132].The unusual γ-linkage between glutamate and cysteine is thought to prevent the GSH from being degraded by aminopeptidases, resulting to its high stability within a cell [132].
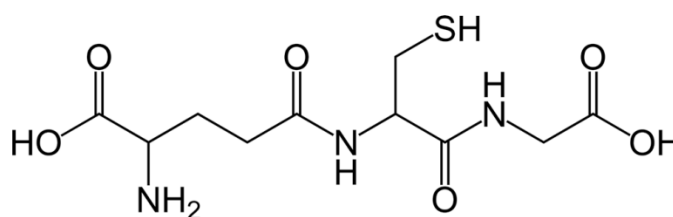
Figure 2.5. Structure of Glutathione.

GSH is synthesized in the cytosol in two steps that require ATP. The first step involves the formation of γ-glutamylcysteine in the presence of enzyme γ-glutamylcysteine synthetase (γ-GCS). This step in the synthesis is the rate limiting step. The next step is the formation of GSH by glutathione synthetase (GS), which utilizes γ-glutamylcysteine and glycine as substrates (Reaction 9) (Figure 2.6).

ATP   ADP   ATP   ADP

Glu + Cys ⟶ γ-glu-cys ⟶ γ-glu-cys-gly (GSH)

γ-GCS   GS

Gly

(Reaction 9)
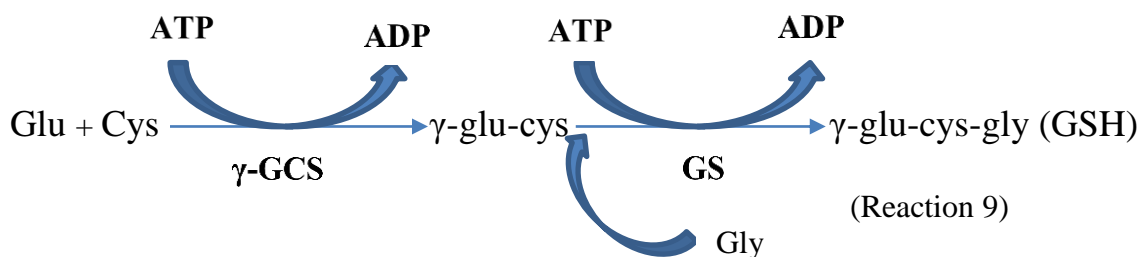
The lens contains a very high concentration of GSH which serves to protect the protein thiols from oxidation to disulfides and, hence, is essential for maintaining the transparency of the tissue [133]. The turnover rate of GSH in the lens was found to be very high, with the compound completely broken down and resynthesized in the tissue about every 48 h [134]. However, the concentration of GSH decreases steadily with age due to the accumulation of oxidants that result from the decreased levels of antioxidant enzymes required for GSH turnover[135]. The γ-GCS (rate limiting enzyme in GSH synthesis) activity in old people falls to one sixteenth of the value found in young ones [136]. With the decreased GSH synthesis, the protein disulfide accumulates causing protein aggregation, insolubilization, and precipitation[137]. This may be an important factor in age-related cataractogenesis [138].

Ascorbic acid (Vitamin C) is a water soluble vitamin that scavenges oxygen free radicals, thereby providing antioxidant capacity. It helps to regenerate Vitamin E (α-tocopherol) that gets oxidized in the presence of lipid peroxidation (Figure 2.7). With age, the plasma levels of Vitamin C have been shown to decrease [139, 140].

The water soluble vitamin, α-Tocopherol (Vitamin E), which is present in the interior of a cell membrane, is the primary antioxidant against oxidant-induced membrane damage. It donates electrons to peroxyl radicals that are generated during lipid peroxidation. α-Tocopherol was shown to prevent pro-inflammatory cytokines, such as IL-1 and IL-6, resulting in protection against myocardial ischemic reperfusion [141]. It was also reported that Vitamin E triggers apoptosis of cancer cells and inhibits free radical formation [142].
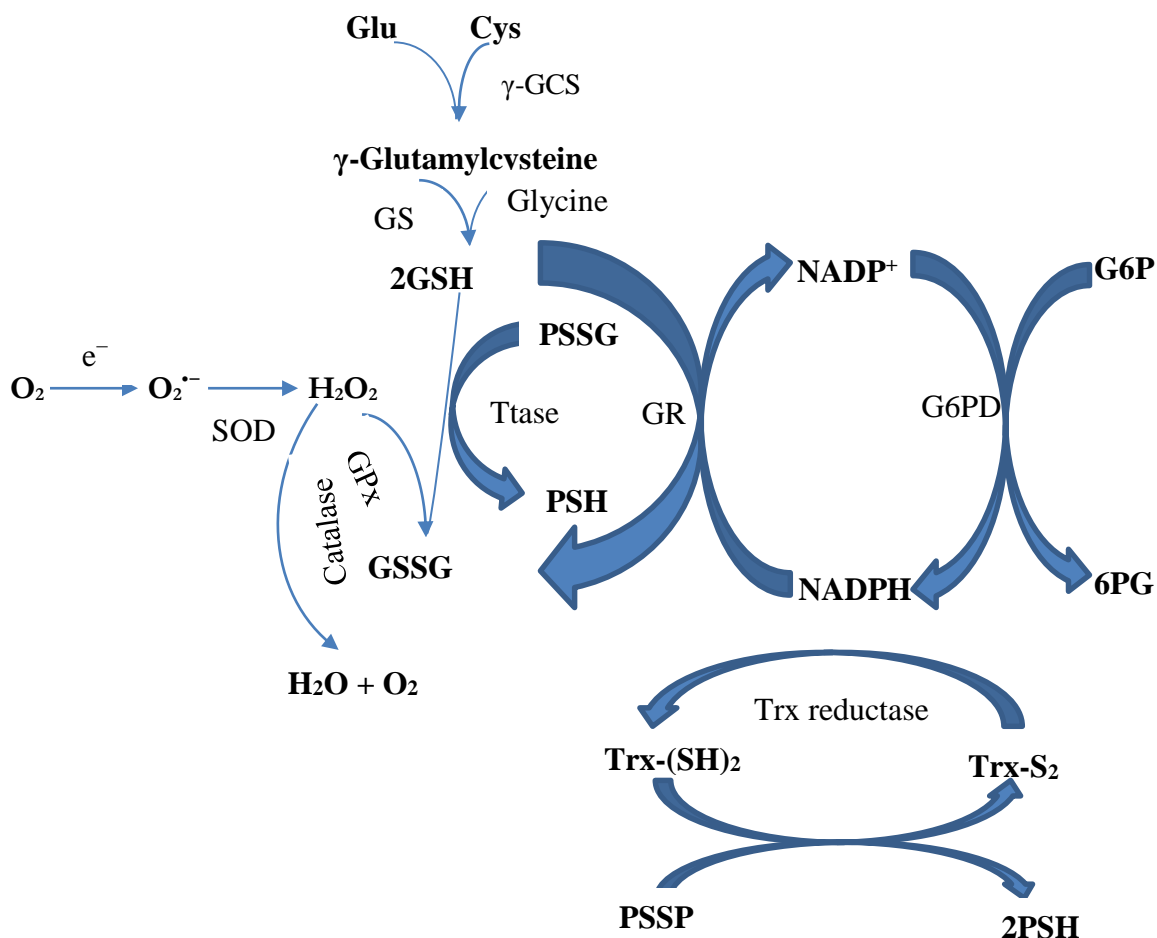
Figure 2.6. Schematic representation of Antioxidant Defense Network. Scheme depicting the basic reactions that take place after the generation of superoxide ($O_2^{\bullet-}$) radicals, and activation of antioxidants to counterbalance the ROS. γ-Glutamylcysteine synthetase (γ-GCS); GSH synthetase (GS); GSH peroxidase (GPx); Thioltransferase (Ttase); GSSG reductase (GR); Glucose-6-phosphate dehydrogenase (G6PD); Glucose-6-phosphate (G6P); 6-phosphogluconate (6PG); Protein thiol disulfides (PSSP); Protein mixed disulfides (PSSG); Protein thiol (PSH).

2.4. CALPAIN ACTIVATION

Calpains (Figure 2.8) are cysteine proteases that are found in cytosol whose enzyme activity depends on calcium (Ca^{2+}). They perform various functions in biological processes, such as signal transduction, cell proliferation, cell cycle progression, differentiation, apoptosis, membrane fusion, and platelet activation [143-147].

Over activation of calpain was found to be implicated in muscular dystrophy, cardiac and cerebral ischemia, platelet aggregation, neurodegenerative diseases, rheumatoid arthritis, and cataract formation [147].
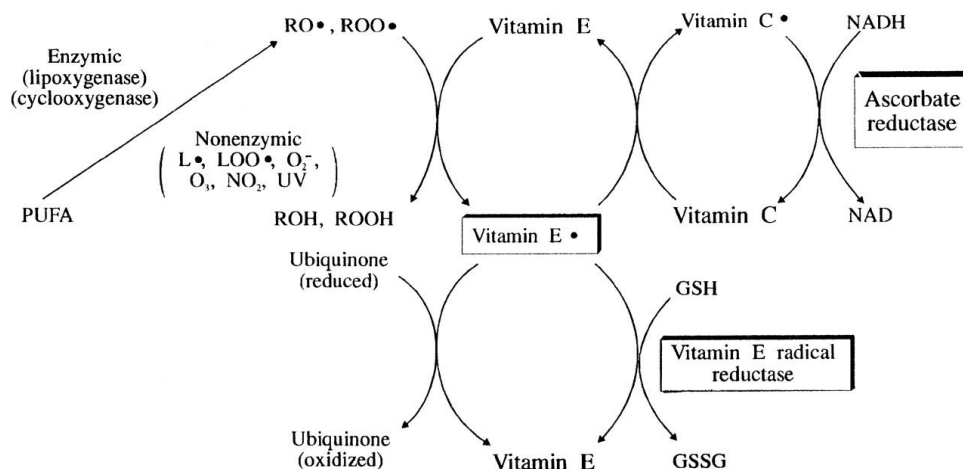
Figure 2.7. Antioxidant Regeneration System.
(Adapted from Alvin C. Chan et al. [148].)

The calpain superfamily contains at least 12 different calpain members of which μ-calpain and m-calpain are the best characterized ones. However, both of these proteins differ in the sensitivities they have toward calcium. For example, μ-calpain is highly sensitive to calcium, even at lower concentrations (5-50 μM), whereas m-calpain has low sensitivity to Ca^{2+}, requiring high concentrations (200-100 μM). Both μ-calpain and m-calpain are heterodimers with a large78-80 kDa catalytic subunit and a small 28kDa regulatory subunit. The large subunit involves four domains (dI-dIV), whereas the small subunit is comprised of two domains (dV and dVI) (Figure 2.8).
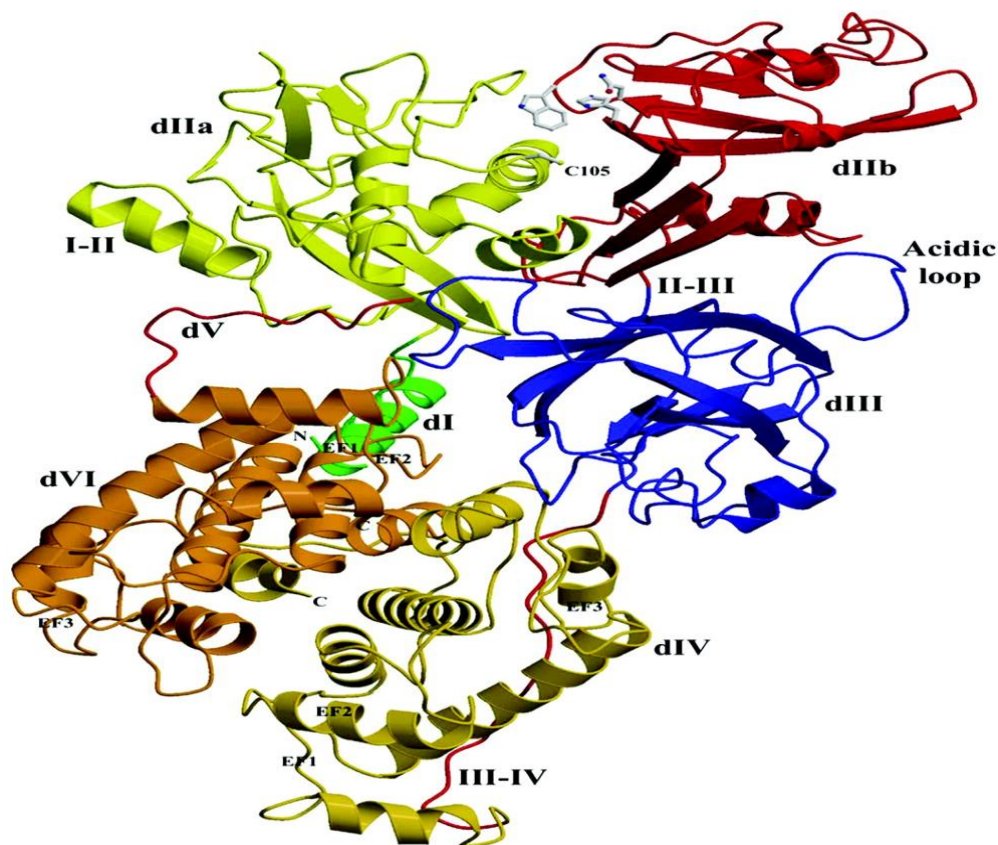
Figure 2.8. Human m-Calpain Crystallographic Structure. The domains are colored differently and are labelled as dI, dII, dIII, dIV, dV, and dVI. The active site Cys, C105 is shown in gray as are His-262, Asn-286, and Trp-288 on domain IIb. (Adapted from Darrel E. Goll et al. [149].)

Domain I consists of α-helix anchored in a cavity of dVI, which stabilizes the circular arrangement of calpain. Domain II consists a catalytic site, which can be divided into two, dIIa and dIIb. Domain III contains an acidic loop that plays a role in Ca^{2+}-promoted activation of calpain [150, 151]. Domains IV and VI are calcium-binding domains with each containing five EF- hand motifs similar to calmodulin protein. Hence, domain dVI is often termed as the calmodulin-like domain [152]. Domain V is unresolved in crystal structure and it contains a cluster of glycine residues in N-terminal.

Calpain exists as an inactive proenzyme in the cytosol which, in response to increased Ca^{2+} levels, gets translocated to the membrane, where it undergoes activation in the presence of Ca^{2+}. In the absence of Ca^{2+}, the sub-domains of domain II (dIIa and dIIb) remain separated due to structural constraints. The activation of calpain is a prerequisite to release the constraints and formation of a functional catalytic site [150, 151, 153, 154]. Activation of m-calpain involves two stages, as shown in Figure 2.9 [153-156]. The first stage is the release of structural constraints imposed by domain interactions. Calcium binding to domains IV, VI, and III releases domain I from domain VI, and domain II from domain III, leading to the dissociation of 28kDa subunit from 78kDa subunit. The second stage involves conformational changes in domain II, which is a consequence of Ca^{2+} binding to the acidic loop in dIII, resulting in the two sub-domains (dIIa and dIIb) come closer to form a catalytic site of the enzyme [150, 157]. However, the rearrangement of the sub-domains of domain dII can occur only after the release of constraints from the first stage [153].

In summary, with increased intracellular calcium levels, the salt bridge interactions unfold (i.e., activation, also called autolysis) releasing the constraints of domain interaction that lead to the dissociation of the smaller subunit from the larger one. This is followed by the rearrangement of domain II, resulting in the formation of a catalytic site and, ultimately, activation of the enzyme that hydrolyzes the structural proteins of the lens. The autolysis (initial activation) of calpain requires high concentrations of intracellular Ca^{2+}, which physiologically is not possible as calcium never reaches the range required for the activation. Thus, there may be other biological factors that sensitize calpain to Ca^{2+} and potentiate autolysis [158]. These factors include

phospholipids [159, 160], such as phosphatidylinositol (that increases calpain sensitivity

to Ca^{2+} by ten-fold), and activator proteins [161, 162] (that increase the sensitivity by

hundred-fold). With involvement of these factors, autolysis can occur under physiological

conditions. This autolyzed calpain is highly sensitive to even very low concentrations of

Ca^{2+} (0.2-0.8 μM) and, hence, autolysis is a very important step before dissociation.
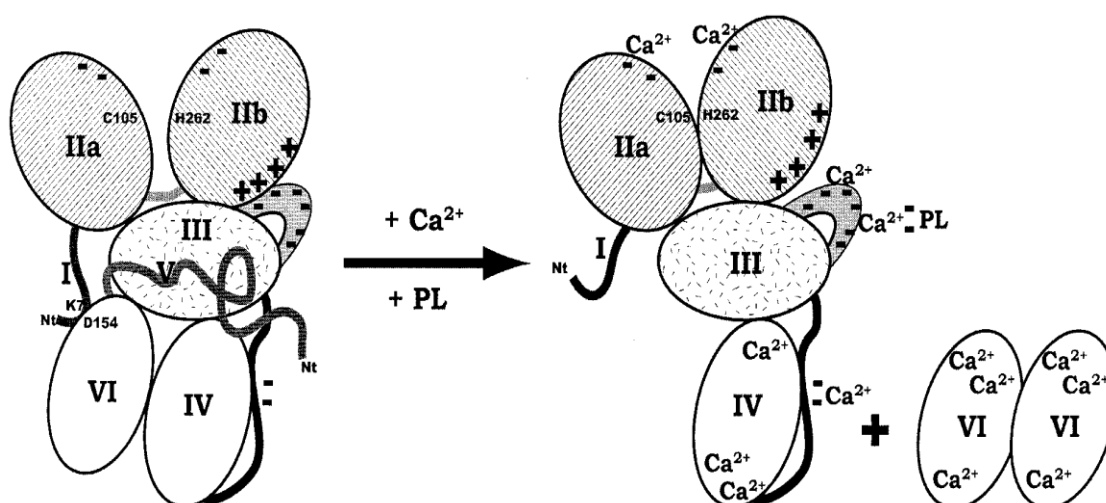
Figure 2.9. Mechanistic representation of Activation of m-Calpain by Ca^{2+}. Ca^{2+} and phospholipids (PL) bind to the acidic loop, resulting in unfolding of the sub-domains of dII that brings the two sub-domains closer, leading to the catalytic site formation and dissociation of the 28kDa from 78kDa. K7 and D154 form a salt bridge in the absence of Ca^{2+}. Cys C105 and His H262 are amino acid residues at the catalytic site; N_t, NH_2-terminal; - and + are the acidic and basic amino acid residues necessary for calcium binding. (Taken from Suzuki et al. [156].)

2.5. (R) - N-ACETYLCYSTEINE AMIDE (NACA).

One approach to treating oxidative stress related problems is by using

antioxidants that neutralize the ROS and restore the redox balance of the system.

However, because of the inefficiency of many compounds in crossing the blood retinal barrier, very few compounds have been tried in the treatment of eye disorders. NACA is a thiol antioxidant that is a derivative of NAC, an FDA approved drug. It has a molecular weight similar to its parent compound, NAC. However, due to the negative charge on NAC, the penetration of this compound through cellular membranes is limited. NACA's characteristics as a drug were improved over NAC by neutralizing the carboxylic acid of NAC, which made the NACA molecule more lipophilic and, therefore, enhanced its ability to penetrate cellular membranes (Figure 2.10). The enhanced ability to penetrate cells allowed NACA to be administered at a lower dose than NAC, giving the drug a greater therapeutic index and lowering the risk of side effects that are traditionally associated with higher doses of NAC [163]. NACA is an excellent source of sulfhydryl groups that can be converted by the cells into metabolites capable of stimulating GSH synthesis [25]. The molecule can also promote intracellular detoxification and act directly as a free radical scavenger. NACA acts as a carrier of NAC and its antioxidant and free radical scavenging abilities are equal to or better than those of NAC [164].
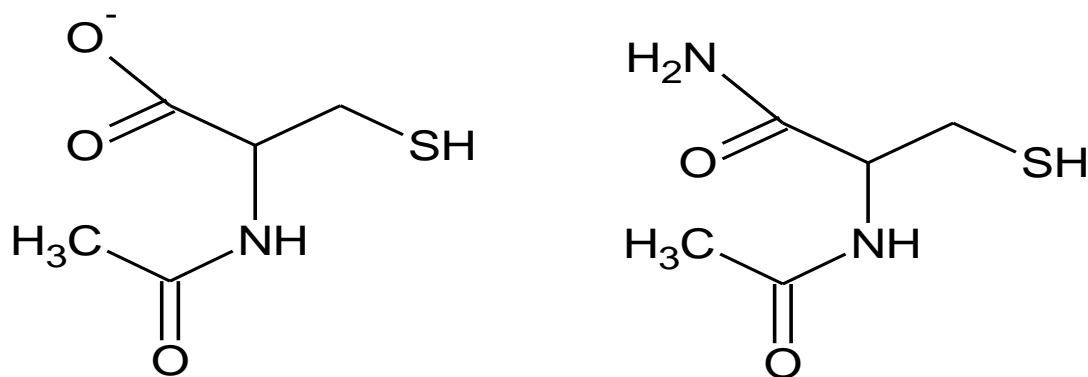
Figure 2.10. Structures of NAC (left) and NACA (right).

With oxidative stress being one of the major contributors of cataracts, many antioxidants were tried previously to prevent/treat cataracts. With this background, NACA as an eye drop formulation has been tried in this study. To evaluate NACA's ability to prevent/treat cataracts, 3 animal models were used with the first model being dexamethasone (Dex) animal study. This is an *ex vivo* study where Dex was used to induce cataracts in the lenses enucleated from Sprague-Dawley rats. The protective role of NACA was evaluated by measuring the oxidative stress parameters. The second study where C57BL/6 mice were used was the acetaminophen (APAP) cataract model. APAP was injected to induce cataracts in mice with NACA being injected 2 h prior to APAP injection. One percent NACA eye drops were administered every day after APAP injection. The study was carried for 2 weeks to evaluate both preventive as well as reversal effects of NACA on APAP-induced cataracts. The third study was performed using sodium selenite as a cataract inducer. In this study, selenite was injected into male Wistar rat pups on postpartum day (PD) 10 to induce cataracts. One percent NACA eye drops were administered starting from PD 15 until the end of week 9 to study the preventive effects of NACA on selenite-induced cataracts. The final study was also performed with sodium selenite as an inducer of cataract. However in this study, NACA was injected (PD 9, 11, 13) prior to selenite injection (PD 10) and 1% percent NACA eye drops were administered from PD 15 until PD 30. The results were indicative of both prevention and reversal role of NACA on selenite cataracts. In summary, NACA could confer a protective effect by providing a substrate for the generation of GSH, thereby, maintains antioxidant levels within the lens. These results indicate that NACA is a potential candidate for development into readily available eye drops to treat cataracts.

3. METHODS

3.1. HPLC ANALYSIS

The levels of GSH, GSSG, GSH/ GSSG ratio, and MDA were measured using HPLC.

3.1.1. Determination of GSH. The levels of GSH in each of the lens samples were determined using HPLC, according to a method developed within our laboratory [165]. Each of the lens samples was first homogenized in a serine borate buffer (pH 7.8) and then centrifuged at 5,000 X g for 10 min at 4 °C. Fifty microliters of the diluted supernatant were added to 200 μl of HPLC grade water and 750 μl of N-(1-pyrenyl) maleimide (NPM) (1 mM in acetonitrile). The resulting solution was incubated at room temperature for 5 min and then the reaction was quenched by the addition of 10 μl of 2 N HCl. The samples were then filtered through a 0.45-μm filter and injected onto the HPLC system. The HPLC system used was the Finnigan Surveyor (Thermo Scientific), which was equipped with an Auto Sampler Plus, LC Pump Plus, and FL Plus Detector. The HPLC column used was a Reliasil ODS-1 C_{18} column (5-μm packing material) with 250 × 4.6 mm i.d. (Orochem Technologies Inc., Naperville, IL, USA). The mobile phase was acetonitrile and water (70:30, v/v) was adjusted to a pH of 2.5 by the addition of 1 ml/L of acetic acid and 1 ml/L of phosphoric acid. The NPM derivative of GSH (Figure 3.1) was eluted from the column isocratically at a flow rate of 1 ml/min. For detection of this derivative, the excitation and emission wavelengths were 330 and 376 nm, respectively.

