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THERAPEUTIC EFFECTS OF N-ACETYLCYSTEINE AMIDE IN THE TREATMENT OF EYE DISORDERS

by

JOSHUA WARREN CAREY

A DISSERTATION

Presented to the Faculty of the Graduate School of the

MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

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ABSTRACT

The loss of vision from age-related eye diseases now affects over 30 million people in the United States and is expected to reach twice that number within the next couple of decades. Among these disorders, age-related macular degeneration and cataract formation represent the largest percentage of these cases. Therapeutic options available for treating age-related macular degeneration are limited and there is no noninvasive form of treatment for cataracts current available. Several studies have indicated that oxidative stress plays an important role in the pathology of these age-related eye disorders. If oxidative stress is responsible for the initiation and progression of these disorders, then antioxidants may play a key role in reducing the severity, halting the progression and, perhaps, even eliminating these disorders. Recent research has shown that an antioxidant called N-acetylcysteine amide (NACA) not only acts as an antioxidant itself but also stimulates the production of glutathione thereby increasing resistance to oxidative stress. To evaluate NACA's ability to provide therapeutic benefits in eye-related disorders, two different experimental models were utilized. The first was an *in vitro* model which examined the oxidative stress and membrane integrity of adult retinal pigmented epithelial ARPE-19 cells in the presence of methamphetamine. The second was an *in vivo* model which examined the cataract generating potential upon the depletion of glutathione levels within the lens. Treatment with N-acetylcystiene amide was applied to each model to determine any beneficial effects during oxidative stress. Overall, the results from this study indicate that NACA treatment represents an effective and alternative treatment to costly cataract surgery and can potentially reduce the health care costs related to age-related eye diseases and greatly improve the quality of life of people affected by these diseases.

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1. INTRODUCTION

Globally, the International Agency for the Prevention of Blindness estimates that over 285 million people suffer from significant visual impairment, including 45 million who are blind. The number of people experiencing a loss in vision is expected to reach twice that number within the next couple of decades. In the United States, it has been estimated that 30 million citizens suffer from significant vision loss, 5 million of which live with severe visual impairment [1]. Visual impairment in the United States is largely due to the effect of age-related eye diseases (AREDs), specifically macular degeneration and cataractogenesis which account for over 70% of the cases involved with the loss of vision [2]. The National Eye Institute estimates that 9 million people in the U.S. currently experience the effects of age-related macular degeneration (nearly 8 million of which suffer from the dry form) and that nearly 20 million citizens have had a cataract develop within at least one eye [3,4].

The increasing number of people affected by these eye-related disorders emphasizes the need for developing therapeutic treatment options. Currently, the therapeutic options available for eye-related diseases are relatively limited. For the wet form of macular degeneration, the only