3.1.2. Measurement of Total GSH and GSSG. Total GSH content was determined by reverse phase HPLC. Each of the lens samples was first homogenized in a

serine borate buffer (pH 7.8) and then centrifuged at 5,000 X g for 10 min at 4 °C. Fifty

microliters of the diluted supernatant were added to 60 µl of NADPH (2 mg/ml) in

nanopure water and 20 µl of 1 unit/ml GR was added, to reduce the GSSG. After 5 min

of incubation at room temperature, the treated samples were diluted with 120 µl H_2O, and

then immediately derivatized with 750 µl of 1.0 mM NPM. The resulting solution was

incubated at room temperature for 5 min and then the reaction was quenched by the

addition of 10 µl of 2 N HCl. These samples were analyzed as detailed for the

determination of GSH using reverse phase HPLC. Data from the original GSH levels and

the total GSH levels in each sample were subsequently used to calculate the levels of

GSSG present in each sample.

NPM   **Thiol**   **NPM-thiol adduct**
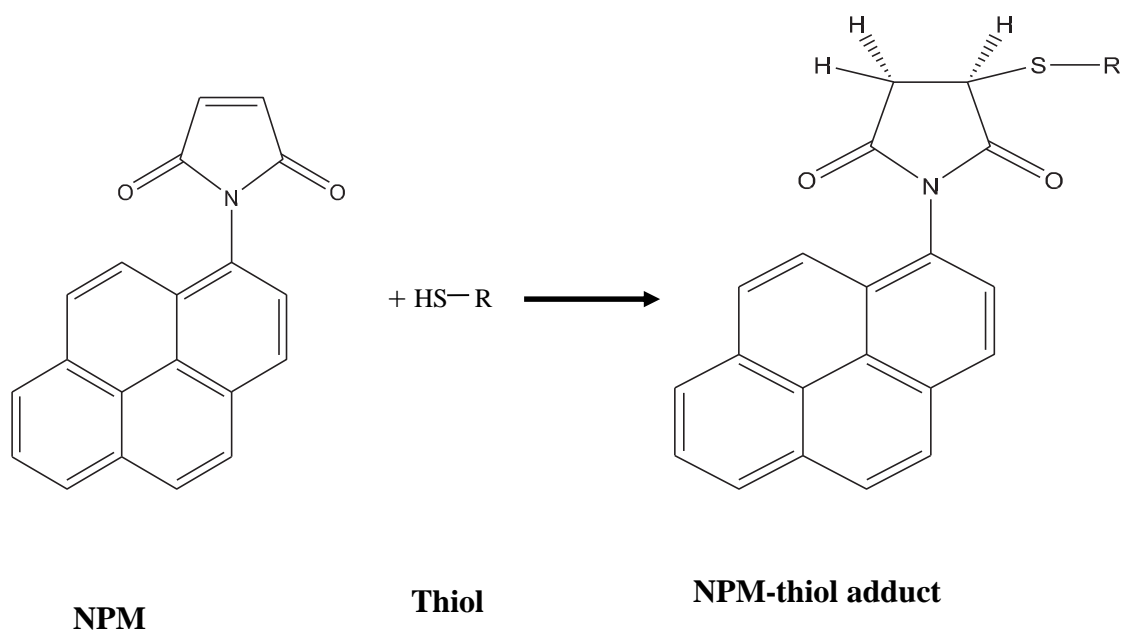
Figure 3.1. NPM derivatization of Thiol Compounds. NPM reacts with free thiols in
solution to form a fluorescent NPM-thiol adduct which has an excitation of 330 nm and
emission of 376 nm.

3.1.3. Determination of MDA. MDA is one of the several low-molecular weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products [166]. It is the most frequently used biomarker of lipid peroxidation. The amount of MDA, a by-product of lipid peroxidation, was determined for each sample according to the method described by Draper [167]. For sample preparation, 350 μl of the lens homogenate were added to 550 μl of 10% trichloro acetic acid (TCA) and 100 μl of 500 ppm butylated hydroxytoluene in methanol. The resulting solution was then boiled for 30 min in a water bath. After cooling in an ice-water bath, the solutions were centrifuged and the supernatant was collected. This supernatant was then added 1:1 with a saturated solution of thiobarbituric acid (TBA). Again, the contents were heated in a boiling water bath for 30 min and then immediately cooled in an ice water bath. The TBA-MDA adduct (Figure 3.2) was then transferred into n-butanol by adding 500 μl of the sample mixture into 1 ml of n-butanol and vortexing each sample for one min. Each sample was then centrifuged at to facilitate the separation of the two phases. The resulting organic layers were first filtered through 0.45-μm filters and then injected onto the HPLC system (Finnigan Surveyor, Thermo Scientific), which was equipped with an Auto Sampler Plus, LC Pump Plus, and FL Plus Detector. The HPLC column used was a Reliasil ODS-1 C_{18} column (5-μm packing material) with 250×4.6 mm i.d. (Orochem Technologies Inc., Naperville, IL, USA). The mobile phase used contained 69.4% sodium phosphate buffer, 30% acetonitrile, and 0.6% tetrahydrofuran. The fluorescent derivative was monitored at an excitation wavelength of 515 nm and an emission wavelength of 550 nm.
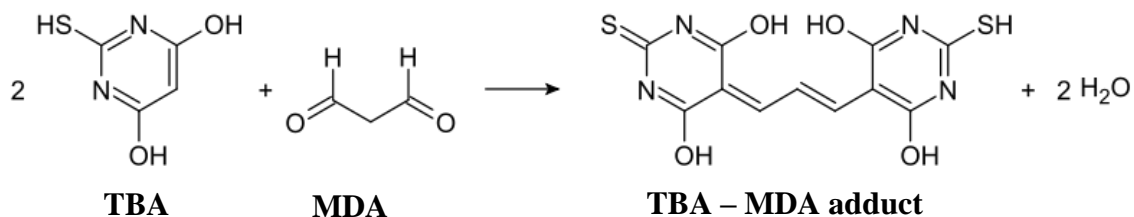
Figure 3.2. Formation of TBA-MDA adduct. In the presence of TBA, MDA forms a fluorescent compound that has an excitation at 515 nm and emission at 550 nm.

3.2. SPECTROPHOTOMETRIC STUDIES

The enzyme activities of GR and Ttase along with the protein levels were measured using UV/Vis spectrophotometer, whereas calcium levels were measured using Atomic absorption spectrophotometer.

3.2.1. GR Activity. GR is the enzyme responsible for recycling GSSG into GSH via a reduction mechanism, utilizing both GSSG and NADPH as a substrate. The activity of this enzyme was determined using a commercial kit from OxisResearch (Portland, OR, USA). The oxidation of NADPH to NADP$^+$ was accompanied by a decrease in absorbance at 340 nm, providing a spectrophotometric means for monitoring the enzyme activity of GR. The activity of GR was determined by adding homogenate to a solution containing both GSSG and NADPH and then recording the absorbance as a function of time at 340 nm. The rate of decrease in the A$_{340}$ was directly proportional to the GR activity in the sample.

3.2.2. Thioltransferase (Ttase) Activity. The Ttase activity was determined by the method of Wang [168]. Briefly, the reaction mixture contained 0.1 M phosphate buffer (pH 7.5), 0.2 mM NADPH, 0.5 mM GSH, and 0.4 units of GR. The reaction mixture (0.8 ml) and an aliquot of a homogenized sample (lens, 0.1 ml) were combined

and then centrifuged to remove particulates. Hydroxyethyl disulfide (20 mM, 0.1 ml) was added to initiate the reaction that was monitored at 340 nm for 2 min. Reaction mixtures without the substrate were used as blanks.

3.2.3. Determination of Calcium Concentration. The lenses from all groups were analyzed to determine the Ca^{2+} concentration, as described by Elanchezhian [169]. Briefly, lenses were placed in acid washed tared glass vials, heated at 100 $^{\circ}$C for 20 h, and dry weights were recorded. Concentrated HCl (0.2 ml) was added to digest the lenses overnight and diluted to 1 ml with deionized water. The samples were then centrifuged at 10,000 X g for 10 min to remove insoluble materials. The supernatant fractions were collected to measure the calcium concentration using an atomic absorption spectrophotometer (model Spectra AA-3100, Perkin Elmer), operated with a slit width of 0.5 nm, and wavelength set at 422.7 nm. Standards were prepared from $CaCO_3$ and deionized water.

3.2.4. Total Protein Determination. Protein levels of the lens homogenates were measured by the Bradford method [170], except for casein zymography and the Western blot where the Bio-Rad DC protein assay was used. This was because the lysis buffer (mentioned in the "Preparation of water-soluble lens proteins" section) contained 1% detergent which would interfere with the results of the Bradford assay.

Procedure for the Bradford method is as follows. Concentrated Coomassie blue (Bio-Rad, Hercules, CA, USA) was diluted 1:5 (v/v) with distilled water. Twenty microliters of diluted lens homogenate were then added to 1 ml of this diluted dye and the solution was then vortexed and allowed to stand at room temperature for 15 min. The absorbance was then measured at 595 nm using a UV spectrophotometer (Shimadzu

Scientific Instruments, Columbia, MD, USA). Bovine serum albumin (BSA) was used as the protein standard.

Procedure for the Bio-Rad DC protein assay is as follows. 'Working reagent A' was prepared by adding 20 µL of reagent S to 1 ml of reagent A. One hundred microliters of diluted lens homogenate was then added to 500 µL of working reagent A and vortexed for proper mixing. To this, 4 ml of reagent B was added, and the solution was vortexed and allowed to stand for 15 min. The absorbance was measured at 750 nm. BSA was used as a protein standard.

3.3. GEL ELECTROPHORETIC STUDIES

SDS-PAGE and Western blots were performed using polyacrylamide gels, whereas, casein zymography was performed using casein zymogram gels.

3.3.1. Casein Zymography. Casein zymography was performed using the method of Raser [171]. Casein zymogram gels were purchased from Bio-Rad. Protein concentration was determined using the Bio-Rad DC protein assay with BSA as a standard. A zymogram sample buffer was added to the supernatant of an equivalent 50 µg of protein, so that the final volume was 30 µL. The gels were prerun at 100 V for 15 min, 4 °C, with a running buffer containing 25 mM Tris-HCl, 0.05% MCE, 192 mM glycine, and 1 mM EDTA (pH 8.3), before samples were loaded into the wells. Twenty five microliters of each sample were loaded and electrophoresed at 100 V (constant) for 2 h at 4 °C in a running buffer. The gels were then incubated in a calcium buffer (20 mM Tris–HCl, 10 mM DTT, 2 mM calcium, pH 7.4) overnight at room temperature with slow shaking. The gels were then stained by coomassie brilliant blue and destained;

achromatic bands of caseinolysis appeared white against a stained background.

3.3.2. Preparation of Water-Soluble Lens Proteins. Rats were euthanized and their eyes were enucleated. Four decapsulated lenses from each group were homogenized in 600 µL of lysis buffer [(Cell Signaling Technology, Inc., Danvers, MA, USA) 20 mM Tris-HCl (pH 7.5); 150 mM NaCl; 1 mM Na_2EDTA; 1 mM EGTA; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM beta glycerophosphate; 1 mM Na_3VO_4; 1 µg/ml leupeptin; 2 mM PMSF] using a glass Dounce homogenizer. The homogenates were centrifuged at 10,000 X g for 15 min at 4 $^{\circ}$C and the recovered supernatants were designated as the water-soluble fraction.

3.3.3. SDS-PAGE of Lens Soluble Proteins. A SDS-PAGE of the soluble lens proteins was performed on a 10% Mini-Protean TGX gel (Cat no: 456-1034, Bio-Rad, Hercules, CA, USA). A 4x Laemmli sample buffer was added to a supernatant, the equivalent of 50 µg of protein, so that the final volume was 30 µL. 25 µl of the sample were loaded and electrophoresed at 100 V (constant) for 2 h at 4 $^{\circ}$C in a running buffer, and then stained with coomassie brilliant blue for 30 min followed by de-staining for 1 h.

3.3.4. Western Blot of m-Calpain. Lens homogenates were prepared in a lysis buffer, and the protein concentration was estimated using a Bio-Rad DC protein assay kit. Briefly, 75 µg of soluble lens proteins were resolved by electrophoresis on 10% Mini-Protean TGX gels (100 V, 2 h) in a running buffer containing 25 mM Tris-HCl, 192 mM glycine, 0.05% 2-mercaptoethanol (MCE), and 0.1% SDS (pH 8.3). The samples were transferred to PVDF membranes by an iBlot® Gel Transfer Device (Life Technologies, Grand Island, NY, USA), followed by the addition of a blocking reagent (blok-CH Chemiluminescent Blocker, Millipore, Billerica, MA, USA). Membranes were

immunoblotted by using the SNAP i.d. 2.0 Protein Detection System (Millipore) with primary antibodies for m-calpain and GAPDH in 1:750 and 1: 1000 dilutions, respectively. Subsequently, the membrane was incubated in the respective secondary antibody (1:1000) for 10 min at room temperature. Final visualization was carried out with the enhanced chemiluminescence kit (Cell Signaling Technology, Inc., Danvers, MA, USA). The protein bands were quantitated by densitometry, where the band intensity ratio of the treated group over the untreated group, or control, was calculated.

3.4. CATARACT EVALUATION

The scale used to grade the intensity of opacification of each lens was defined as follows: clear lens, grade 0; lens with slight opacity, grade 1; lens with partial nuclear opacity, grade 2; lens with dense nuclear opacity, grade 3. All rat pups/mice received a drop of 2.5% phenylephrine hydrochloride and a drop of 1% tropicamide ophthalmic solution in each eye to initiate mydriasis, and were placed in a dark room for 1 h before the examination. The animals were put to sleep by placing them in an isoflurane/oxygen chamber that is well regulated for 2-5 mins (leaving them for more time will ultimately lead to death of the animal). The lenses of each rat/mouse were then observed using a slit-lamp microscope at $10\times$ magnification. The degree of opacity in each lens was determined and verified by a certified ophthalmologist (Dr. Humeyra Karacal, Ophthalmologist, Washington University School of Medicine in Saint Louis) and then documented using a digital camera.

3.5. WATER CONTENT

For this experiment, first the weight of each clean vial (V_1) was recorded, lenses were placed into these vials, and the weight of the lens along with the vial (V_2) was recorded. The difference between these two gave the weight of the wet lens (V_3). The dry weight of the lens (V_4) was measured after heating at 100 ^{o}C in an oven for 24 h. The difference between the wet weight of the lens (V_3) and the dry weight of the lens (V_4) gave the water content of the lenses among different groups.

3.6. STATISTICAL ANALYSIS

All reported values were represented as the mean ± S.E of quadruplets. Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Statistical significance was ascertained by one-way analysis of variance, followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were considered significant.

4. N-ACETYLCYSTEINE AMIDE PROTECTS AGAINST DEXAMETHASONE-INDUCED CATARACT RELATED CHANGES IN CULTURED RAT LENSES

Glucocorticoids (GCs) are steroid hormones that play a role in physiological processes and are widely used as immunosuppressive and anti-inflammatory agents in the treatment of many clinical conditions, including rheumatoid arthritis, asthma, autoimmune diseases, and various ocular diseases [172, 173]. However, their clinical use is restricted due to a wide range of complications associated with their long-term topical and systemic use. One of the ocular complications of glucocorticoid toxicity is the development of posterior subcapsular cataracts (PSCs) [174-179]. Unfortunately, certain patients cannot avoid long-term steroid therapy and, therefore, development of adjunctive therapeutics for the prevention of steroid-induced cataracts is highly desirable.

Oxidative stress and depletion of GSH are implicated in the etiopathogenesis of glucocorticoid-induced cataracts [10, 11, 180-183]. Epidemiological studies have documented loss of GSH in various types of cataracts [184-186]. A decrease in GSH levels after glucocorticoid exposure [187, 188], along with an increase in the levels of lipid peroxide in the lens [188], have been reported. Furthermore, protection by antioxidants, like Vitamin E and ascorbic acid [178, 189], against damage caused by a soluble corticosteroid suggests the role of oxidative stress in steroid-induced cataract formation.

Promising results with NACA [22-24, 26, 28, 190, 191] in various oxidative stress-related disorders encouraged us to investigate the protective role of NACA in the prevention of dexamethasone (DEX), a synthetic glucocorticoid, induced cataracts. Our data showed that NACA inhibits Dex-induced cataract formation by limiting lipid

peroxidation and increasing the ratio of GSH/GSSH in a lens. NACA can potentially be developed into a promising adjunctive therapeutic option for patients undergoing therapy with glucocorticoids.

4.1. EXPERIMENTAL DESIGN

Lactating female Sprague-Dawley rats, with 30 male 14-day-old pups, were purchased from the breeding facility at Charles River and were housed in a temperature- (~22 °C) and humidity- (~55%) controlled animal facility, with a 12 h/12 h light/dark cycle. The animals had unlimited access to rodent chow and water and were utilized after 3 days of acclimatization. All animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and guidelines of the Animal Care and Use Protocol Review Committee at the Missouri University of Science and Technology. For rat lens culture, eyes from 21-day-old rats were enucleated to expel the lens using plastic-coated forceps and fine scissors. Eyes were immediately transferred to Dulbecco's MEM (pH 7.2; Sigma, St. Louis, MO), containing 0.1% bovine serum albumin (BSA; GibcoBRL, Grand Island, NY) and antibiotic solution (GibcoBRL; 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) [192]. The culture media was changed daily. Approximately 24 h after the preparation of organ cultures, clear lenses were selected and were randomly divided into four groups with 8 lenses in each group: (1) control group (Dulbecco's Modified Eagle Medium (DMEM)), (2) Dex group (5 µM Dex dissolved in DMEM), (3) NACA-only group (50 µM NACA in DMEM), and (4) NACA pretreatment group (pretreatment with 50µM NACA for 6 h, followed by 5 µM Dex only for 18 h in DMEM). Lenses in each group were cultured in

the DMEM media (as detailed above) for 7 days at 37°C under 5% CO_2. Old media was replaced with fresh media every day and the lenses were evaluated daily, using a dissecting microscope and photographed to check for the development of opacity.

4.2. RESULTS

The results indicate the effects of NACA and Dex on various oxidative stress parameters. The change in these parameters in the NACA-only group were similar to those observed in the control group. Hence the levels of NACA were not reported in the bar graphs.

4.2.1. Prevention of Dex-Induced Cataracts by NACA. To examine the effects of Dex on the lens, an *ex vivo* rat lens model was used. Rat lenses were isolated and were treated in an organ culture to directly cause steroid-induced cataracts. The lenses were incubated with 5 µM Dex and morphologic changes in the whole lenses were recorded photographically using a dissection microscope. Opacity first appeared at day 3 in the lenses incubated with Dex. All the 8 lenses in the Dex group developed cataracts by day 7. In contrast, only 25% of the lenses developed cataracts in the NACA pretreatment group. Untreated control lenses remained transparent until day 7 (Figure 4.1).

4.2.2. Effects of NACA on Intracellular GSH Levels in Dex-Treated Cultured Rat Lens. To support the hypothesis that GSH depletion would induce cataract formation in the Dex-treated group, the levels of intracellular GSH were measured. Figure 4.2 shows the effect of Dex on lens GSH levels in the presence and absence of NACA. A 7-day exposure to 5µM of Dex decreased the GSH level to 21% of that of the control. A pretreatment with 50 µM of NACA increased the GSH level although not significantly.

4.2.3. Effect of NACA on Oxidized Glutathione (GSSG) Levels and GSH/GSSG Ratio in Dex-Treated Cultured Rat Lens. GSSG levels in the lens of the Dex-only group were found to have significantly increased by approximately three times the GSSG levels in the control group. The amounts of GSSG found in the lenses with NACA pretreatment were significantly lower as compared to the Dex-only group and were close to that of the control group. A graph showing these results is in Figure 4.3. An interesting result was obtained by observing the ratio between the GSH and the GSSG levels in the lenses of each group. As expected, the control group was found to have the highest ratio of GSH to GSSG. The ratio dropped to about 6% of control in the Dex-only group (Figure 4.4). However, NACA pretreatment increased this ratio significantly to approximately 50% of the control group value.

4.2.4. Effect of NACA on GR Activity in Dex-Treated Cultured Rat Lens. GR is a key antioxidant enzyme involved in maintenance of cellular GSH homeostasis. It reduces GSSG back to the reduced form, GSH. A significant reduction in the activity of GR was observed upon Dex treatment. However, NACA pretreatment increased the activity of GR (Figure 4.5).

4.2.5. Effect of NACA on LPO Byproduct: MDA. MDA was used as an index of LPO. Dex-treated lens had significantly higher levels of MDA, as compared to those of the control (Figure 4.6). Pretreatment with 50 µM of NACA completely reduced this increase, with MDA levels becoming nearly the same as those of the control, and with a p value of <0.05, as compared to that of the control. The NACA-only treated group showed no significant difference when compared to the control.

4.3. DISCUSSION

A posterior sub-capsular cataract is one of the ocular complications of glucocorticoid (GC) toxicity. Despite a well-established link between the use of GCs and cataracts, treatment with GCs cannot be avoided in some cases. GC-induced cataract formation is directly attributed to oxidative stress that occurs within the lens. Oxidation, which can be caused by an overabundance of oxidative stress generators, such as molecular oxygen, hydrogen peroxide, and free radicals, produces a major insult upon the lens, which can lead to the loss of GSH, lipid peroxidation, and a decrease in antioxidant enzyme activity [193-195]. GSH is an indispensable and primary lenticular antioxidant [183]. A wide body of evidence indicates loss of GSH because of its oxidation to GSSG, since its levels increase drastically once cataracts develop. Therefore, an alternative method for treating or preventing the occurrence of GC-induced cataracts would be through the use of a GSH prodrug. With this background, we evaluated the effects of a novel antioxidant and a potent GSH prodrug, N-acetylcysteine amide (NACA), in the prevention of cataracts induced by Dex in rat lenses. Results from morphological observations indicated that NACA was able to reduce the opacification of the lens within this *ex vivo* Dex-induced cataract model (Figure 4.1).

As discussed earlier, GSH is an essential lenticular antioxidant and is present in high concentrations in the lens, providing a first line of defense against oxidative damage [5], as well as playing an important role in antioxidant defense and redox regulation [9]. Results from this study indicate that treatment with Dex decreased lenticular GSH and the GSH/GSSG ratio significantly. In addition, it increased GSSG levels significantly. However, pretreatment with NACA significantly increased the ratio of GSH/GSSG by

decreasing the levels of GSSG. The ratio of reduced to oxidized glutathione (GSH/GSSG) serves as a representative marker of the antioxidative capacity of a cell. Depletion of GSH could be due to several possible mechanisms, including, but not limited to the efflux of GSH from the lens, sequestration of GSH by incorporation into mixed disulfide aggregates, enhanced consumption of GSH in the process of detoxification of ROS, and downregulation of enzymes involved in GSH biosynthesis. Another possible explanation for the decrease in GSH levels under oxidative stress is the reduced GR activity. Some studies have indicated that loss of GSH will directly affect the activity of the GSH-dependent enzyme GR. This enzyme plays an important role in GSH homeostasis. It has been reported that, under oxidative stress, the protein sulfhydryl (protein–SH) groups are lost [196], which are essential for enzyme activity [197]. Under such circumstances, GSH is not regenerated, so depletion of GSH indicates that the tissue is undergoing oxidative stress. The decreased activity of GR seen in this study with Dex treatment supports the hypothesis of GSH depletion upon treatment with Dex. A decrease in the lenticular GSH levels has been observed upon treatment with GCs [10, 187, 198]. *In vivo* treatment of rabbits with the Dex eye drops, as used for cataract surgery, reduced GSH levels in the lens [198]. In addition, free radical scavengers have also been reported to prevent GC-induced cataract formation [199-201] by increasing GSH levels and decreasing lipid peroxides (LPOs).

Decreasing the levels of GSH, when ROS are present can trigger a cascade of further oxidative damage. LPO has been associated with the formation of cataracts in patients [202-204]. The extent of LPO was determined in this study by measuring the amount of MDA (a by-product of LPO) within the lens. Unavailability of GSH as a

substrate for GPx stalled the process of LPOs decomposition and, thus, increased the levels of MDA in the Dex-treated group. NACA supplied an adequate amount of GSH as a substrate for GPx to effectively decompose LPOs in the rats, reducing MDA levels (Figure 4.6). The multiple roles of NACA in preventing cataract formation include direct scavenging of free radicals, providing cysteine for GSH synthesis, and nonenzymatic reduction of the preexisting toxic GSSG into GSH.

These results suggest that NACA can prevent the formation of Dex-induced cataracts by directly and indirectly maintaining the GSH/GSSG ratio in healthy lenses, allowing the lens to better cope with oxidative stress. NACA could confer a protective effect by providing a substrate for the generation of GSH, maintaining antioxidant levels within the lens and, possibly, through disulfide-exchange mechanisms. Treatment with NACA may prove to have a major therapeutic role in Dex-induced cataracts. In future studies, the focus will be on the prophylactic role of NACA in GC-induced cataract formation and investigate the development of a topical formulation for the application of this antioxidant in an *in vivo* model.
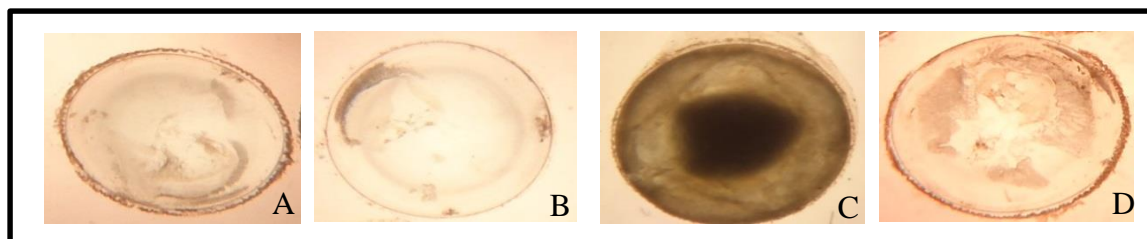
Figure 4.1. Appearance of Cultured Rat Lens with Dex and NACA at Day 7. Opacity was observed only in the Dex group at day 7 (Figure 1.C), which was significantly prevented by pretreatment with NACA. A: Control; B: NACA –only; C: 5 µM Dex; D: 5 µM Dex + 50 µM NACA.
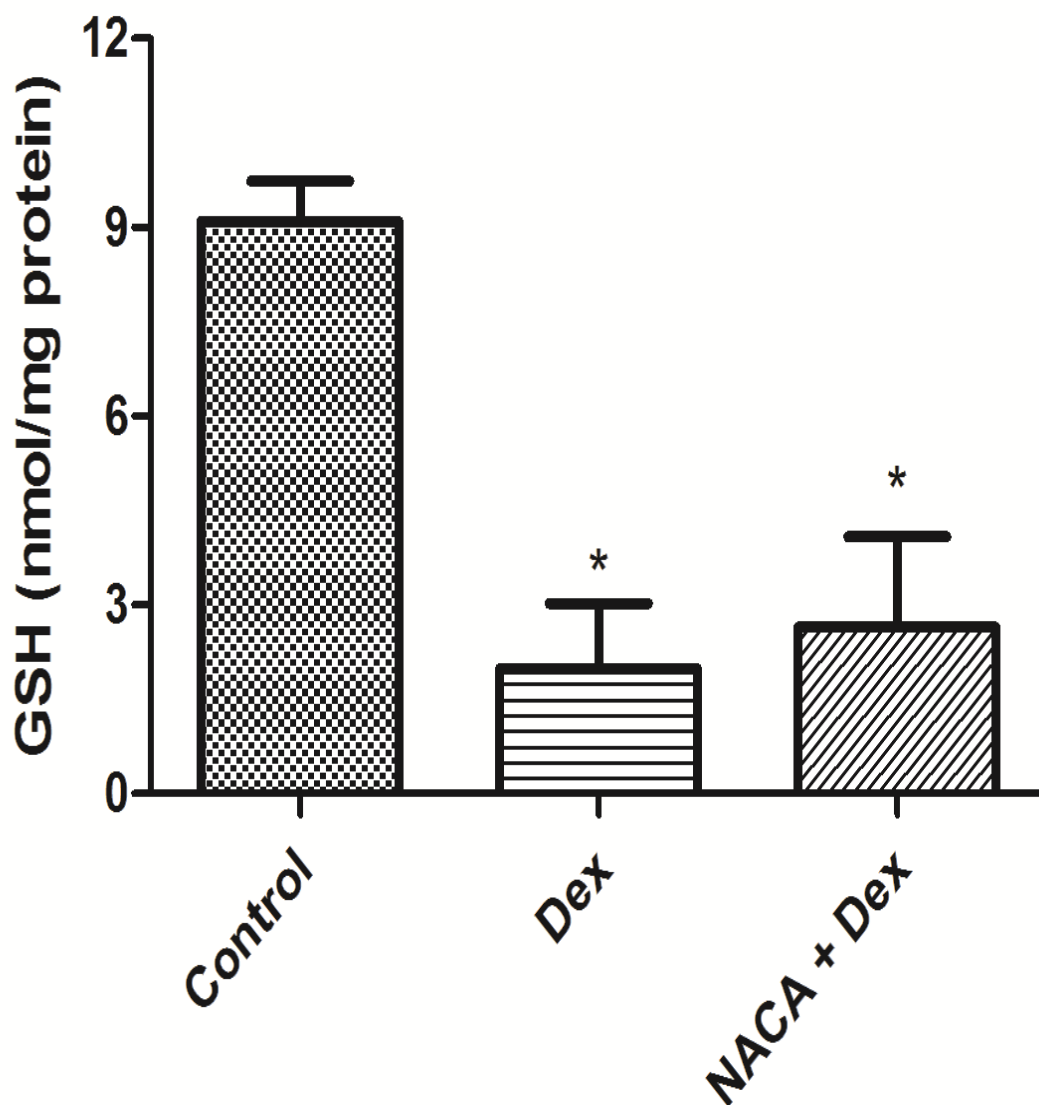
Figure 4.2. Intracellular GSH Levels in Lens after Treatment with Dex and NACA. GSH levels were measured after 7 days of treatment for control, NACA, Dex, and Dex + NACA groups. Exposure to Dex (5 µM) significantly decreased intracellular GSH levels. Pretreatment with NACA (50 µM) prevented such a dramatic decrease. The NACA-only treated group showed no significant difference when compared to the control. *$p \leq 0.05$ compared to the control group, and #$p \leq 0.05$ compared to the Dex group.
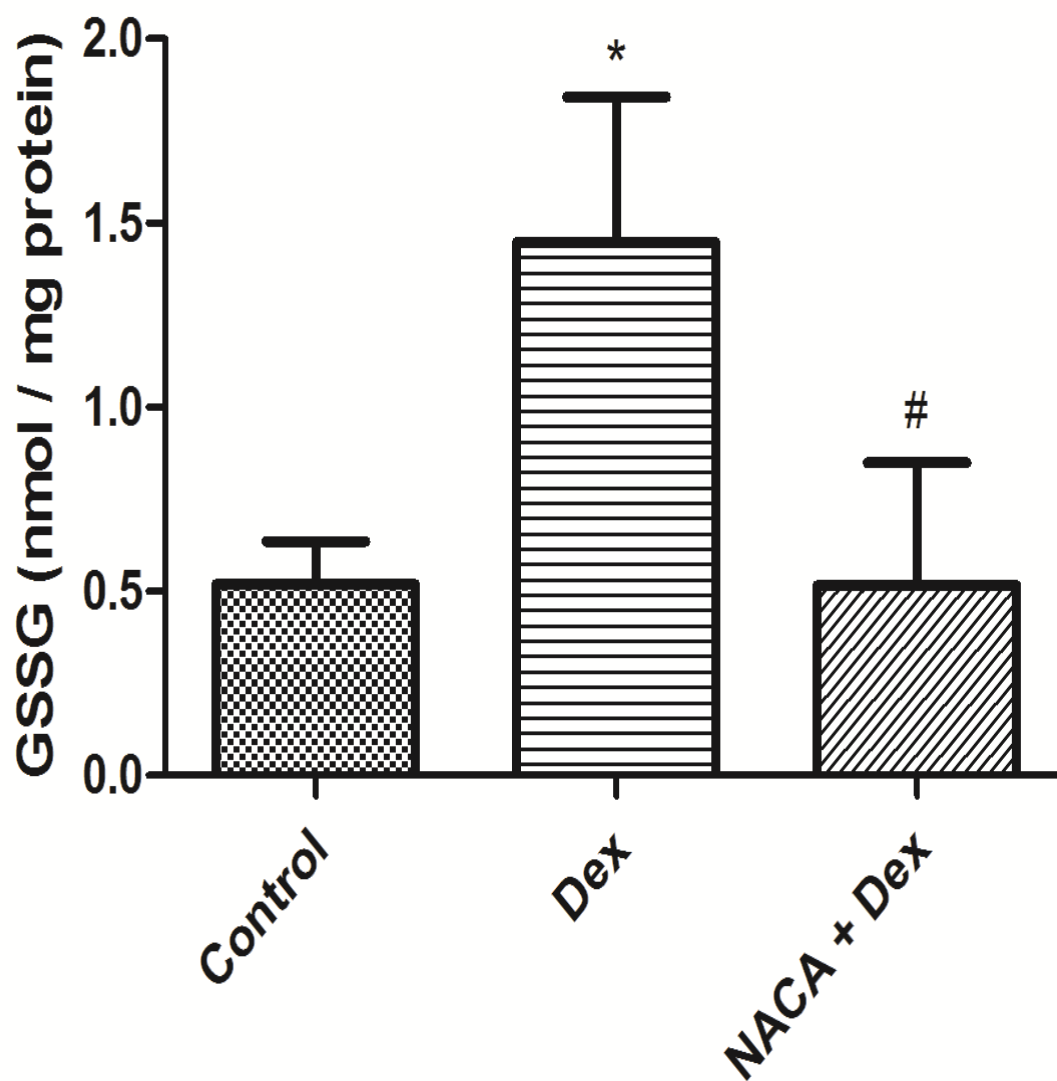
Figure 4.3. Intracellular GSSG Levels in Lens after Treatment with Dex and NACA. The GSSG level was significantly higher in the Dex-only group than in the control. This GSSG level was significantly reduced upon pretreatment with NACA. The NACA-only treated group showed no significant difference when compared to the control. *p ≤ 0.05 compared to the control group, and #p ≤ 0.05 compared to the Dex group.
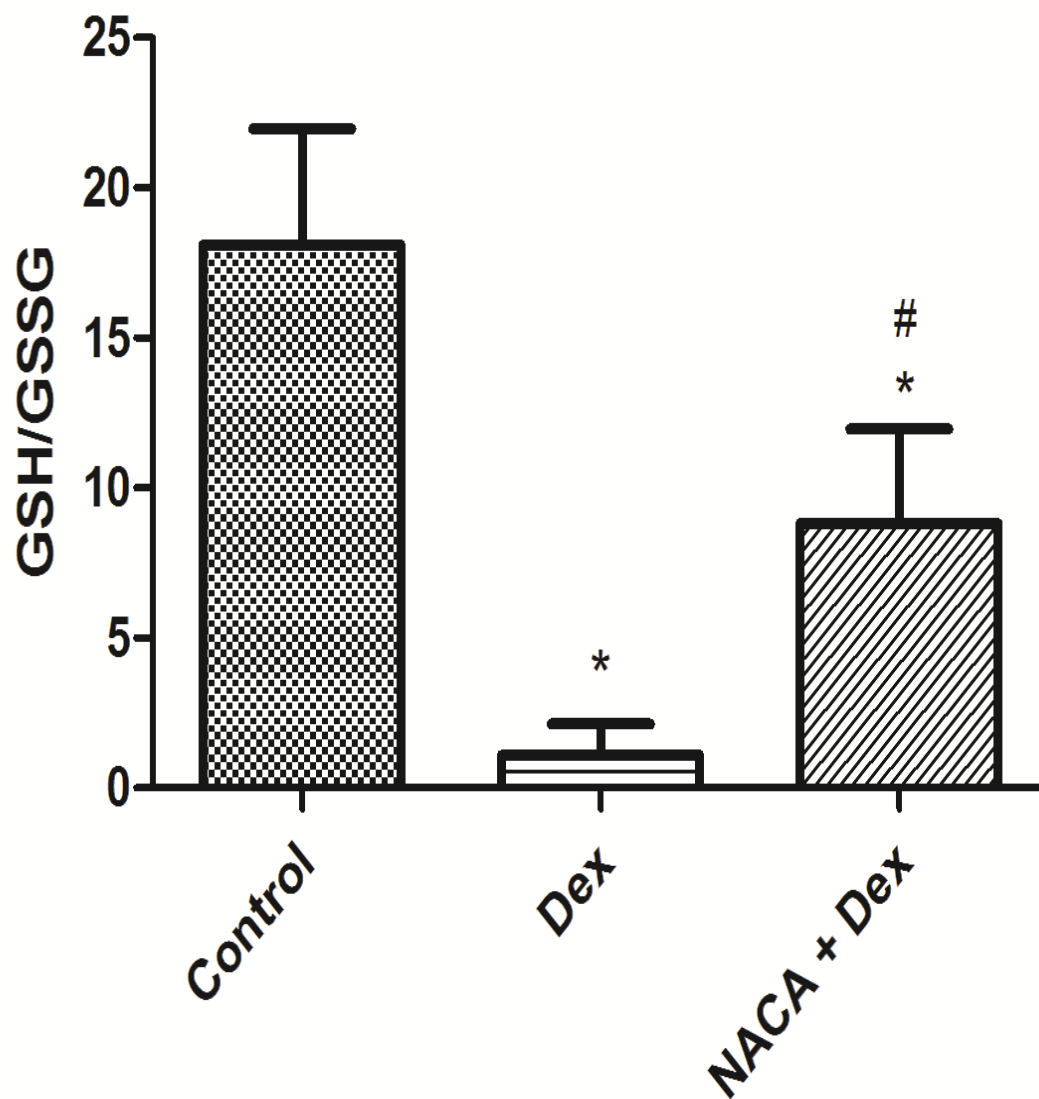
Figure 4.4. GSH/GSSG Ratio in Lens after Treatment with Dex and NACA. The GSH/GSSG ratio in the Dex only group was significantly lower than in the control group. This GSH/GSSG ratio was significantly increased upon pretreatment with 50uM NACA. The NACA-only treated group showed no significant difference when compared to the control. *p ≤ 0.05 compared to the control group, and #p ≤ 0.05 compared to the Dex group.
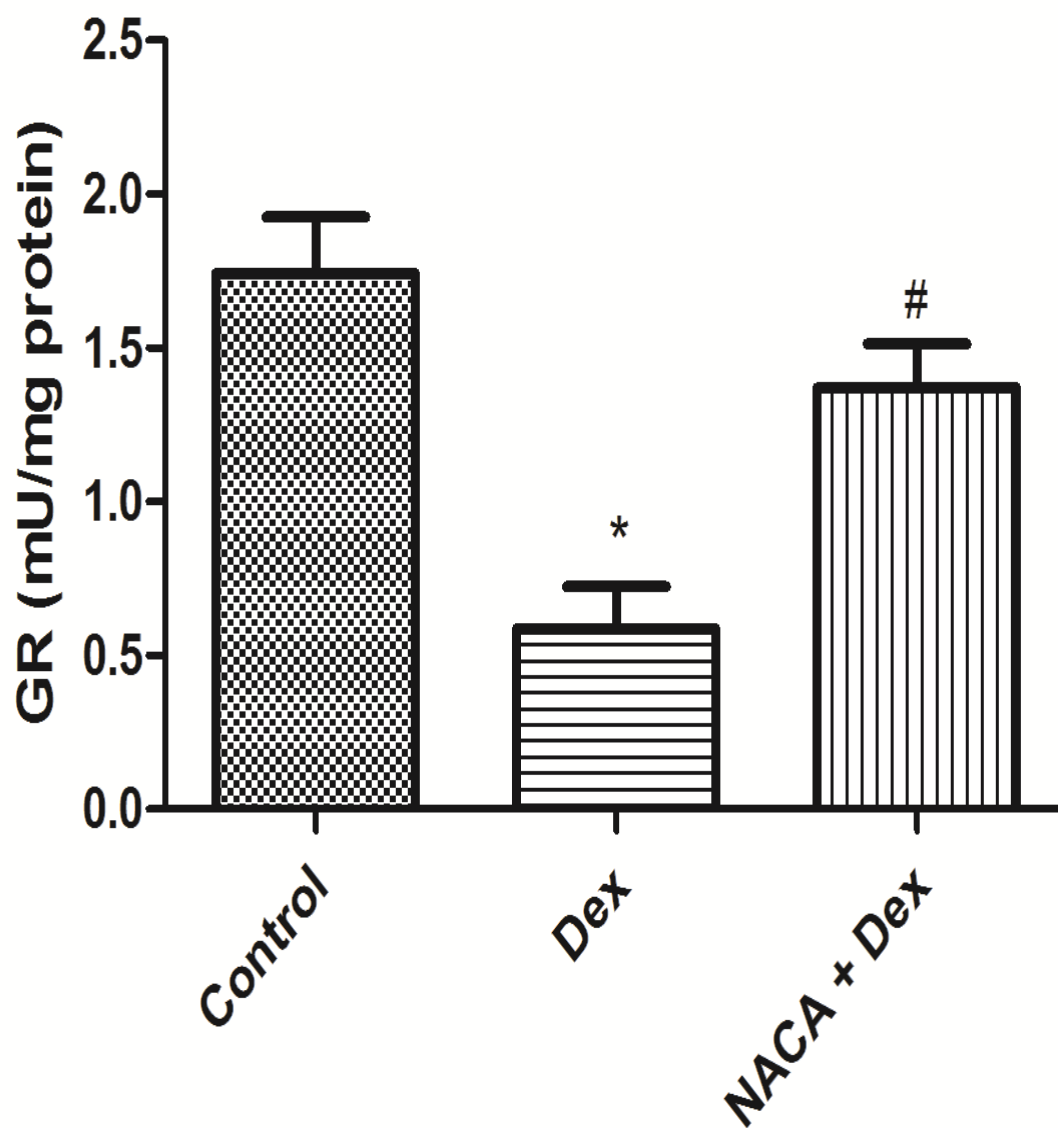
Figure 4.5. GR Activity in Lens after Treatment with Dex and NACA. GR activity was significantly lower in the Dex-only group than in the control group, while NACA pretreatment increased its activity. The NACA-only treated group showed no significant difference when compared to the control. *p ≤ 0.05 compared to the control group, and #p ≤ 0.05 compared to the Dex group.
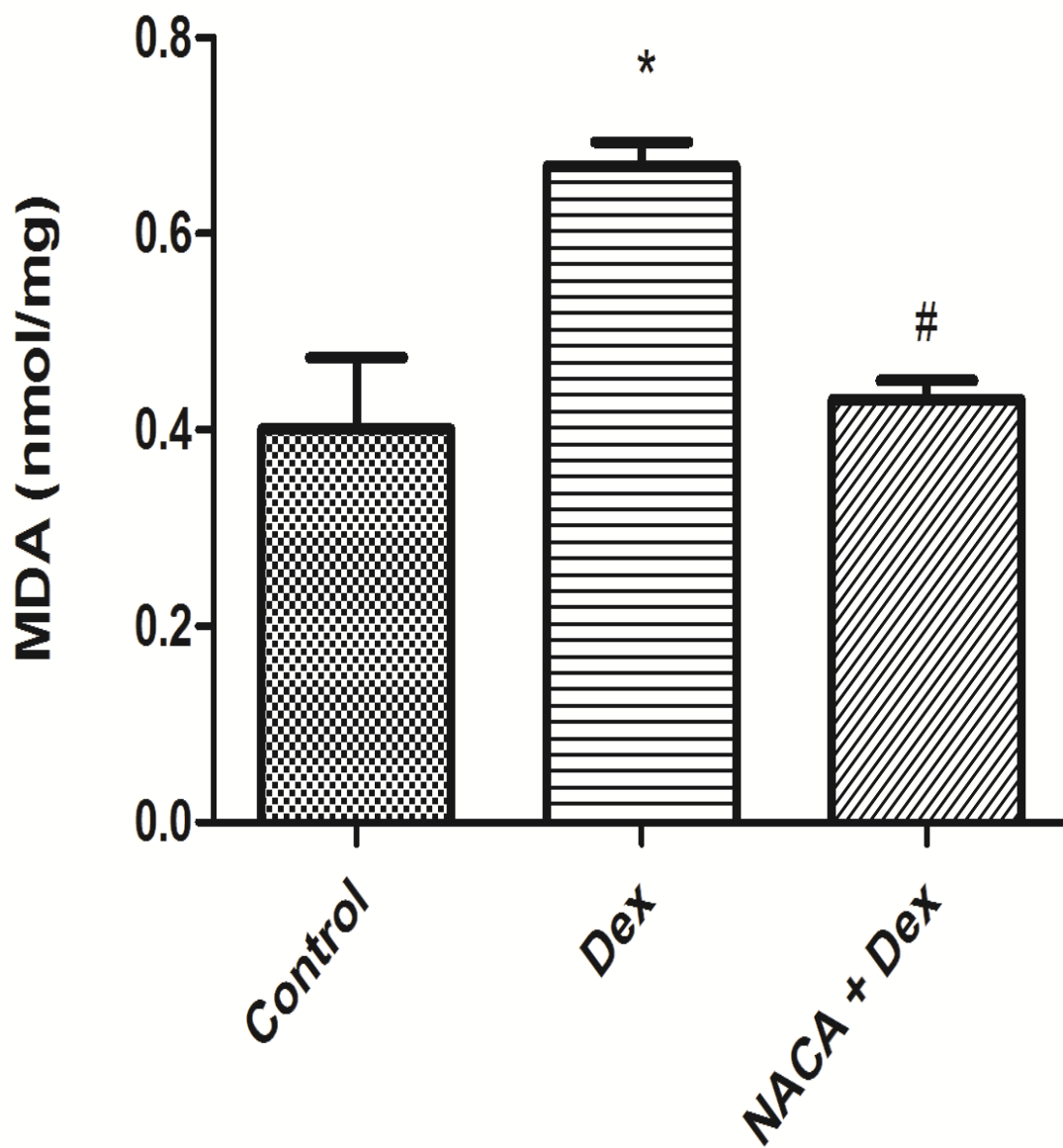
Figure 4.6. MDA Levels in Rat Lens after Treatment with Dex and NACA. It was found that, after 7 days of Dex treatment, the MDA levels significantly increased. Dex (5 μM) induced a significant increase in the MDA level. Pretreatment with 50 μM of NACA decreased lipid peroxidation significantly. *$p \leq 0.05$ compared to the control group, and #$p \leq 0.05$ compared to the Dex group.

5. EFFECTS OF N-ACETYLCYSTEINE AMIDE EYE DROPS ON ACETAMINOPHEN-INDUCED CATARACTS IN C57BL/6 MICE

Acetaminophen (N-acetyl-p-aminophenol; APAP), one of the most widely prescribed medications in the world is an analgesic and antipyretic drug which is considered to be safe when used at a therapeutic dose [205]. However, at high doses, APAP may cause severe damage to the liver [206-218], and has been reported to be the most important cause of acute liver failure in many nations [214, 219-221]. In addition to liver failure, APAP also has an adverse effect on kidneys [222], the pancreas resulting in pancreatitis [223], and the lens causing cataracts [224-229]. APAP gets transformed to a reactive and toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI) in the presence of hepatic cytochrome P450 (CYP) enzymes (Figure 5.1) [205, 230-232]. At a therapeutic dose of APAP, this NAPQI undergoes conjugation with GSH and gets eliminated from the body; however, at a higher dose, NAPQI depletes the GSH stores by approximately 90% , subsequently binding to the cysteine residues of other proteins, leading to the formation of APAP-protein adducts, and thereby causing toxicity [233-235]. Qian and Shichi [236] suggested that the "NAPQI formed from the biotransformation of APAP by CYP is transported by blood circulation to the eye, secreted with the aqueous humor, flows to the anterior chamber, and causes damage to the lens epithelium". Histological studies revealed structural and functional changes of mitochondria in mice injected intracamerally with NAPQI [237] and increased intracellular free calcium levels, leading to the activation of calpains [226]. Mitochondrial dysfunction leads to increased production of ROS that regulates the activities of enzymes and also the expression levels of proteins leading to cataract.
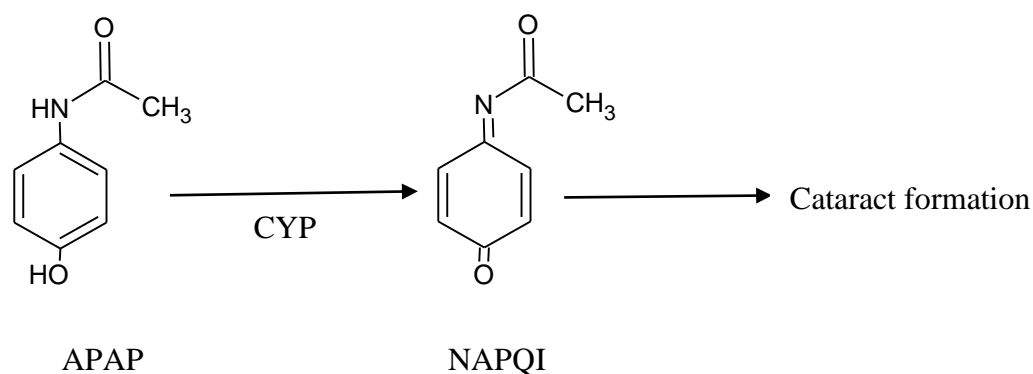
Figure 5.1. APAP Biotransformation to NAPQI

Antioxidants are becoming more widely used in preventing as well as treating oxidative stress related disorders. As oxidative stress and GSH depletion were reported as important events in the development of cataracts by APAP, use of GSH prodrugs could slow down or prevent the cataract progress. Previous *in vivo* studies using different prodrugs such as mercaptoethanol/L-cysteine mixed disulfide (CySSMe) [228], diallyl disulfide + N-acetylcysteine [227] have been shown to be successful in preventing APAP-induced cataract formation.

In this study, the role of NACA in preventing APAP-induced cataracts in C57BL/6 mice was evaluated. Cataracts were induced by intraperitoneal injection of β-naphthoflavone (CYP inducer) into C57BL/6 mice, 3 days prior to APAP injection. To evaluate NACA's ability to prevent cataract formation, NACA was injected intraperitoneally 2 h prior to APAP injection and NACA eye drops were continued until the conclusion of the experiment. The results suggest that NACA prevented cataract formation by increasing the GSH levels, GSH/GSSG ratio, GR and Ttase activities, while decreasing the levels of GSSG and MDA.

5.1. EXPERIMENTAL DESIGN

Sixteen C57BL/6 mice (male, 4-6 weeks of age, each weighing 20-24 g) were purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A) and were housed in a temperature- (~22 °C) and humidity- (~55%) controlled animal facility, with a 12 h/12 h light/dark cycle. The animals had unlimited access to rodent chow and water and were utilized after 3 days of acclimatization. All animal procedures were conducted under an animal protocol approved by the Institutional Animal Care and Use Committee of the Missouri University of Science and Technology. The mice were divided into four groups, (1) control, (2) APAP-only, (3) NACA-only, and (4) NACA + APAP. Three days prior to APAP injection, β-naphthoflavone (37.5 mM) was prepared in warm corn oil and injected intraperitoneally (0.02 ml/g body wt.) into the mice belonging to the APAP-only and the NACA + APAP groups to induce CYP [228]. Warm corn oil was injected in the control and the NACA-only groups. APAP was also prepared in warm corn oil and was injected intraperitoneally (0.15 mmol/ml, administered at a level of 0.02 ml/g body wt.) into the APAP-only and the NACA + APAP groups. All mice in the NACA-only and the NACA + APAP groups received an intraperitoneal injection of NACA (250 mg/kg body wt., prepared in 25 mM phosphate buffer, pH 7.4), 2 h before APAP injection , whereas, phosphate buffer was injected in the control and the APAP-only groups. Cataracts were observed within the first 5 h of APAP injection in the APAP-only group. One percent NACA eye drops (prepared in 25 mM phosphate buffer, pH 7.4) were continued for 2 weeks before sacrificing the animals. Grading of the cataract formation was performed with the help of a slit microscope on the last day of eye drops, along with picture documentation. All mice were anesthetized 2-3 h after administration of the last NACA

or buffer eye drop by intraperitoneal injection with a 40% urethane solution (0.1 ml/10 g

body wt.). After sacrifice, their lenses were harvested, and then immediately placed on

dry ice. Samples were stored at a temperature of −80 °C for further analysis. The lenses

were then analyzed for oxidative stress parameters, such as GSH levels (reduced and

oxidized), MDA levels, GR and Ttase activities.

5.2. RESULTS

The results indicate the effects of NACA and APAP on various oxidative stress

parameters.

5.2.1. Effects of APAP and NACA on Cataract Formation in the Lens.

Administration of an injection of 0.2 ml/g body wt. of 0.15 mmol/ml of APAP was

significant enough to cause the development of cataracts. Upon examination with a slit-

lamp microscope, it was also found that all of the mice within the APAP-only group

developed cataracts and were classified as grade 3. However, in the mice treated with

NACA in conjunction with APAP, it was found that only 25% of the lenses had shown

opacity. The majority of lenses were of a clarity similar to that seen in the control group.

Lenses observed within the control and the NACA-only groups were completely clear.

The grading of the lens in all of the groups is tabulated in Table 5.1, and the slit-lamp

pictures of representative lenticular opacities observed for each group are shown in

Figure 5.2.

5.2.2. Effects of NACA on GSH and GSSG Levels in the Lens. GSH levels in

the lenses of the rat pups in the APAP-only group were found to be significantly ($p <$

0.05) lower than those of the lenses in the control and the NACA-only groups. However,

Table 5.1. Classification of Degree of Cataract Formation in Mice after NACA Treatment

Group (n=4)	Grade 0	Grade 1	Grade 2	Grade 3
Control	4	0	0	0
NACA-only	4	0	0	0
APAP-only	0	0	0	4
NACA+APAP	3	1	0	0

treatment with NACA in the NACA + APAP group (Figure 5.3) significantly ($p < 0.05$) increased the GSH levels compared to that of the APAP-only group.

GSSG levels in the lenses of the APAP-only group were found to be significantly ($p < 0.05$) increased by approximately ten times the GSSG levels in the control group. However, treatment with NACA in the NACA + APAP group had significantly ($p < 0.05$) decreased the GSSG levels similar to those of the control. The GSSG levels in the NACA-only group were found to be similar to those of the control group (Figure 5.4).

A similar trend was observed with the ratio between the GSH and the GSSG levels in the lenses of each group. As expected, the control group was found to have the highest ratio of GSH to GSSG. A significant ($p < 0.05$) reduction (approximately 90%) of the ratio GSH to GSSG was observed in the APAP-only group when compared to those of the control group. However, an increase in the GSH to GSSG ratio was seen in the NACA + APAP group when compared to the APAP-only group, but was not significantly different from that of the control group lenses (Figure 5.5).

5.2.3. Effects of NACA on GR and Ttase Activities in the Lens. The activities of two major antioxidant enzymes: GR, and Ttase were measured within the lens. Significant ($p < 0.05$) reductions in GR, and Ttase activities were observed in the lenses of animals injected with APAP in comparison with those of the control group and the NACA-only group. However, the mice that were treated with NACA showed increased enzyme activities, compared to the APAP-only group (Figure 5.6 and 5.7). No significant differences between GR, and Ttase activities for the control and the NACA-only groups were determined.

5.2.4. Effects of NACA on Lipid Peroxidation in the Lens. The levels of MDA, the byproduct of lipid peroxidation, were measured in the lens samples and were consistently high in lens containing cataracts. The results suggest a significant ($p < 0.05$) twofold increase in MDA levels in the APAP-only group compared to the control group. However, MDA levels in the lenses of the mice belonging to the NACA + APAP group showed a significantly larger ($p < 0.05$) reduction than those in the APAP-only group and approached levels similar to those of control (Figure 5.8).

5.3. DISCUSSION

The cytotoxicity of APAP depends on the metabolic transformation of APAP to NAPQI by cytochrome P450 enzymes (CYP) [205, 230-232, 238]. At normal levels of APAP, the CYP enzyme activation of APAP and its conversion to NAPQI is not enough to cause a cataract as it is eliminated from the body by conjugating with GSH, whereas at higher doses of APAP and a CYP inducer (such as β-naphthoflavone), the NAPQI that is formed from biotransformation of APAP reaches the eye through blood circulation, gets

secreted with aqueous humor and damages the lens epithelium, resulting in a cataract [224-229]. Studies have demonstrated that NAPQI covalently binds to cellular organelles [206] such as mitochondria [239], and similar to cyanide, NAPQI disrupts the mitochondrial membrane causing the loss of mitochondrial membrane potential and depletion of ATP [240]. The loss of mitochondrial membrane integrity results in increased ROS generation, leading to oxidative stress which can lead to the loss of GSH, lipid peroxidation, decreased antioxidant enzyme activities and dysregulation of signal transduction pathways [193-195].

With oxidative stress and GSH depletion being involved in the etiology of an APAP-induced cataract, an antioxidant that can replenish GSH levels can be tried to treat this APAP-induced cataract. Based on this, we evaluated the effects of a novel antioxidant and a potent thiol-exchange compound NACA to prevent as well as treat cataracts induced by APAP. Morphological examination (Figure 5.1) of the lenses indicated that NACA was able to prevent the formation of cataracts in the NACA + APAP group in about 75% of the mice, whereas a grade 1 cataract was observed in one mouse (Table 5.1). However, all the mice in APAP-only group had grade 3 cataracts.

As discussed earlier, GSH being an important antioxidant in the lens, provides the first line of defense against oxidative damage caused by ROS [5]. It is present in unusually high concentrations in the lens and is responsible for lens transparency [133]. Previous studies have indicated that depletion of GSH levels precedes the formation of cataracts [130, 132, 133, 136, 137, 182, 241], resulting in oxidation of protein sulfhydryl groups and, thereby, causing cataracts [203]. Results from this study indicated a significant decrease of GSH levels in the lenses of the APAP-only group compared to

that of the control group. On the other hand, NACA being a GSH prodrug, increased the

GSH levels in the NACA + APAP group (Figure 5.2). This might be due to NACA's

ability to donate sulfhydryl groups for GSH synthesis [191] and also through reduction of

GSSG to GSH, by nonenzymatic thiol disulfide exchange [24]. These results are in

agreement with previous results that have reported a decrease in GSH levels in the lenses

upon APAP induction of cataracts in mice [227, 228].

GSSG is an oxidized form of GSH which accumulates with increased oxidative

stress in the cells. Results indicated significantly high levels of GSSG in the lenses of the

APAP-only group (Figure 5.3) compared to that of the control group lenses, resulting in a

decreased GSH/GSSG ratio (Figure 5.4). However, treatment with NACA in the NACA

+ APAP group decreased the GSSG levels significantly and, thereby, increased the

GSH/GSSG ratio in comparison with the APAP-only group. This could be due to

decreased GR activity in the APAP-only group (Figure 5.5). The GR catalyzes the

NADPH dependent reduction of GSSG to GSH, thereby maintaining the intracellular

GSH pool and reduced environment inside the cell, which is essential for the proper

functioning of proteins [242]. With decreased GR activity in the APAP-only group, when

compared to the control group, the conversion of GSSG to GSH could have been

hindered leading to the accumulation of high GSSG levels in the APAP-only group. On

the other hand, NACA treatment increased the GR activity in the lenses of the NACA +

APAP group resulting in decreased GSSG levels.

Decreased GSH would lead to accumulation of free radicals in the lenses resulting

in lipid peroxidation. Lipid peroxidation has been associated with cataract formation

[203, 204, 243]. The unsaturated bonds in fatty acids undergo lipid peroxidation in the

presence of free radicals and form stable by-products, such as MDA, which are used as markers of lipid peroxidation. Lenses from the APAP-only group had significantly higher levels of MDA (Figure 5.7), compared to those of the control group lenses, indicating increased lipid peroxidation in the APAP-only group. However in the NACA + APAP group, treatment with NACA resulted in significant reduction of lipid peroxidation induced by APAP, in comparison to that of the APAP-only group, by providing adequate amounts of GSH, a substrate for GPx, an enzyme that catalyzes the conversion of hydrogen peroxide to water.

The Ttase is an enzyme which catalyzes the conversion of protein mixed disulfide (PSSG) back to protein thiol (PSH), by using GSH as one of the substrates. During cataract formation, oxidative stress builds up, due to diminished levels of GSH, and leads to protein-thiol mixed disulfide formation such as crystallin-thiol mixed disulfides, which then precipitate within the lens causing a cataract. [244]. This may be due to decreased Ttase activity as a consequence of lowered GSH levels during cataract development. Depletion of Ttase activity in cataract formation has been previously reported [168, 245, 246]. Mice injected with APAP had a significantly lowered Ttase enzyme activity, as compared to control and, thus, leading to the precipitation of crystallin proteins, whereas treatment with NACA in the NACA + APAP group reversed the Ttase enzyme activity (Figure 5.6). This reversal of Ttase activity by NACA in the NACA + APAP is neither significantly different from that of the control group nor the APAP-only group.

These results suggest that NACA can prevent the formation of APAP- induced cataracts in C57BL/6 mice. This can be attributed to its antioxidant properties that result in enhanced levels of GSH, decreased levels of MDA and GSSG, and increased Ttase,

and GR enzyme activities. NACA could confer a protective effect by providing a

substrate for the generation of GSH, maintaining antioxidant levels within the lens and,

possibly, through disulfide-exchange mechanisms. Treatment with NACA may prove to

have a major therapeutic role in Dex-induced cataracts. In future studies, the focus will be

on evaluating NACA's ability to reverse the grade of cataracts using *in vivo* studies.

Control   NACA-only

APAP-only   NACA + APAP
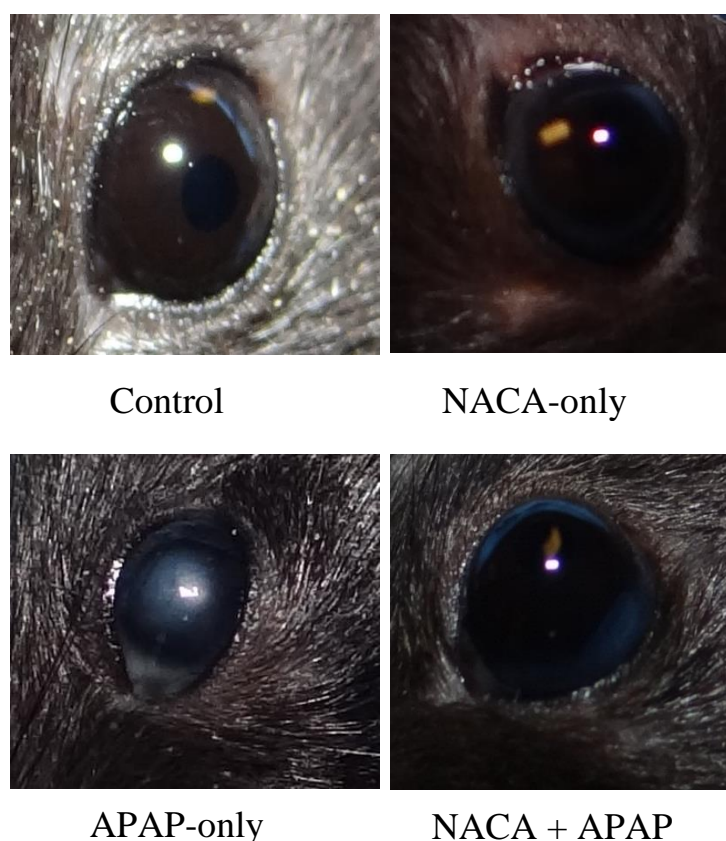
Figure 5.2. Slit Lamp Images of Eyes. These pictures were taken a day before sacrifice. A representative picture of the lenses observed for each group is shown. (A) Lenses from control group were found to be clear with no detectable cataracts. (B) Lenses from NACA-only group exhibited similar results as that of control, with no detectable cataracts. (C) Lenses from APAP-only group exhibited dense nuclear cataracts (100%). (D) 75% of lenses from NACA + APAP group exhibited no cataract (grade 0), whereas, 25% of lenses have developed grade 1 cataracts.
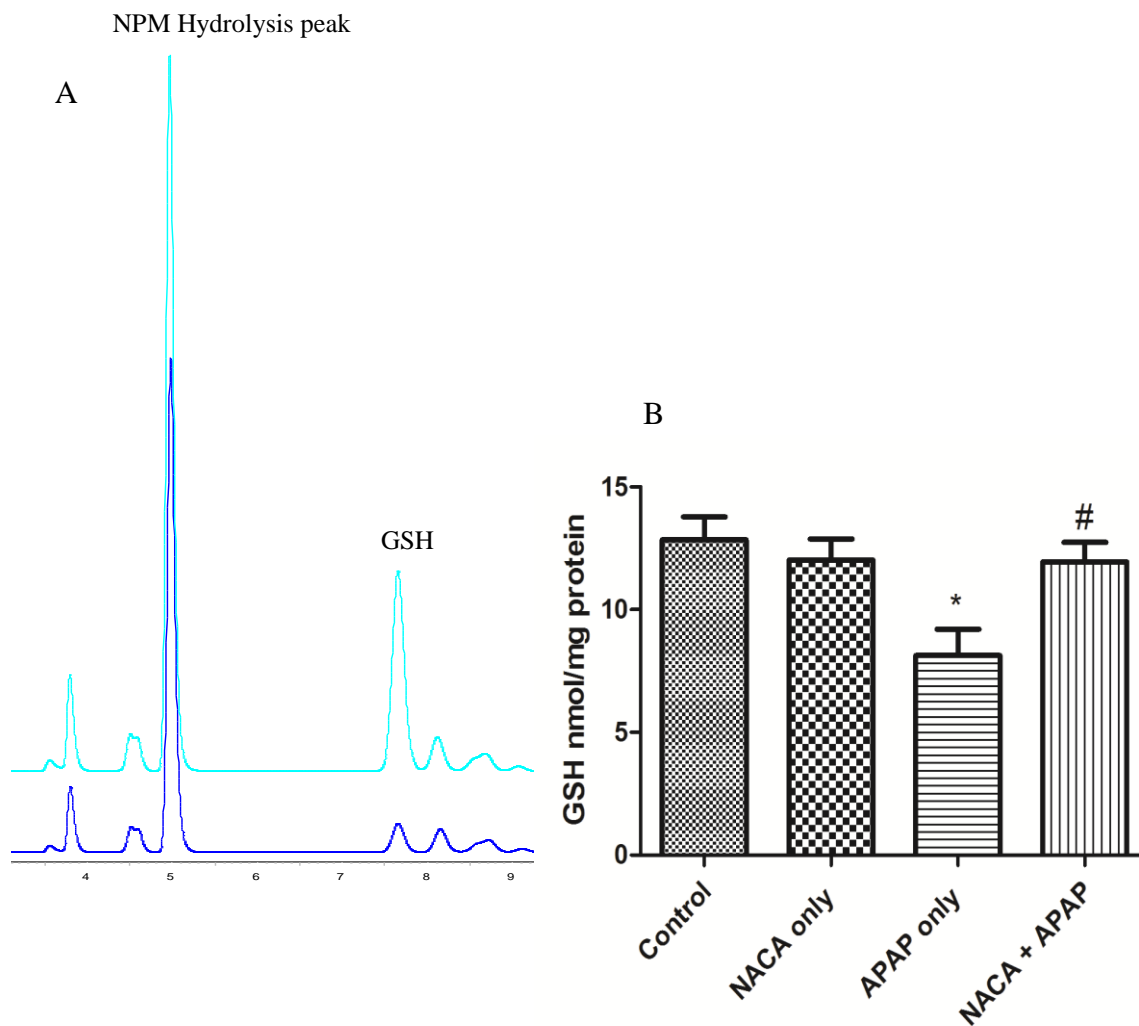
Figure 5.3. GSH Levels in the Lens. A: Chromatograms of GSH- dark blue chromatogram indicates APAP only, light blue chromatogram indicates NACA + APAP. B: Bar graph representation of the GSH level in the NACA-only group was similar to that of the control; exposure to APAP significantly decreased the amount of GSH within the lens. A significant increase in GSH levels was observed with NACA treatment in NACA + APAP group. '*' refers to significant difference from the control and '#' refers to the significant difference from the APAP-only group.
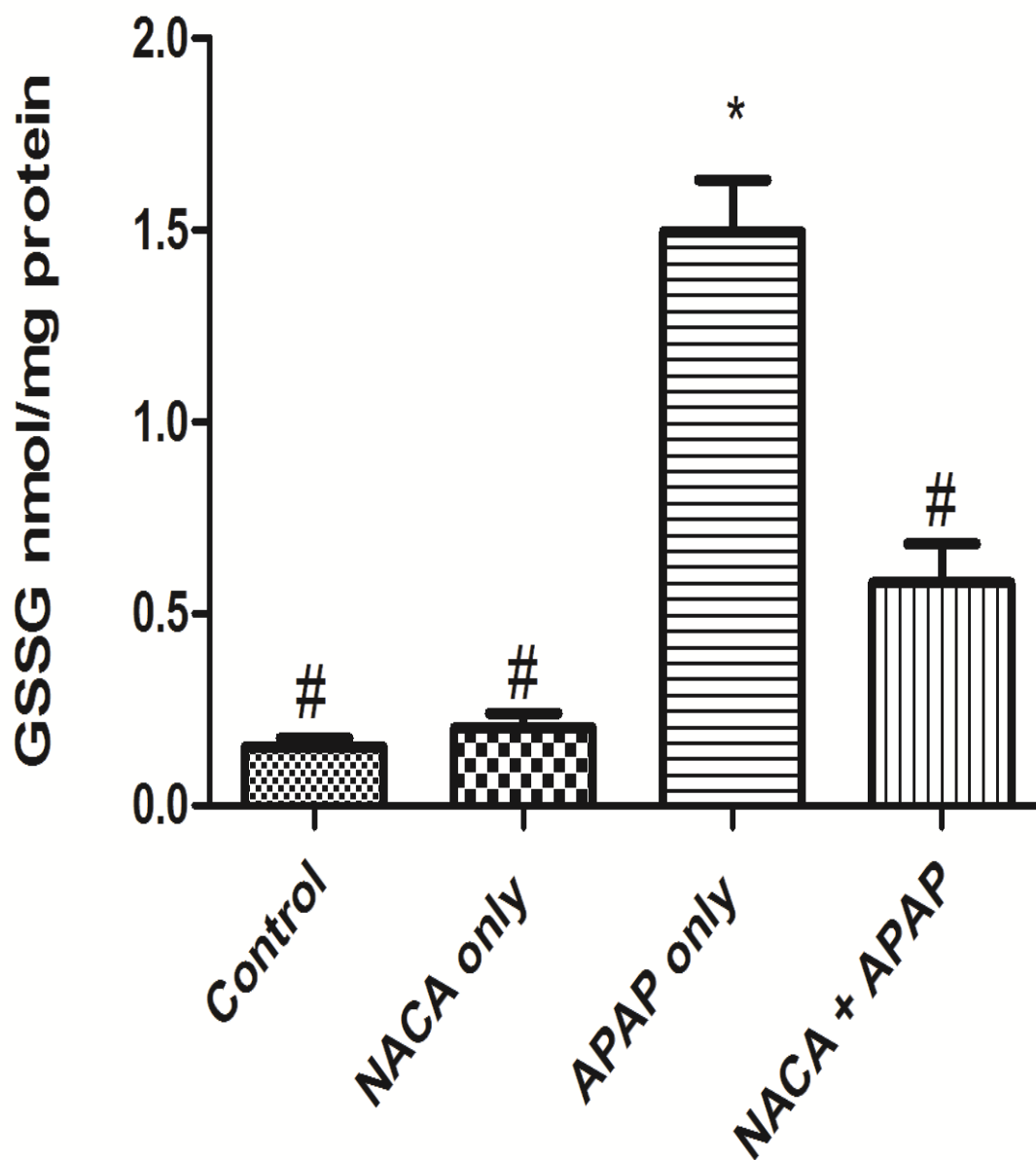
Figure 5.4. GSSG Levels in the Lens. The GSSG level in the NACA-only group was similar to that of the control; exposure to APAP significantly increased the amount of GSSG within the lens. A significant decrease in GSSG levels was observed with NACA treatment of the NACA + APAP group. '*' refers to significant difference from the control group and '#' refers to the significant difference from the APAP-only group.
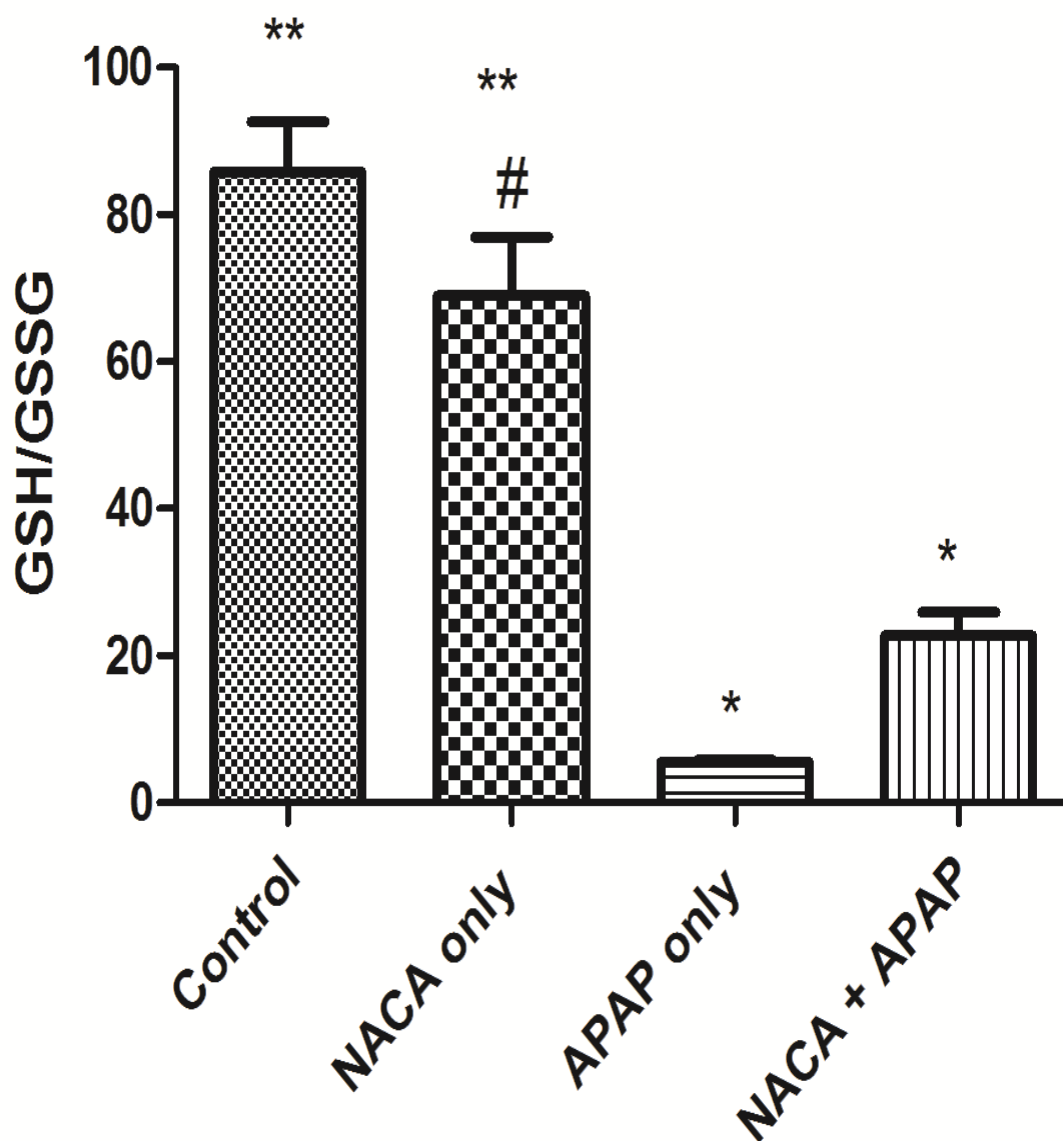
Figure 5.5. GSH/GSSG Ratios in the Lens. An accurate measure of the redox status within the lens can be obtained with the ratio between GSH to GSSG. The higher the value of this ratio, the more of a reduced environment was present within the lens. The APAP-only group had a significantly lower ratio than the control group. NACA treatment was found to increase this ratio but not to the amount determined within the control group. '*' refers to significant difference from the control group, '#' refers to significant difference from the APAP-only group, and '**' indicates significant difference from the APAP + NACA group.
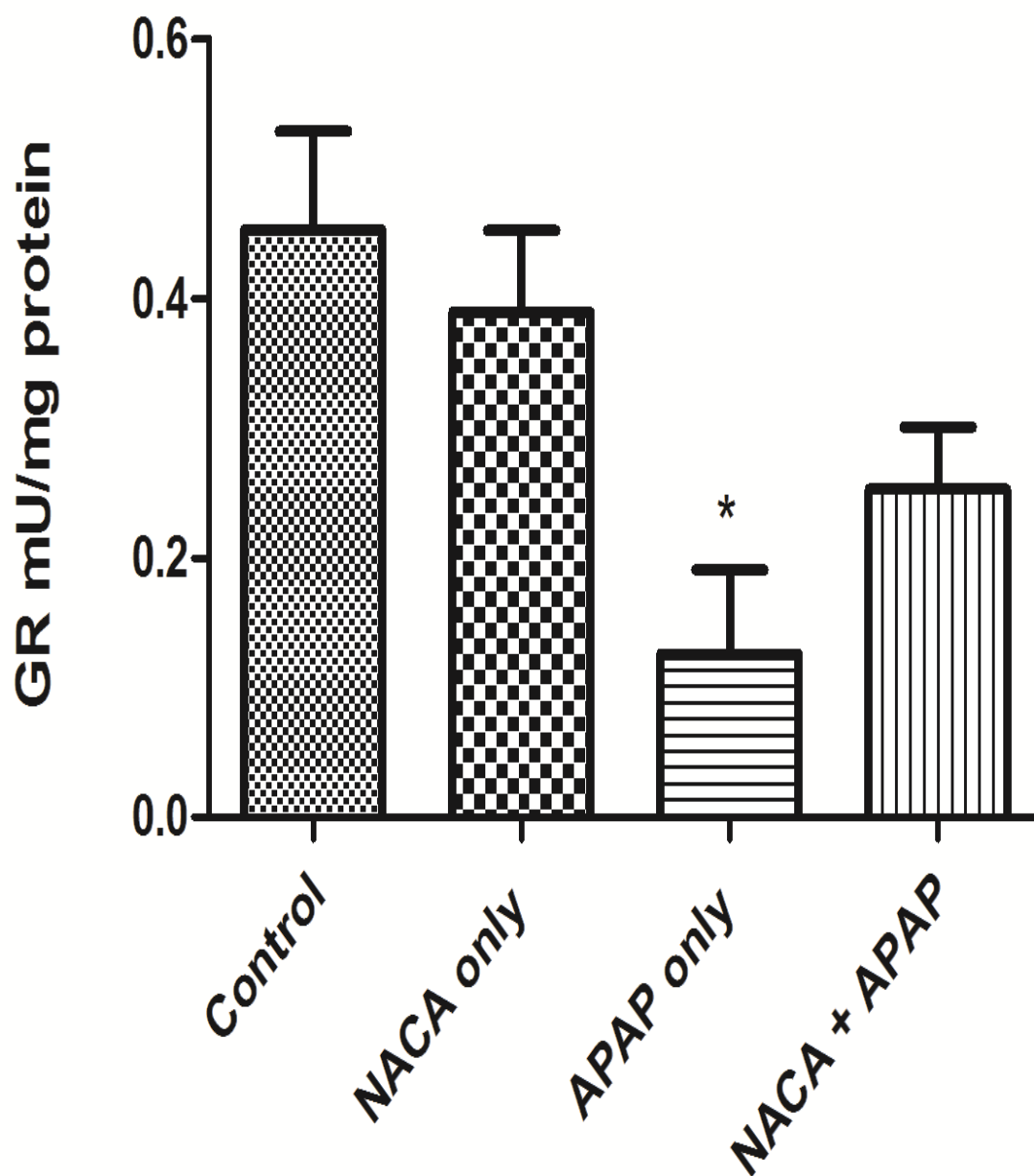
Figure 5.6. GR Activity in the Lens. GR is essential for maintaining the levels of GSH in the cell. The results of GR activity in the lens indicated a significantly lower GR activity in the lenses of the APAP-only group than in the control group, while NACA treatment in the NACA + APAP group increased its activity but not to that of the control. The NACA-only treated group showed no significant difference when compared to the control. * refers to significant difference compared to the control group.
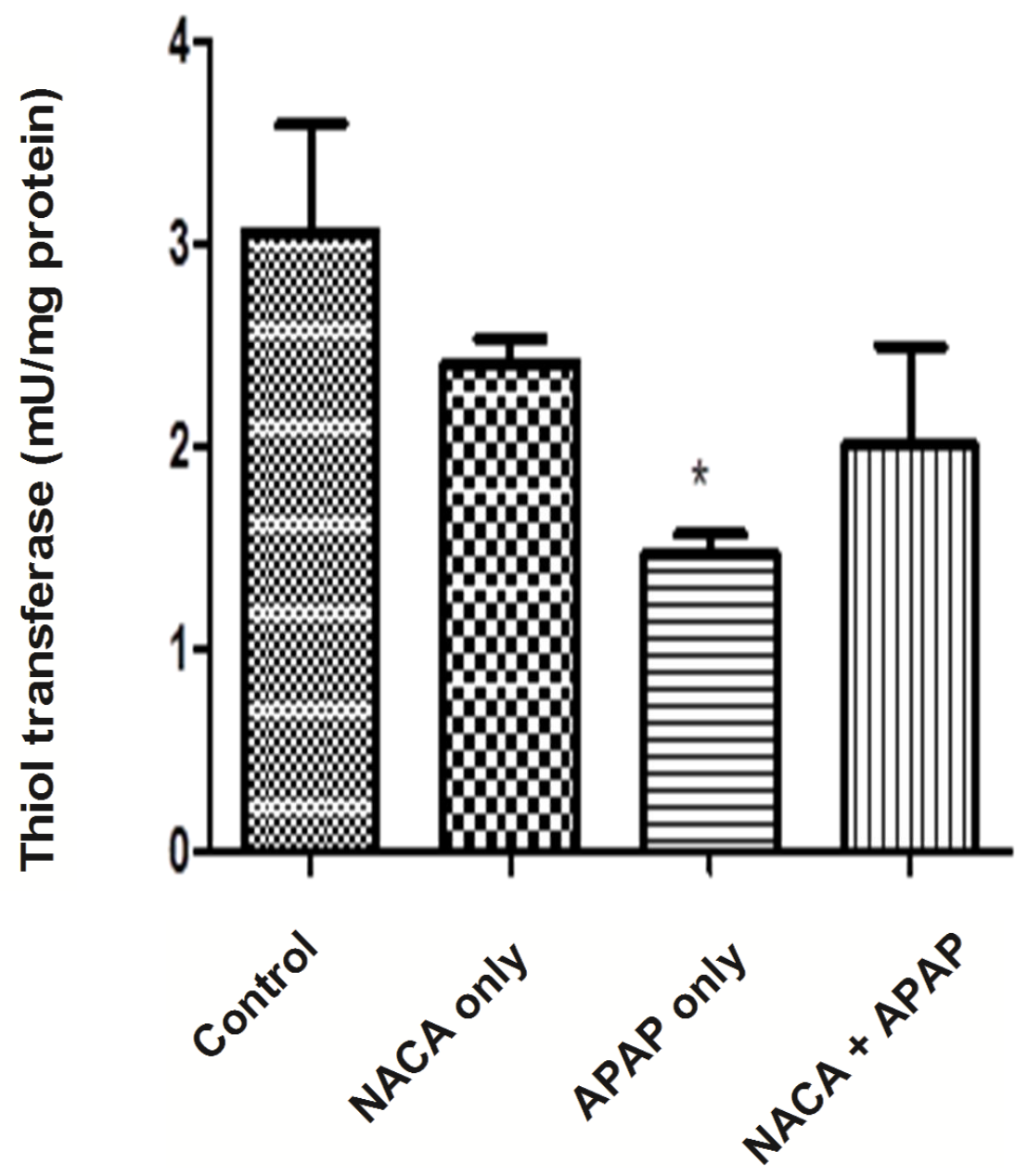
Figure 5.7. Thioltransferase Activity in the Lens. Ttase activity was found to be significantly lower in the APAP-only group than in the control group, while NACA treatment in the NACA + APAP group increased its activity but not to that of the control. The NACA-only treated group showed no significant difference when compared to the control. * refers to significant difference compared to the control group.
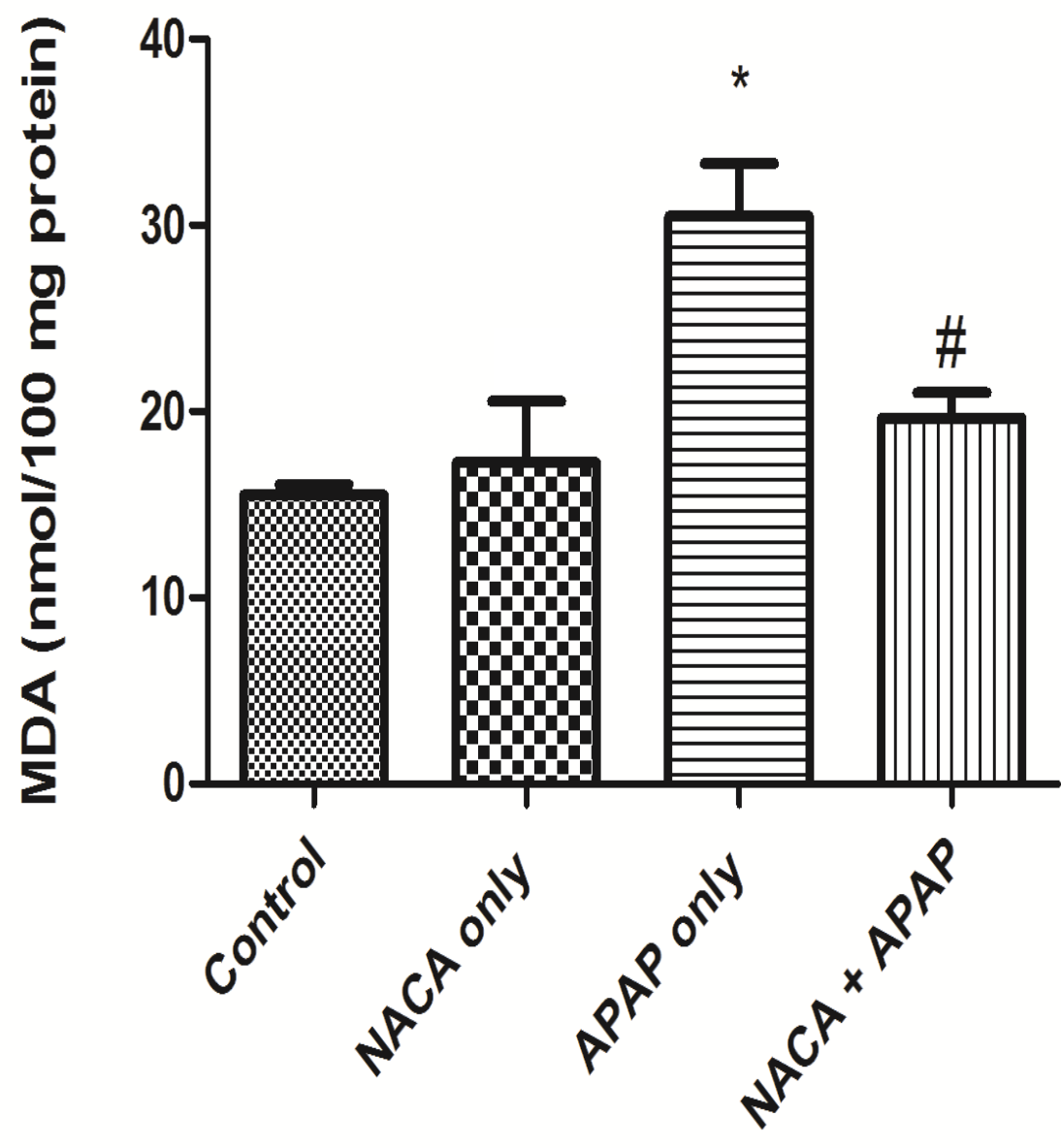
Figure 5.8. MDA Levels in the Lens. These Results indicate that the amount of MDA in the lenses of the NACA-only group were very similar to those of the control group, while the APAP-only group showed elevated levels of MDA. Treatment with NACA in the NACA + APAP group was able to retain MDA levels similar to that of the control. '*' refers to significant difference compared to the control group and '#' refers to significant difference from the APAP-only group.

6. EFFECTS OF N-ACETYLCYSTEINE AMIDE EYE DROPS ON PREVENTION AND REVERSAL OF SODIUM SELENITE-INDUCED CATARACTS IN WISTAR RATS

A cataract is a cloudiness of the lens region in the eye which attributes to more than 50% of the blindness in the world. The selenite-induced cataract model is the most commonly used model to study cataracts, as it partially mimics the senile nuclear cataracts in humans where oxidative stress plays a major role [247]. Furthermore, Shearer proposed the involvement of oxidative stress in selenite-induced cataracts and the sequence of events leading to cataract formation [247]. It was hypothesized that the oxidative damage caused by selenite possibly involves oxidation of critical sulfhydryl groups on calcium ATPase or ion channels, leading to altered metabolism in lens epithelium and also in the loss of calcium ATPase activity and, hence, resulting in an accumulation of calcium in the nucleus, which could cause activation of m-calpain. Activation of m-calpain initiates the proteolysis of β-crystallins leading to the in-solubilization of proteolyzed β- and α-crystallins; co-precipitating γ-crystallins; and ultimately leading to the formation of cataracts.

Since oxidative stress is implicated in cataract formation, a logical approach to counteract cataractogenesis would be the use of an effective lens permeating antioxidant that would neutralize and alleviate cumulative oxidative damage. Several compounds with antioxidant properties have been reported to prevent selenite-induced cataracts such as resveratrol [248], saffron [249], ellagic acid [250], garlic [251], melatonin [252], and drevogenin D [253], caffeine [254], ebselen [255] in *in vivo*, lycopene [256], and N-acetyl-L-carnitine [169, 257, 258] in both *in vitro* and *in vivo*, ocimum sanctum in *in vivo*

and *ex vivo* [259] as well. In fact, N-acetylcysteine (NAC, a GSH prodrug) has previously been tried in selenite-induced cataracts in *in vivo* [260] and was shown to prevent oxidative damage to the lens, slowing down the cataractogenesis.

Therefore, in this study, the effect of NACA eye drops in preventing as well as reversing the selenite-induced cataract in Wistar rat pups was evaluated. This chapter involves two parts and in both parts a single intraperitoneal injection of sodium selenite (Na_2SeO_3, 19-30 μmol/kg body wt.) was given on post-partum day 10 to induce cataracts in Wistar rats. In part one, 1% NACA eye drops were administered once a day starting from the day rat pups opened their eyes (PD 15) and continued until the end of week 9. Morphological examination of the rat eyes was performed at both week 5 and week 9. Oxidative stress parameters were evaluated to check the effect of NACA eye drops in reversing the cataracts induced by sodium selenite. In part two, intraperitoneal injection of NACA (250 mg/kg body wt.) was administered on day 9, 11, and 13 to assess the preventive effect of NACA, and 1% NACA eye drops were given once a day, starting PD 15 (the day rat pups opened their eyes) until PD 30 to check NACA's ability to reverse cataract intensity. Morphological examination of the rat eyes was performed on PD 30. Oxidative stress parameters, such as GSH, GSSG, GSH/GSSG, MDA, and calcium levels in rat lenses, were determined. GR and Ttase enzyme activities were also quantified. Casein zymography and Western blots were performed to study the activity and levels of m-calpain. The results suggest NACA's ability to protect against the cataracts induced by sodium selenite. Hence, NACA can potentially be developed into a promising therapeutic option for prevention and reversal of cataract formation.

6.1. EFFECTS OF N-ACETYLCYSTEINE AMIDE ON REVERING SELENITE-INDUCED CATARACTS IN WISTAR RATS

This study demonstrates the oxidative stress induced by an injection of sodium selenite and the protective effects of NACA eye drops against the oxidative stress.

6.1.1. Experimental Design. Lactating female Wistar rats with 2-day-old 40 male pups were purchased from the breeding facility at Charles River and were housed in a temperature- (~22 °C) and humidity- (~55%) controlled animal facility, with a 12-h light and dark cycle. The animals had unlimited access to rodent chow and water and were used for experiments after a week of acclimation. All animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and by the Animal Care and Use Protocol Review Committee at the Missouri University of Science and Technology. The rats were divided into four groups: (1) control, (2) NACA-only, (3) Na_2SeO_3-only, and (4) Na_2SeO_3 + NACA, such that each group had 16 male pups. The pups in the Na_2SeO_3-only group and the NACA + Na_2SeO_3 were injected intraperitoneally with a single dose of sodium selenite (19 µmol/kg body wt.) on post-partum day 10, whereas 25 mM of phosphate buffer (pH 7.4) was injected into the pups from the control group and NACA-only group. Upon the opening of the rat pups eyes, cataracts were evaluated using a slit-lamp microscope for each of the animals. Twenty microliters of 1% NACA eye drop solution (prepared in sterilized 25 mM phosphate buffer pH 7.4) were administered once a day in each eye of the rat pups, starting from PD 15 until the end of week 9. Lenses of each rat were examined every week to grade the intensity of cataracts. Half of the rats from each group were sacrificed at week 5 and the lens removed, rinsed with sodium borate buffer, and immediately transferred into liquid nitrogen. Samples were stored at a temperature of -80 °C for further analysis. The lenses

were then analyzed to determine if NACA showed any improvement in oxidative stress parameters, including GSH (reduced and oxidized), GR, and MDA. The remaining half of the animals were sacrificed at the end of week 9, and then processed and analyzed as described for week 5 lenses (Table 6.1).

6.1.2. Results. The results indicate the effects of Na_2SeO_3 and NACA eye drops on various oxidative stress parameters.

6.1.2.1. Effects of NACA on cataract formation in the lens. A single injection of 19 μmol/kg body wt. of Na_2SeO_3 on PD 10 was significant enough to cause the development of cataracts by the time the rat pups opened their eyes. Upon examination with a slit-lamp microscope at week 5, it was found that all rats within the Na_2SeO_3-only group developed cataracts, with one out of sixteen (6.25%) developing grade 1 cataracts, three rats (18.75%) had grade 2 cataracts, and the remaining twelve of the sixteen (75%) developing grade 3 cataracts. However, treatment with NACA eye drops in the Na_2SeO_3 + NACA group did not show any reduction in cataract intensity; one rat out of sixteen (6.25%) had grade 1 cataract, four rats (25%) had grade 2 cataracts and eleven rats (68.75%) had grade 3 cataracts. All of the rat lenses in the control and the NACA-only groups had no signs of cataracts. Hence, NACA eye drops were continued for a longer time period to study the reversal effect of NACA on the intensity of cataract grade. Continuation of NACA eye drops in the Na_2SeO_3 + NACA group until the end of week 9 resulted in a clear reduction in cataract intensity; three rats out of eight (37.5%) had grade 1 cataracts, five rats (62.5%) had grade 2 cataracts, and none of the rats had grade 3 cataracts. Whereas in the Na_2SeO_3-only group, five rats (62.5%) out of eight showed grade 3 cataracts and the remaining three rats (37.5%) showed grade 2 cataracts.

This morphological examination indicated that prolonged administration (9 weeks) of NACA eye drops can reverse the intensity of cataract grade. The grading of the lens in all of the groups is tabulated in Table 6.2, and the slit-lamp pictures of representative lenticular opacities observed for each group are shown in Figure 6.1

6.1.2.2. Effects of NACA on GSH, GSSG and GSH/GSSG ratio in the lens.

The Lens GSH level is a good indication of redox status in a rat's eye. At week 5, the GSH levels in the lenses of the rat pups in the Na_2SeO_3-only group were found to be significantly ($p < 0.05$) lower than those in the lenses in the control and the NACA-only groups. However, treatment with NACA in the Na_2SeO_3 + NACA group did not increase the levels of GSH significantly when compared to that of the Na_2SeO_3-only group (Figure 6.2.A). The GSH levels in the lenses of the control and the NACA-only group showed no significant difference.

Interestingly, at the end of week 9, the GSH levels in the lenses of the Na_2SeO_3-only group reached back to the levels observed in the control group. While NACA treatment in the lenses of the Na_2SeO_3 + NACA group increased the GSH levels compared to those of the remaining groups (Figure 6.2.B).

At week 5, the lenses of the Na_2SeO_3-only group showed increased GSSG levels when compared to the lenses of the control group, but not significantly higher, whereas, treatment with NACA in the Na_2SeO_3 + NACA group decreased the GSSG levels significantly ($p < 0.05$), when compared to the Na_2SeO_3-only group lenses (Figure 6.3.A). At the end of week 9, the GSSG levels in the lenses of the Na_2SeO_3-only group were neither significantly higher than those of the control group lenses nor those with NACA treatment in the Na_2SeO_3 + NACA group that significantly reduced the GSSG

levels compared to those of the Na$_2$SeO$_3$-only group (Figure 6.3.B).

At week 5, the GSH/GSSG ratio in the lenses of the Na$_2$SeO$_3$-only group was found to be not significantly different from that of the lenses of the control group. Whereas, NACA treatment in the Na$_2$SeO$_3$ + NACA group showed a significantly increased GSH/GSSG ratio compared to that of the lenses of the Na$_2$SeO$_3$-only group (Figure 6.4.A). The same trend was observed even at the end of week 9; however, NACA treatment in the Na$_2$SeO$_3$ + NACA group showed a significantly increased GSH/GSSG ratio compared to that of the lenses of the Na$_2$SeO$_3$-only group and also the control group lenses (Figure 6.4.B).

6.1.2.3. Effects of NACA on MDA levels in the lens. Lipid peroxidation was measured based on the levels of MDA, the byproduct of lipid peroxidation. Results of week 5 indicated that the amount of MDA in the lenses of the Na$_2$SeO$_3$-only group was significantly ($p < 0.05$) elevated when compared to the lenses of the control group. Whereas NACA treatment in the Na$_2$SeO$_3$ + NACA group showed decreased MDA levels, which were close to the levels observed in the control group lenses (Figure 6.5.A).

At the end of week 9, the amount of MDA in the lenses of the Na$_2$SeO$_3$-only group was found to be significantly ($p < 0.05$) higher (two and a half times more) than that in the lenses of the control group. However, NACA treatment in the Na$_2$SeO$_3$ + NACA group lenses showed significantly ($p < 0.05$) reduced levels of MDA when compared to the lenses of the Na$_2$SeO$_3$-only group (Figure 6.5.B) with values that were nearly the same as those of the control.

6.1.2.4. Effects of NACA on GR activity in the lens. GR is an enzyme that catalyzes the conversion of GSSG to GSH in the presence of NADPH. Results of GR

at week 5 indicated that the activity of this antioxidant enzyme in the lenses of the Na_2SeO_3-only group was significantly ($p < 0.05$) higher than that in the control group lenses. However, NACA treatment in the Na_2SeO_3 + NACA group showed a significantly ($p < 0.05$) decreased activity of GR than that of the Na_2SeO_3-only group, with the activity approaching close to the activity observed in the control group lenses (Figure 6.6.A). Interestingly, at week 9, the GR activity in the lenses of the Na_2SeO_3-only group was close to the activity observed in the control group lenses. Whereas, NACA treatment in the Na_2SeO_3 + NACA group showed a significantly ($p < 0.05$) increased activity of GR, when compared to that of the lenses in the Na_2SeO_3-only group (Figure 6.6.B).

Table 6.1. Classification of Treatment Groups studied

Groups	Animals	Injection at PD 10	Eye Drop at PD 14
Control	16	Phosphate buffer (pH 7.4) injection	Phosphate buffer (pH 7.4)
NACA-only	16	Phosphate buffer (pH 7.4) injection	1% NACA
Na_2SeO_3-only	16	Sodium selenite (19 µmol/kg body wt.) injection	Phosphate buffer (pH 7.4)
Na_2SeO_3 + NACA	16	Sodium selenite (19 µmol/kg body wt.) injection	1% NACA

Week 5
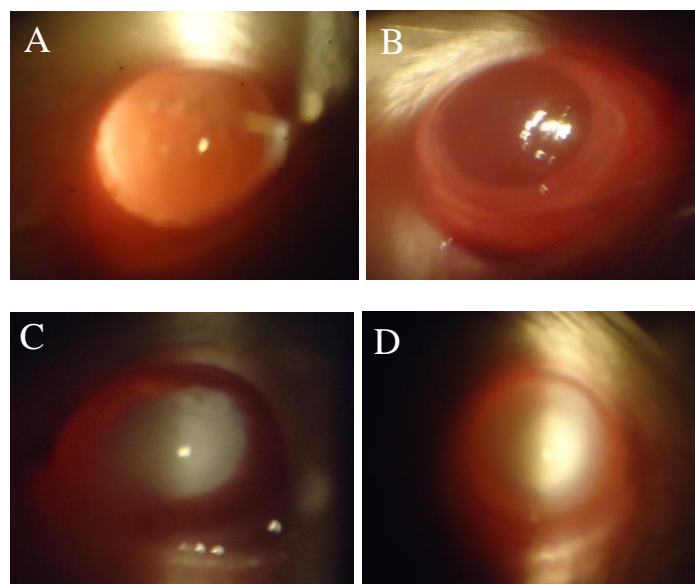
Week 9
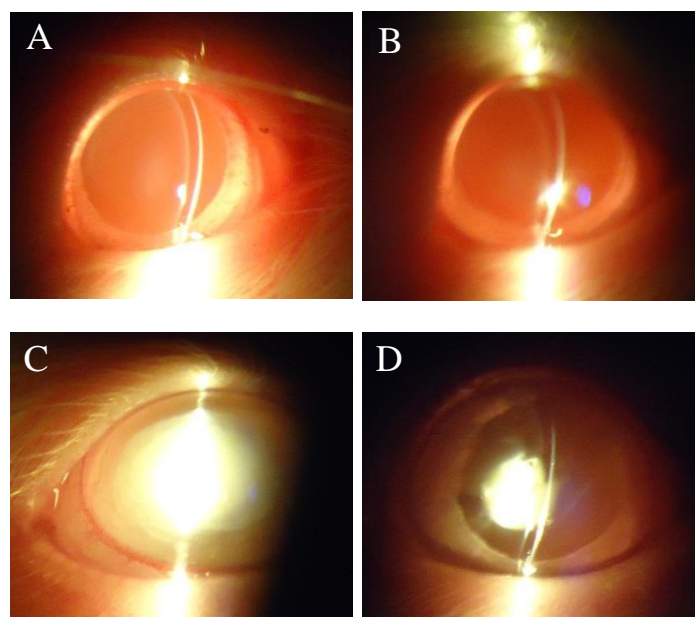
Figure 6.1. Slit Lamp Images of Rat Eyes at Week 5 and Week 9. A: Control group, B: NACA-only group, C: Na_2SeO_3-only group, D: Na_2SeO_3 + NACA. In comparison to the Na_2SeO_3-only group, the eyes of rat pups in the Na_2SeO_3 + NACA did not show any reduction in cataract grade at week 5. Continuation of NACA eye drops until week 9, resulted in 0% grade 3 cataracts, reversing them to grade 2 and grade 1.

Table 6.2. Classification of Degree of Cataract Formation in Rat Pups after NACA Treatment. The summary of the degree of cataracts developed by lenses in each group. The degrees of opacity are defined as follows: grade 0, normal transparent lens; grade 1, initial signs of cataract; grade 2, partial nuclear cataract; grade 3, dense nuclear cataract. 'n' represents the number of animals utilized in each group.

Week 5

Group (n=16)	Grade 0	Grade 1	Grade 2	Grade 3
Control	16	0	0	0
NACA-only	16	0	0	0
Na_2SeO_3-only	0	1	3	12
Na_2SeO_3 + NACA	0	1	4	11

Week 9

Group (n=8)	Grade 0	Grade 1	Grade 2	Grade 3
Control	8	0	0	0
NACA-only	8	0	0	0
Na_2SeO_3-only	0	0	3	5
Na_2SeO_3 + NACA	0	3	5	0

A
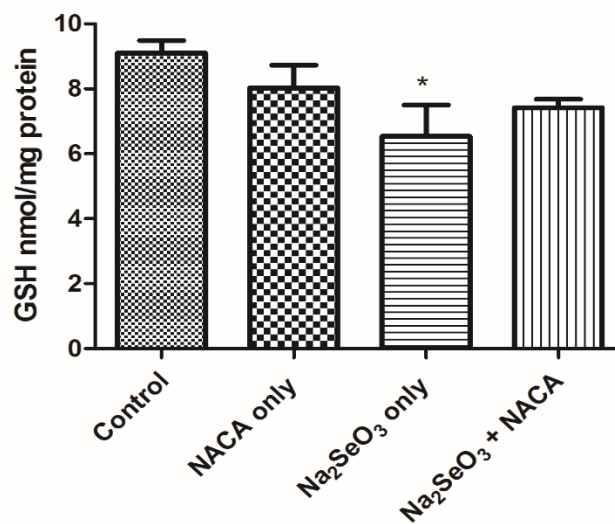
B
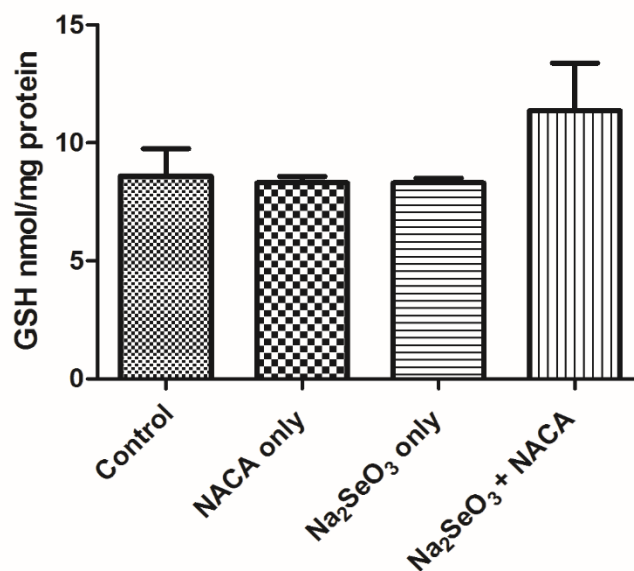
Figure 6.2. GSH Levels in the Lens. Comparison of GSH levels of A: week 5, and B: week 9. At week 5, the GSH levels in the lenses of Na_2SeO_3-only group were significantly lower than the control group lenses. At week 9, GSH levels in the Na_2SeO_3-only group reached a level similar to that in the control group lenses. At week 5, NACA treatment in the Na_2SeO_3 + NACA group did not show any improvement in GSH levels; however, NACA treatment in the Na_2SeO_3 + NACA group lenses, until week 9, showed an increase in the GSH levels compared to those in the remaining groups. '*' refers to significant difference from the control group.

A
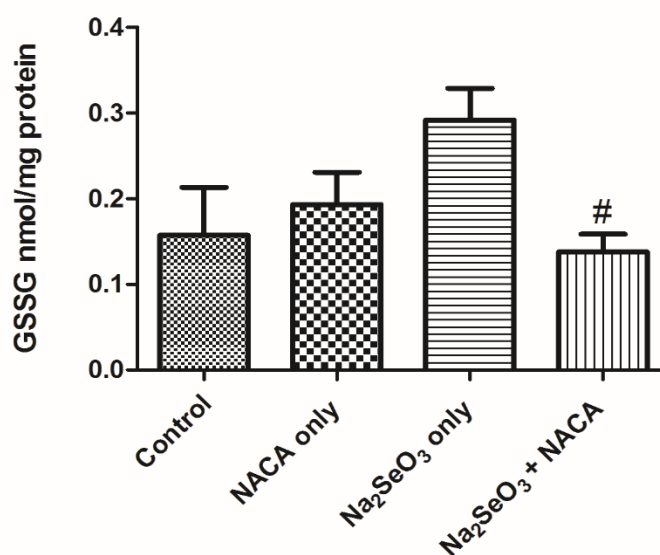
B
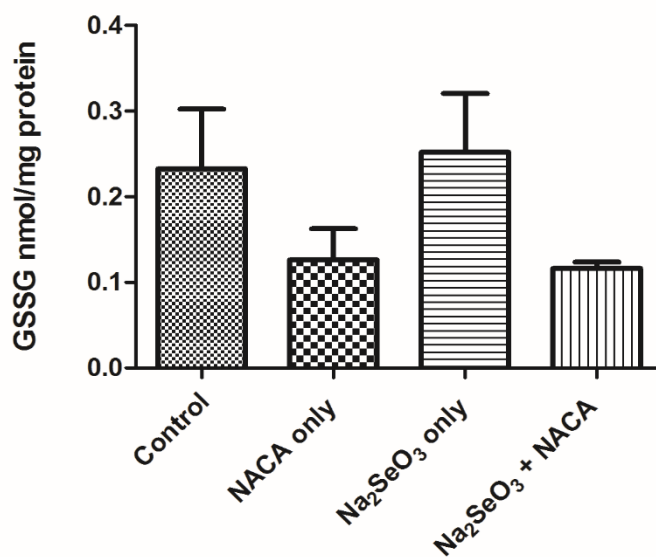
Figure 6.3. GSSG Levels in the Lens. Comparison of GSSG levels of A: week 5, and B: week 9. At week 5, the GSSG levels in the Na_2SeO_3-only group were higher than those of the control group lenses. Whereas, NACA treatment in the lenses of Na_2SeO_3 + NACA group significantly reduced the levels of GSSG when compared to the Na_2SeO_3-only group. At week 9, reductions of GSSG levels were observed in the lenses of the Na_2SeO_3 + NACA group when compared to the Na_2SeO_3-only group. '#' refers to significant difference from the Na_2SeO_3-only group.

A
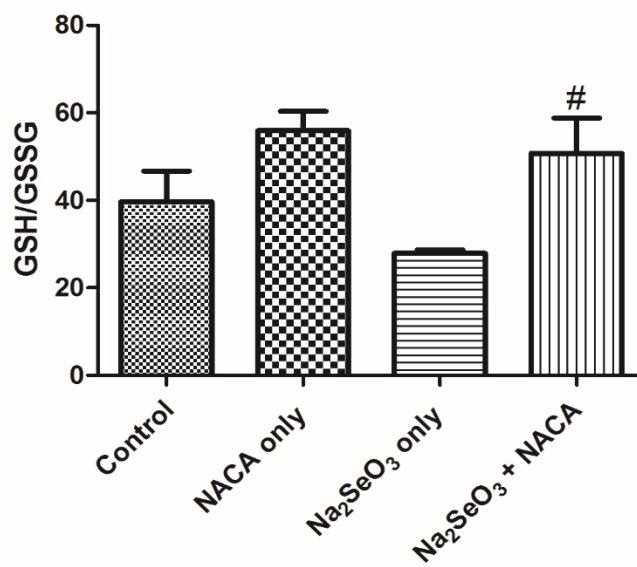
B
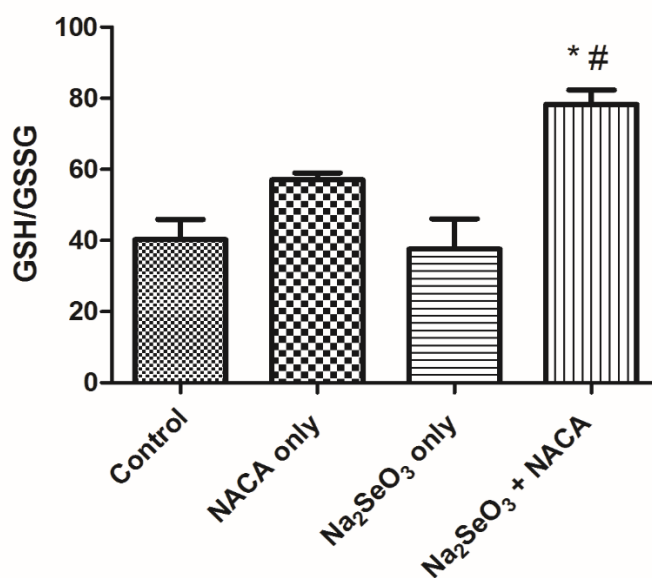
Figure 6.4. GSH/GSSG Ratio in the Lens. Comparison of GSH/GSSG ratios of A: week 5, and B: week 9. At week 5, there was a reduction of the GSH/GSSG ratio in the lenses of the Na_2SeO_3-only group compared to the control group; treatment with NACA increased this ratio significantly in comparison to the Na_2SeO_3-only group. A similar trend was observed also at week 9. '*' refers to significant difference from the control group, '#' refers to significant difference from the Na_2SeO_3-only group.

A
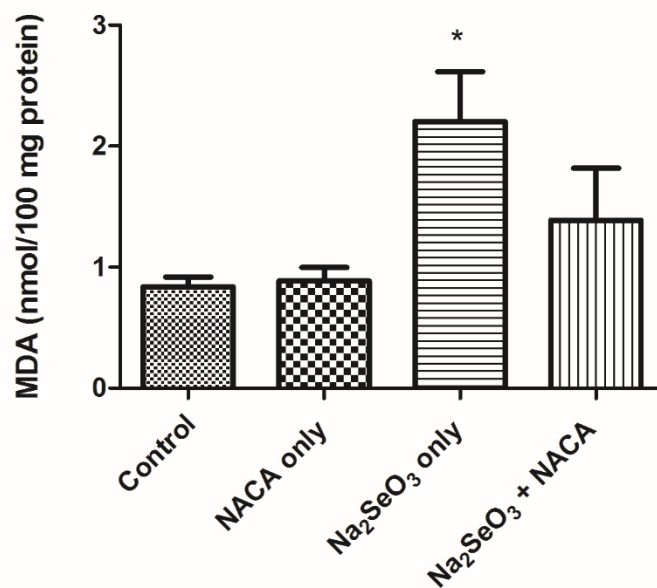
B
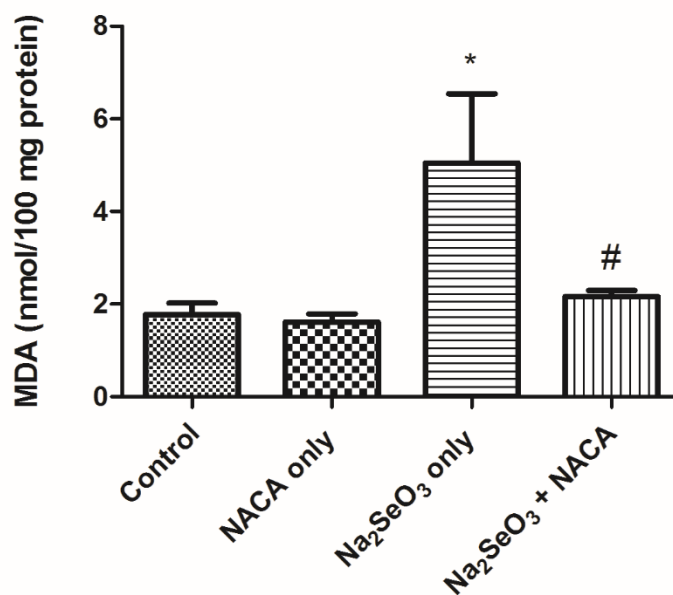
Figure 6.5. MDA Levels in the Lens. Comparison of MDA levels of A: week 5, and B: week 9. At week 5, MDA levels in the lenses of Na_2SeO_3-only group were significantly higher than those of the control group. NACA restored the MDA levels close to the control. The same trend was observed also at week 9, with NACA having significantly reduced the MDA levels when compared to the Na_2SeO_3-only group. '*' refers to significant difference from the control group, '#' refers to significant difference from the Na_2SeO_3-only group.

A
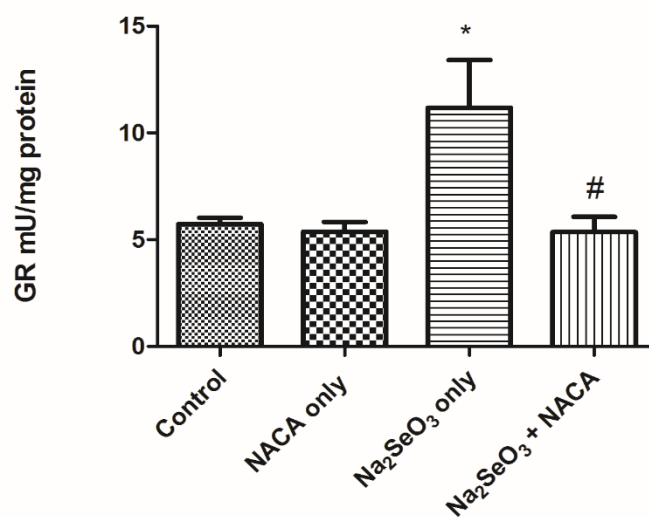
B
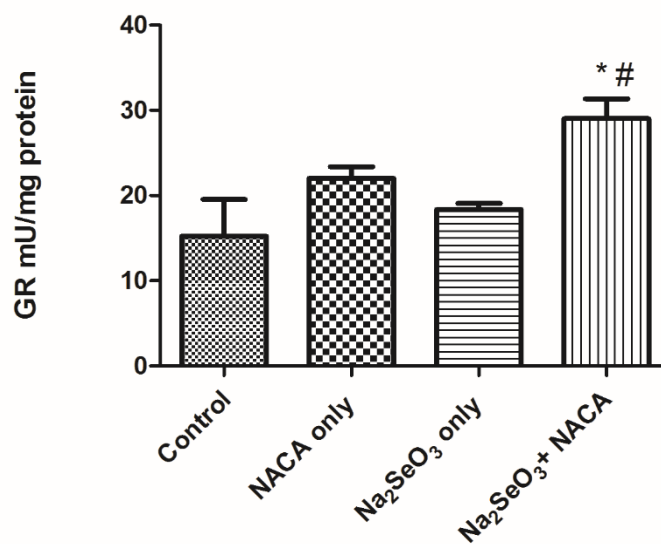
Figure 6.6. GR Activity in the Lens. Comparison of GR activity of A: week 5, and B: week 9. At week 5, GR activity in the Na_2SeO_3-only group lenses was significantly higher than that in the control group lenses. In contrast, NACA treatment significantly reduced the GR activity in comparison to the the Na_2SeO_3-only group. At week 9, the GR activity in the lenses of the Na_2SeO_3-only group was close to the control group; however, NACA treatment significantly increased the GR activity compared to those of the control and the Na_2SeO_3-only groups. '*' refers to significant difference from the control group, '#' refers to significant difference from the Na_2SeO_3-only group.

6.2. EFFECTS OF N-ACETYLCYSTEINE AMIDE ON PREVENTION AND REVERSAL OF SELENITE-INDUCED CATARACTS IN WISTAR RATS

This study was performed to investigate the preventive as well as the reversal effects of NACA on the cataracts induced by a single injection of sodium selenite.

6.2.1. Experimental Design. Lactating female Wistar rats, with 40 male 2-day-old pups, were purchased from the breeding facility at Charles River and were housed in a temperature- (~22 °C) and humidity- (~55%) controlled animal facility, with a 12-h light and dark cycle. The animals had unlimited access to rodent chow and water and were used for experiments after a week of acclimation. All animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and by the Animal Care and Use Protocol Review Committee at the Missouri University of Science and Technology. The rats were divided into four groups: (1) control, (2) NACA-only, (3) Na_2SeO_3-only, and (4) NACA + Na_2SeO_3, such that each group had one lactating female rat with 10 male pups. All rat pups in the NACA and NACA + Na_2SeO_3 groups received an intraperitoneal injection of NACA (250 mg/kg body wt.) once a day on PD 9, 11, and 13 to check the preventive effect of NACA on selenite-induced cataracts, whereas the control and Na_2SeO_3 groups received an intraperitoneal injection of phosphate buffer (pH 7.4) . On PD 10, all rat pups in the Na_2SeO_3-only and the NACA + Na_2SeO_3 groups received an intraperitoneal injection of sodium selenite (19 µmol/kg body wt.), whereas the control and the NACA-only groups received equal volumes of phosphate buffer (pH 7.4). One percent NACA eye drops, prepared in phosphate buffer (pH 7.4), were started from PD 15 (the day that the rat pups opened their eyes) through PD 30 to check for the reversal effect of NACA on the grade of cataracts in the NACA +

Na$_2$SeO$_3$ group (Table 6.3). Grading of the cataract formation was performed with the use of a slit lamp microscope on PD 15, before starting the eye drops, and on the last day (PD 30) before sacrificing the pups. All rats were anesthetized 2-3 h after administration of the last NACA or buffer eye drops, by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (15 mg/kg). All rat pups were massed at the beginning and the end of the study. After sacrifice, their lenses were harvested, and then immediately placed on dry ice. Samples were stored at a temperature of −80 °C for further analysis.

Table 6.3. Classification of Treatment Groups studied

Groups	Injections on PD 9,11,13	Injection on PD 10	Eye Drop from PD10
Control	Buffer	Buffer	Buffer (pH 7.4)
NACA-only	NACA	Buffer	1% NACA
Na$_2$SeO$_3$-only	Buffer	Na$_2$SeO$_3$	Buffer (pH 7.4)
NACA + Na$_2$SeO$_3$	NACA	Na$_2$SeO$_3$	1% NACA

6.2.2. Results. The results indicate the effects of NACA and Na$_2$SeO$_3$ on various oxidative stress parameters.

6.2.2.1. Effects of NACA on cataract intensity in the lens. An injection of 19 μmol/kg body wt. of Na_2SeO_3 administered on postpartum day 10 was significant enough to cause the development of cataracts by the time the rat pups opened their eyes. Upon examination with a slit-lamp microscope, it was found that all rat pups within the Na_2SeO_3 group developed cataracts, with two out of ten (20%) developing grade 3 cataracts and the remaining eight out of ten (80%) developing grade 4 cataracts. In comparison, treatment with NACA intraperitoneal injections decreased the severity of cataract formation: eight rats out of ten (80%) developed grade 3 cataracts, while one rat formed a grade 1 cataract, and one rat did not develop any cataract (grade 0). These results indicated that NACA was successful in preventing cataract formation. By the end of week 4, there was no change in the grades of the cataracts in the Na_2SeO_3-injected rat pups [eight out of ten (80%) rats had grade 4 cataracts and two rats had grade 3 cataracts]. However, treatment with NACA eye drops decreased the severity of cataract formation, with five rats out of the ten (50%) having grade 3 cataracts, two rats had grade 2 cataracts, two rats had grade 1 cataracts, and one rat had no cataract (grade 0). The grading of the lens in all of the groups is tabulated in Table 6.4, and the slit-lamp pictures of representative lenticular opacities observed for each group are shown in Figure 6.7.

6.2.2.2. Effects of NACA on GSH and GSSG levels in the lens. GSH levels in the lenses of the rat pups in the Na_2SeO_3-only group were found to be significantly ($p < 0.05$) lower than those of the lenses in the control and the NACA-only groups. However, treatment with NACA in the NACA + Na_2SeO_3 group (Figure 6.8.A) significantly ($p < 0.05$) increased the GSH levels compared to that of the Na_2SeO_3-only group.

GSSG levels in the lenses of the rat pups in the Na_2SeO_3-only group were found to be significantly ($p < 0.05$) higher than those of the lenses in the control group. However, NACA treatment (NACA + Na_2SeO_3 group) was not able to reduce GSSG levels back to the control level (Figure 6.8.B).

The ratio of GSH and GSSG in the lenses of rat pups in the Na_2SeO_3-only group were found to be significantly ($p < 0.05$) lower than that of the lenses in the control group, whereas, the GSH/GSSG ratio in the NACA treatment group (NACA + Na_2SeO_3) was not significantly different from that of the Na_2SeO_3-only group or the control group lenses (Figure 6.8.C).

Table 6.4. Classification of Degree of Cataract Formation in Rat Pups after NACA administration at Weeks 2 and 4. Dense cataract formation was prevented with NACA injection in NACA + Na_2SeO_3 at the end of week 2 when the rats opened their eyes. Continuation of NACA eye drops until the end of week 4 decreased the number of rats in grade 2 cataract, reversing them to grade 1 cataract.

Groups (n=10)	Grade of Cataracts (week 2)				Grade of Cataracts (week 4)			
	0	1	2	3	0	1	2	3
Control	10	0	0	0	10	0	0	0
NACA-only	10	0	0	0	10	0	0	0
Na_2SeO_3-only	0	0	2	8	0	0	2	8
NACA + Na_2SeO_3 group	1	1	8	0	1	4	5	0

6.2.2.3. Effects of NACA on MDA levels. MDA levels in the lenses of the rat pups in the Na_2SeO_3-only group were found to be significantly ($p < 0.05$) higher than that of the control group lenses. However, NACA treatment in the lenses of the NACA + Na_2SeO_3 group showed a significant decrease ($p < 0.05$), with MDA levels becoming nearly the same as those of the control (Figure 6.9).

6.2.2.4. Effect of NACA on GR and Ttase activities. The GR activity in the lenses of the Na_2SeO_3-only group was found to be significantly ($p < 0.05$) higher than those of the control, the NACA-only, and the NACA + Na_2SeO_3 groups.

The Ttase activity in the lenses of the Na_2SeO_3-only group was found to be significantly ($p < 0.05$) lower than that of the control group lenses. However, Ttase activity in the NACA treatment group (NACA + Na_2SeO_3) was not significantly different from that of the Na_2SeO_3-only group or the control group lenses (Table 6.5).

6.2.2.5. Effects of NACA on water content in the lens. The water content in the lenses of the Na_2SeO_3-only group was found to be significantly ($p < 0.05$) lower than that of the control and the NACA-only groups. However, NACA treatment significantly increased the water content in the lenses of the NACA + Na_2SeO_3 group ($p < 0.05$) compared to that of the Na_2SeO_3-only group (Figure 6.10).

6.2.2.6. Effects of NACA on calcium levels in the lens. Lenses in rat pups that received Na_2SeO_3 alone showed significantly ($p < 0.05$) higher levels of calcium than the lenses in the control and the NACA-only groups. However, treatment with NACA showed a significant ($p < 0.05$) reduction in the levels of calcium compared to that of the Na_2SeO_3-only group, with the levels becoming nearly the same as those of the control (Figure 6.11).

Table 6.5. Activities of Antioxidant Enzymes in Rat Pups. NACA treatment in the NACA + Na_2SeO_3 group significantly decreased the GR activity when compared to the GR activity of Na_2SeO_3 group lenses. Although NACA increased the thioltransferase activity in NACA + Na_2SeO_3 group, it was not significantly high when compared to that of the Na_2SeO_3 group. Statistical analysis was performed by one-way analysis of variance, followed by Tukey's multiple comparison tests. *$p < 0.05$ compared to the control group and #$p < 0.05$ compared to the Na_2SeO_3 group. Values are reported as mean (n=4) ± S.E.

Groups(n=4)	GR	Ttase
Control	0.77 ± 0.16	2.0 ± 0.28
NACA	0.92 ± 0.06	1.8 ± 0.20
Na_2SeO_3	$1.3 \pm 0.23^{*}$	$1.2 \pm 0.19^{*}$
NACA + Na_2SeO_3	$0.88 \pm 0.16^{\#}$	1.8 ± 0.35

6.2.2.7. Effects of NACA on casein zymography. The change in the lens m-calpain activity among the groups was measured by casein zymography. With the evidence of a rise in calcium levels, activation of the calcium dependent protease (m-calpain) was investigated by observing calcium-activated calpain-mediated lysis of casein on the gel. The casein zymogram showed a less intense band of m-calpain in the lenses of the Na_2SeO_3-only group. The intensity of the band in the NACA treatment (NACA + Na_2SeO_3) group was close to that of the control group (Figure 6.12.A).

6.2.2.8. Effects of NACA on the Western blot of m-Calpain. To support our results of casein zymography, Western blot analysis of m-calpain protein was performed in the soluble protein fractions of lenses. A less intense band of m-calpain protein was observed in the lenses of the Na_2SeO_3-only group compared to the control

group lenses. However, the lenses in the NACA treatment (NACA + Na_2SeO_3) group showed a more intense band when compared with the Na_2SeO_3-only group (Figure 6.12.B and 6.12.C).

6.2.2.9. Effects of NACA on SDS-PAGE of lens soluble proteins. SDS-PAGE of soluble lens proteins from the Na_2SeO_3-only group showed the disappearance of a number of bands in the molecular weight region of crystallin proteins (19-31 kDa), whereas in the lenses of the NACA treatment (NACA + Na_2SeO_3) group, crystallin protein bands were preserved (Fig. 6.13). This indicated that Na_2SeO_3 caused the precipitation of crystallin proteins, making them insoluble.
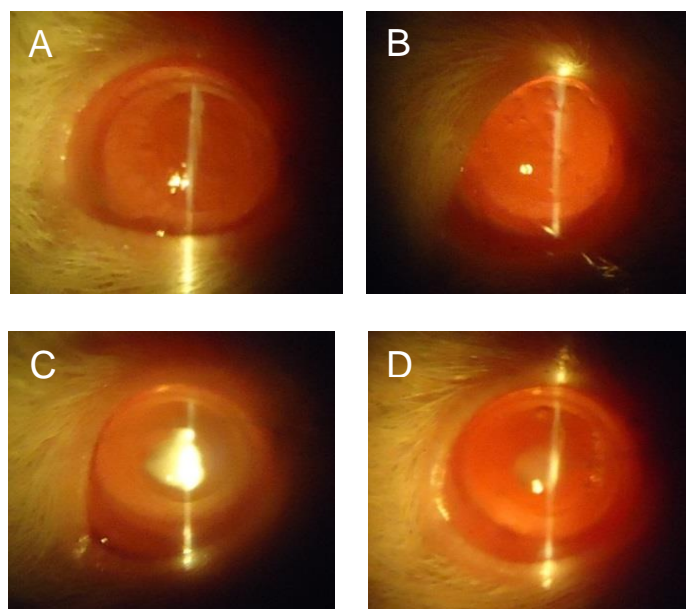
Figure 6.7. Slit Lamp Images of Eyes. These pictures were taken at week 4 (1 day before sacrifice). A representative picture of the lenses observed for each group is shown. A: Lenses from the control group were found to be clear with no detectable cataracts; B: Lenses from the NACA group exhibited similar results to that of the control, with no detectable cataracts; C: Lenses from Na_2SeO_3 group exhibited dense nuclear cataracts (80%); D: Lenses from NACA + Na_2SeO_3 group exhibited a lower grade of cataracts.

A

B

C
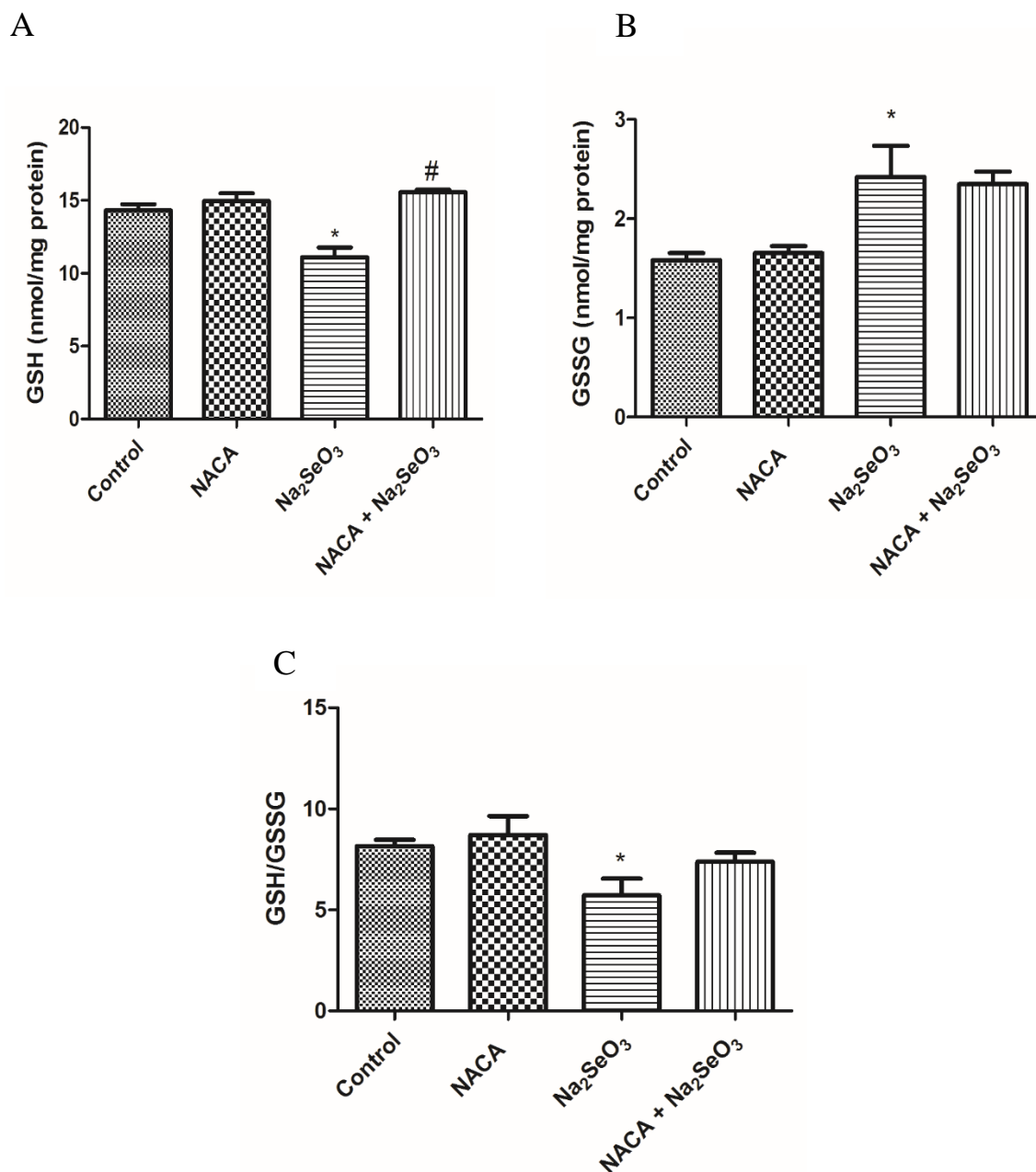
Figure 6.8. Effect of NACA on (A) GSH, (B) GSSG, and (C) GSH/GSSG in Lenses of Rat Pups injected with Na_2SeO_3. A: Treatment with NACA significantly increased the GSH levels when compared with the Na_2SeO_3 group. B: Treatment with NACA didn't show a significant decrease in GSSG levels. C: Treatment with NACA increased the GSH/GSSG ratio, but it was neither significantly different from the control nor the Na_2SeO_3 group lenses. Statistical analysis was performed by one-way analysis of variance, followed by Tukey's multiple comparison tests. *$p < 0.05$ compared to the control group and #$p < 0.05$ compared to the Na_2SeO_3 group. Values are reported as mean (n=4) ± S.E.
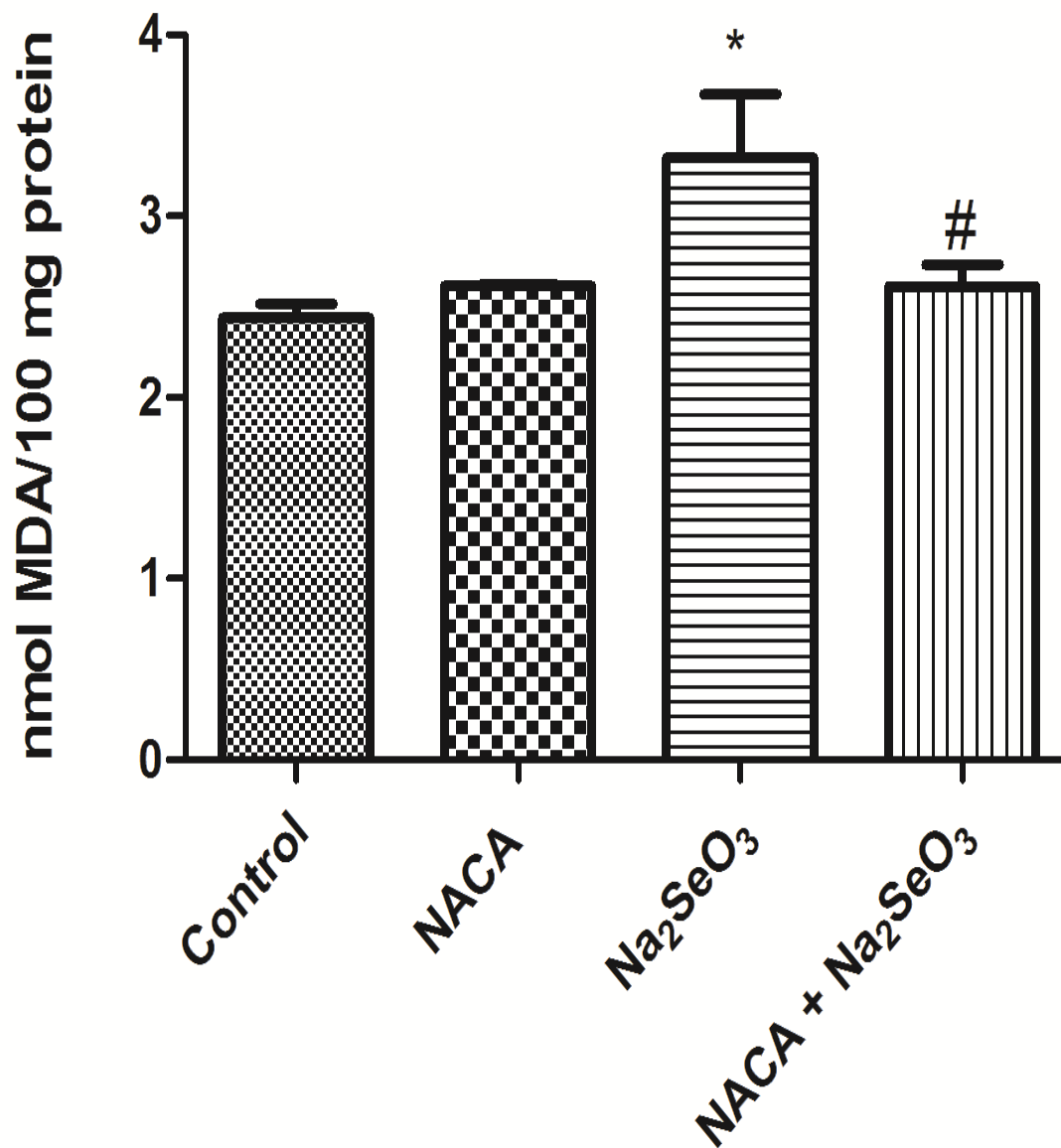
Figure 6.9. Effect of NACA on MDA Levels in Rat Pups injected with Na_2SeO_3. Lenses from sodium selenite injected rat pups showed significantly higher levels of MDA compared to the control, whereas treatment with NACA significantly decreased the MDA levels. Statistical analysis was performed by one-way analysis of variance, followed by Tukey's multiple comparison tests. *p < 0.05 compared to the control group and #p < 0.05 compared to the Na_2SeO_3 group. Values are reported as mean (n=4) ± S.E.
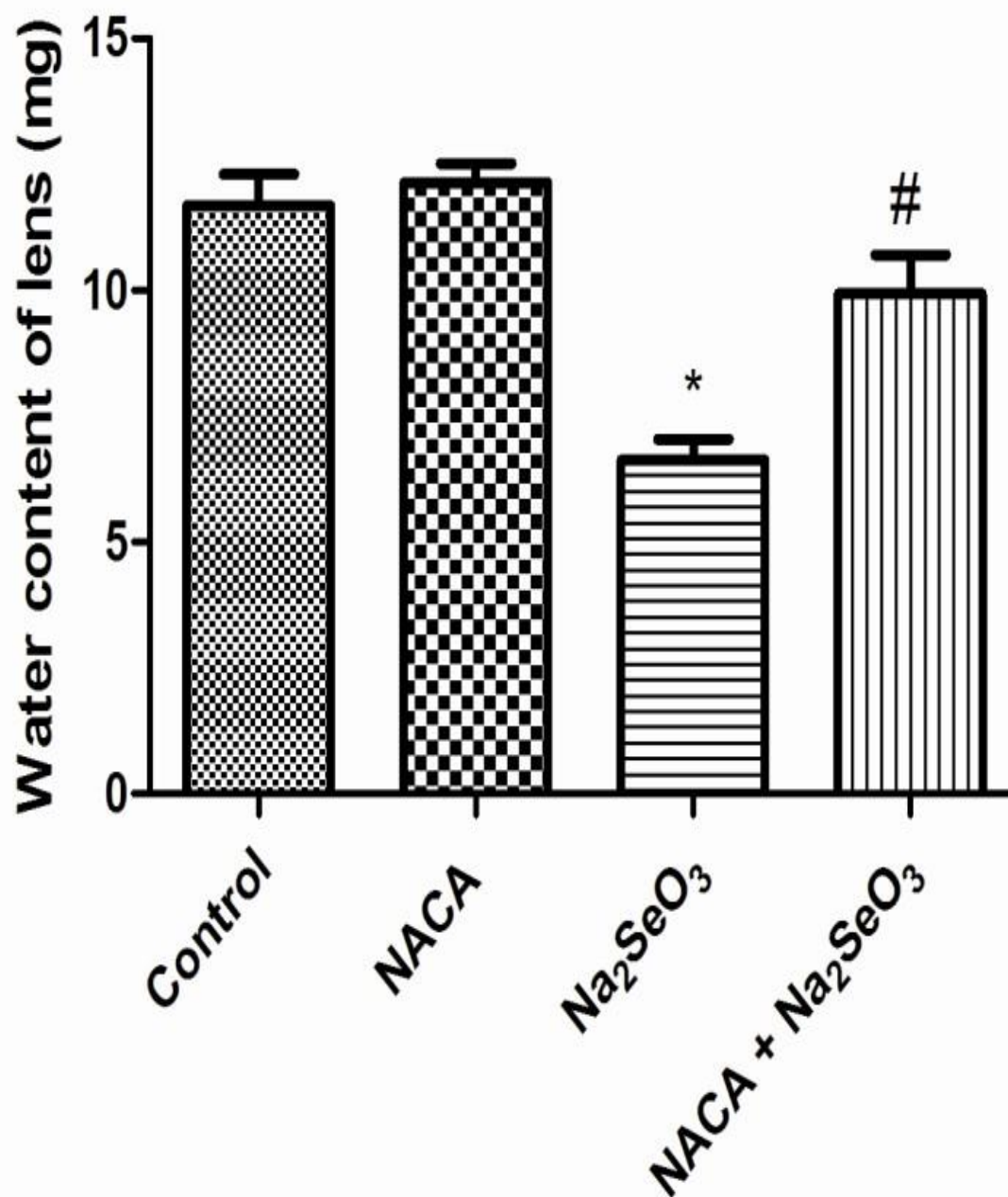
Figure 6.10. Effect of NACA on the Water Content in Rat Pups injected with Na_2SeO_3. Lenses from sodium selenite injected rat pups showed significantly decreased levels of water content compared to that of the control, whereas treatment with NACA significantly increased the water content. *p < 0.05 compared to the control group and #p < 0.05 compared to the Na2SeO3 group. Values are reported as mean (n=4) ± SD.
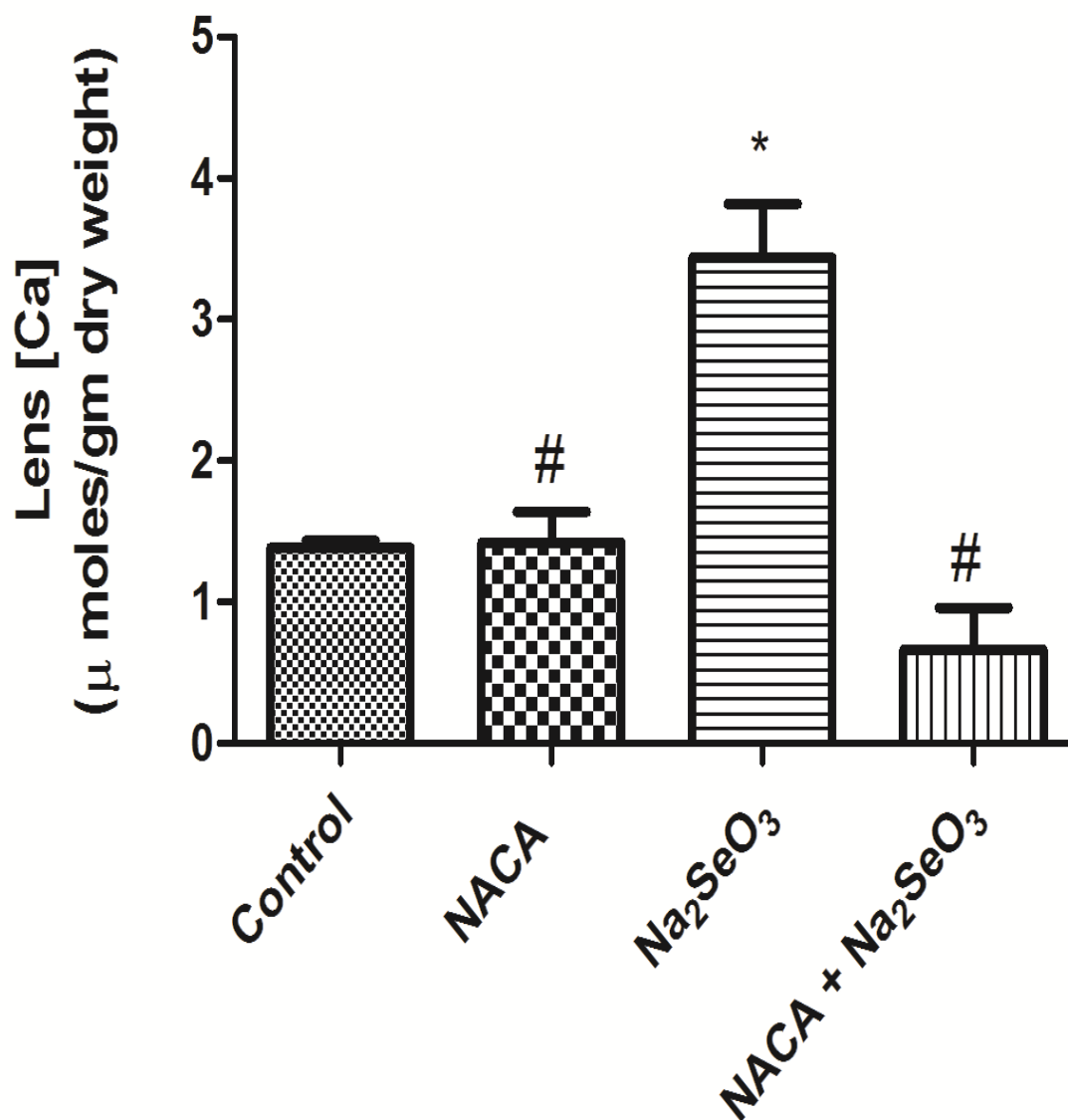
Figure 6.11. Effect of NACA on Calcium Levels in Rat Pups injected with Na_2SeO_3. Lens from sodium selenite injected rat pups showed significantly higher calcium levels compared to that of the control, whereas treatment with NACA significantly decreased the calcium levels. Statistical analysis was performed by one-way analysis of variance, followed by Tukey's multiple comparison tests. *$p < 0.05$ compared to the control group and #$p < 0.05$ compared to the Na_2SeO_3 group. Values are reported as mean (n=4) ± S.E.
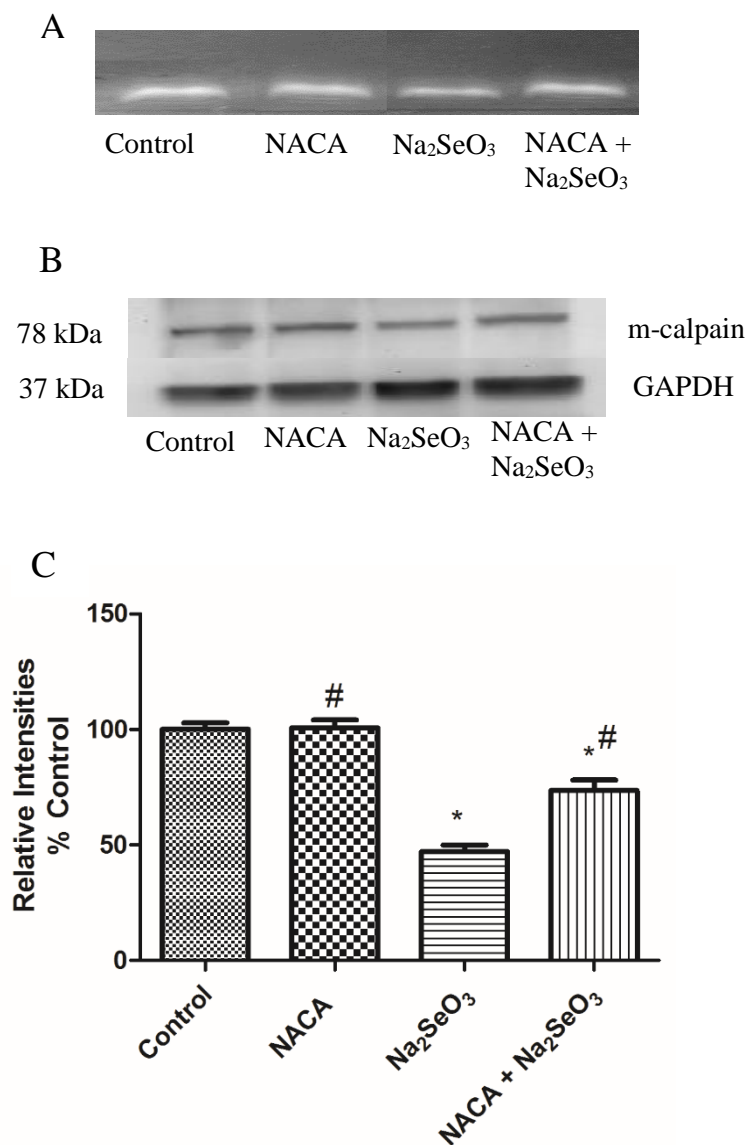
Figure 6.12. Effect of NACA on (A) Casein Zymography, (B) Western Blot, and (C) Relative Densitometry Intensities of m-Calpain Protein in Rat Pups injected with Na_2SeO_3. A: Lens from sodium selenite injected rat pups showed a decreased band intensity of m-calpain indicating decreased m-calpain activity compared to the control. However, treatment with NACA preserved the m-calpain activity; B: Western blot from sodium selenite injected rat pups showed a band intensity of m-calpain compared to control; C: Western blot results normalized to the levels of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein. The graphs represent relative densitometric analysis of treated group lenses over the control group lenses. *$p < 0.05$ compared to the control group and #$p < 0.05$ compared to the Na_2SeO_3 group. The graph is representative of triplicate gels and the values are reported as mean ± S.E.
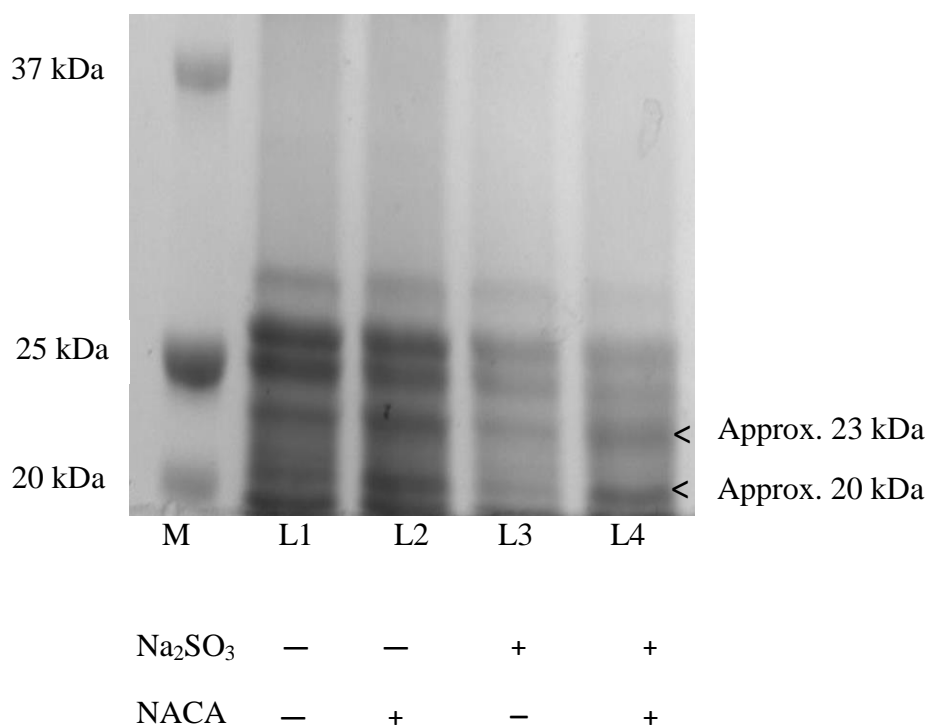
Figure 6.13. Effects of NACA on SDS-PAGE of Crystallin Proteins. L1 represents control, L2 represents NACA, L3 represents Na_2SeO_3, and L4 represents NACA + Na_2SeO_3 groups. Lens from sodium selenite injected rat pups showed decreased crystallin band intensities compared to the control, whereas treatment with NACA preserved the crystallin band intensities.

6.3. DISCUSSION

Cataract blindness is a major cause of preventive blindness worldwide, especially in the developing countries of Africa and Asia [261]. The only effective treatment currently available for cataracts is the surgical removal of the opacified lens and replacement with an artificial lens to restore sight. Although cataract surgery is considered to be a very successful procedure in terms of visual outcome; the cost of surgery, need for trained personnel and surgeons, and postsurgical complications, limit the worldwide availability and accessibility to this procedure. Hence, alternative

prevention and treatment procedures are worthy of investigation [262].

Oxidative stress is an imbalance between the rate of oxidant production and its degradation [263]. Substantial supporting evidence suggested that ROS and oxidative damage were involved in the development of cataracts [264, 265]. Cataract formation has multifactorial etiology. Free radical-induced oxidative stress, resulting in the depletion of antioxidant defense systems in the lens, is considered to be a major factor in the formation of cataracts. The lens depends on a balanced redox state for maintaining its transparency, and a high content of GSH in the lens is believed to play a key role in achieving this [133, 183, 266], whereas decreased levels of GSH in the lens leads to free radical accumulation, resulting in lipid peroxidation and decreased antioxidant enzyme activity [193, 194], which all lead to cataract development.

The sodium selenite cataract model has received a lot of attention as it provides a number of general similarities to human cataracts, such as reduced GSH, increased calcium, increased lipid peroxidation, decreased water soluble proteins, protein insolubilization, precipitation, and proteolysis. Hence, a selenite cataract model is considered to be a good model for initial drug trials [247]. Based on this, a number of anticataractogenic agents have been tested against sodium selenite-induced cataracts. With this background, the present study was focused on investigating the preventive and reversal effects of NACA on selenite-induced cataracts.

Wistar rat pups were injected with sodium selenite on day 10 and the reversal effect of NACA on selenite-induced cataract was studied by administering 1% NACA eye drops starting from postpartum day 15 until the end of the 9th week. Morphological examination of rat lenses at week 5 in the Na_2SeO_3 + NACA group showed no reduction

in cataract intensity, when compared to that in the Na_2SeO_3-only group. But continuation of 1% NACA eye drops, until the end of week 9, resulted in a reduction of cataract intensity with all of the grade 3 cataracts in the Na_2SeO_3 + NACA group having reverted to grade 2 (Table 6.2).

A comparative observation of GSH levels, GSSG levels, and the GSH/GSSG ratio in the Na_2SeO_3-only group at week 5 and week 9 showed no significant difference from the control group lenses, even in the presence of dense nuclear cataracts. This could be because of the regeneration of GSH in the Na_2SeO_3-only group (Figure 6.2). This can be explained by comparing the GR activities between the control group and the Na_2SeO_3-only group lenses. GR is an enzyme that catalyzes the conversion of GSSG back to GSH. A significant increase in GR activity was seen in the Na_2SeO_3-only group with relative to the control group, indicating the activation of lens defense systems against the oxidative stress induced by selenite (Figure 6.6). This could be the reason for increased GSH levels in the Na_2SeO_3-only group. These results are in agreement with previous studies [184, 267, 268]. According to Wang et al., "the concentration of total lens GSH dropped to approximately 25-30% of normal within 12-24 h post-sodium selenite injection, and GSH levels recovered completely by 20-25 day post selenite injection, despite the fact that a single cataractogenic dose of selenite appeared to be cleared from the lens within 1 week". This indicates that a selenite cataract model is not a good model for studies that requires longer durations of testing. Interestingly, NACA treatment in the NACA + Na_2SeO_3 group showed a significant increase in the GSH/GSSG ratio at both week 5 and week 9, when compared to the Na_2SeO_3-only group (Figure 6.4). Furthermore, NACA treatment also showed a significant reduction in the GSSG levels at week 5; however, at

week 9, no significant reduction was observed with regard to GSSG levels in comparison to that of the Na_2SeO_3-only group (Figure 6.3).

Lipid peroxidation is a very important oxidative stress parameter in selenite-induced cataracts [203, 204, 243]. Lenses from the Na_2SeO_3-only group showed significantly increased levels of MDA at both week 5 and week 9, in comparison to those of the control group indicating that, although the GSH levels in the Na_2SeO_3-only group recovered to the control levels, the lipid peroxidation caused by selenite might be an important factor contributing to the opacities observed in the Na_2SeO_3-only group. However, NACA treatment in the NACA + Na_2SeO_3 group have restored the MDA levels back to control. The increase in the GSH/GSSG ratio and a decrease in MDA levels with NACA treatment in the NACA + Na_2SeO_3 group support the decreased cataract intensities in the NACA + Na_2SeO_3 group (Figure 6.5).

Based on the above results, the experimental design was modified by shortening the project to one month. This was done by administering NACA (250 mg/kg body wt.) intraperitoneal injections on postpartum days 9, 11, and 13. Selenite (19 µmol/kg body wt.) was injected intraperitoneally on postpartum day 10, while NACA eye drops were administered starting postpartum day 15 (rat pups opened their eyes on day 15) until postpartum day 30. This study was performed to investigate the preventive as well as reversal role of NACA on selenite-induced cataracts. Rat lenses from each group were examined and photographed on postpartum day 15 when they opened their eyes, and also on day 30 before sacrificing the rats to report the cataract intensities. Results from morphological observation indicated that NACA was able to prevent, as well as reverse the density of cataracts in the NACA + Na_2SeO_3 group (Table 6.4).

As discussed earlier, GSH is the most important antioxidant present in the lens; it is the first line of defense against oxidative stress [5]. Results from this study showed a significant decrease in GSH levels in the lenses of the Na_2SeO_3-only group (Figure 6.8.A) when compared to those of the control group, indicating oxidative stress. In addition to a decrease in GSH, increase in intracellular GSSG (Figure 6.8.B) levels were observed in the lenses of the Na_2SeO_3-only group. Furthermore, the ratio of GSH/GSSG, which serves as a representative marker of the antioxidative capacity of a cell, decreased in the lenses of the Na_2SeO_3-only. Treatment with NACA significantly increased the GSH levels in the NACA treated group. This indicated that NACA had replenished the GSH levels and prevented oxidative stress. The protective effects of NACA are probably mediated by its ability to supply cysteine for GSH biosynthesis, in addition to reducing extracellular cystine to cysteine [191], and by conversion of GSSG to GSH by non-enzymatic thiol disulfide exchange [24]. These results are in agreement with previous studies in animal models which reported a decrease in GSH levels upon Na_2SeO_3 treatment [184, 250, 255, 269-272].

However, significant improvement in the GSH/GSSG ratio was not observed in the NACA treated sodium selenite group (NACA + Na_2SeO_3) (Figure 6.8.C). Furthermore, changes in the levels of GSH and GSSG were seen to affect the activity of the GR. This enzyme plays an important role in GSH homeostasis: it regenerates GSH from GSSG. Increased activity of GR in the lenses of the Na_2SeO_3-only group could be attributed to the activation of the lens antioxidant defense network against a change in the redox status. However, this increase in activity of GR was not sufficient to convert all of the GSSG to GSH and resulted in high levels of GSSG leading to a lower GSH/GSSG

ratio. Furthermore, NACA treatment increased the levels of GSH and restored GR activity (Table 6.5).

Lipid peroxidation has been associated with cataract formation [203, 204, 243]. The double bonds in fatty acids undergo lipid peroxidation in the presence of free radicals and form stable by-products, such as malondialdehyde (MDA), which are used as markers of lipid peroxidation. Lenses from the Na_2SeO_3-only group had increased levels of MDA (Figure 6.9), compared to those of the control group lenses, indicating increased lipid peroxidation in the Na_2SeO_3-only group. These results were in line with other studies which have reported increased lipid peroxidation upon treatment with Na_2SeO_3 [23, 249, 250, 255, 258, 269, 270]. NACA was able to reduce the lipid peroxidation induced by Na_2SeO_3, by providing adequate amounts of GSH, a substrate for GPx, an enzyme that catalyzes the conversion of hydrogen peroxide to water.

A key GSH-dependent enzyme, Ttase, catalyzes the conversion of PSSG back to PSH, by using GSH as one of the substrates. During cataract formation, oxidative stress builds up, due to diminished levels of GSH, and leads to protein-thiol mixed disulfide formation. Crystallin-thiol mixed disulfide then precipitates within the lens causing cataracts [244]. We speculate that this may be due to decreased Ttase activity as a consequence of lowered GSH levels during cataract development. Depletion of Ttase activity in cataract formation has been previously reported [168, 245, 246]. Rat pups injected with Na_2SeO_3 showed decreased Ttase enzyme activity, as compared to control, leading to the precipitation of crystallin proteins, whereas treatment with NACA restored the Ttase enzyme activity close to that of the control (Table 6.5).

A wide body of evidence indicates the association of cataracts with lens hardness [273, 274]. According to Tabandeh et al, lens hardness might be because of the dehydration of the lens resulting in increased accumulation of water insoluble proteins at the center of the nucleus, leading to compaction of lens fibers [274]. The result from this study is in agreement with the above findings. Lenses from the Na_2SeO_3-only group indicated a drop in water content and NACA treatment reversed water levels close to those of the control (Figure 6.10). Reduced water content may probably be associated with changes in the interaction between protein chains that cause in-solubilization and precipitation of proteins, leading to lens hardness.

To estimate proteolysis of lens crystallin proteins, SDS-PAGE of the water soluble protein fraction was performed. Previous studies have indicated that crystallin proteins lie in a molecular weight range of 19-31 kDa, with α-crystallin at approximately 19-20 kDa, β-crystallin at 23-31 kDa, and γ-crystallin at 21-23 kDa [184]. Intensities of the crystallin bands in lenses of the Na_2SeO_3-only group were significantly lower compared to those of the control group, whereas treatment of selenite-challenged rats with NACA resulted in considerable preservation of the intensity of the crystallin bands, with molecular sizes of around 20 kDa and 23-25 kDa (Figure 6.13). This suggests an inhibition of crystallin proteolysis by NACA and, hence, prevention of precipitation.

Precipitation of proteins can also result from calcium uptake in the lens. High concentrations of calcium in the lens nucleus of selenite cataracts have been previously reported by Hightower et al [275], which may have been due to inhibition of Ca-ATPase activity [276]. Accumulation of calcium in the nucleus of the lens leads to m-calpain activation, proteolysis, in-solubilization, and precipitation of crystallins. Lenses from the

Na_2SeO_3-only group showed a 2.5 fold increase in calcium levels when compared to the levels in the control. These results are in agreement with other studies which previously reported elevated calcium levels in the lenses of selenite-induced cataracts [169, 184, 275, 277]. Treatment of selenite-challenged rats with NACA (NACA + Na_2SeO_3 group) prevented such an increase, and maintained the lens calcium levels close to those of control (Figure 6.11).

Calpains are cysteine proteinases whose activity depends on calcium concentrations. Based on the calcium levels required to activate these calpains, two predominant forms of calpains have been reported, μ-calpain (micromolar concentrations of calcium) and m-calpain (millimolar concentration of calcium) [149]. Increased calcium levels lead to dissociation of m-calpain subunits, resulting in activation of m-calpain that hydrolyzes the lens substrates such as crystallins leading to cataractogenesis. However, activation of m-calpain is followed by degradative autolysis of m-calpains [253]. In the present study, casein zymography was performed to check m-calpain activation. Contrary to the expectations based on increased calcium levels, lenses from the Na_2SeO_3-only group showed decreased m-calpain activity with a less intense band (Figure 6.12.A). These results could be attributed to the degradative autolysis of m-calpain subsequent to their activation. These results were further supported by performing Western blots of m-calpain. A similar trend with decreased m-calpain levels were observed in the Na_2SeO_3-only group (Figure 6.12.B and 6.12.C). Decreased m-calpain activity and levels can thus be attributed to degradative autolysis following activation of m-calpain [169, 275, 278, 279].

In summary, the data from the selenite study indicated that oxidative stress plays a role in cataract formation and, particularly in the GSH system and, therefore, the use of a GSH prodrug might prove beneficial in preventing and reversing cataract formation. The data supported our hypothesis that NACA protects by increasing GSH, reducing MDA levels, and restoring enzyme activities together with the calcium levels. Our present and future studies may eventually help ophthalmologists prevent cataract formation in high-risk populations and non-surgically treat early stage cataracts, producing favorable patient outcomes while decreasing medical costs.

7. CONCLUSIONS

With establishment of oxidative stress as one of the primary reasons for cataract development, the research has shifted towards finding a non-invasive method to alleviate oxidative stress that results in cataracts. One logical approach would be through the administration of therapeutic doses of antioxidants. In recent years, a significant number of studies have reported the positive effects of antioxidants in treating cataracts. NACA, is a GSH prodrug and supplies cysteine for *de novo* synthesis of GSH, in addition it is an excellent source of thiol groups and is able to convert GSSG back to GSH by non-enzymatic thiol disulfide exchange. Furthermore, NACA also supplies cysteine from cystine, aiding in intracellular GSH biosynthesis. NACA promotes intracellular detoxification by scavenging free radicals and chelating copper ions. The purpose of these experiments was to investigate the potential anticataractogenic effects of NACA in both *in vivo* and *ex-vivo* models. The results described here suggest that NACA provides a protective effect against cataracts.

In the *ex vivo* study, Dex was used to induce cataracts and the protective effect of NACA was demonstrated against cataracts induced by Dex. Lenses grown in Dex medium showed posterior cataracts, whereas the lenses in the NACA treatment (NACA + Dex) group showed a decreased intensity of posterior cataracts. The results from oxidative stress parameters indicated that NACA was able to reduce the extent of oxidative damage by returning the levels back to control. More studies on Dex-induced cataracts are needed to determine whether NACA can treat Dex-induced cataracts.

An APAP-induced cataract model was used to demonstrate the protective role of NACA in mice. The morphological examination and the results from oxidative stress parameters of lenses from each group indicated that NACA was able to prevent the formation of dense nuclear cataracts. However, an APAP model was not a direct cataract model since it required an inducer such as β-naphthoflavone to activate CYP, which is a pre-requisite for cataract development. Therefore, we chose to study the therapeutic potential of NACA further in a more appropriate animal model.

Sodium selenite-induced cataract model is a reproducible model with many similarities to human cataracts and is considered as one of the most favored models to perform early drug trials. However, the main drawback with this model is GSH regeneration within 25 days post selenite injection, which limits this model to be applicable only for studies that require fewer than 30 days of drug trial. The prevention and reversal effects of NACA on sodium selenite-induced cataracts can be attributed to its ability to increase the levels of GSH and the GSH/GSSG ratio, and decrease the levels of MDA and calcium, while restoring enzyme activities.

The data suggest that NACA has the potential to significantly improve patient health and the clinical care of cataracts. Preventing, as well as reversing cataract formation, would mean that, in many cases, surgery could be avoided completely. Further development of pharmacological agents like NACA may eventually help ophthalmologists prevent cataract formation in high-risk populations and non-surgically treat early stage cataracts while shifting the emphasis towards pharmacological rather than more expensive surgical intervention, producing favorable patient outcomes while decreasing medical costs.

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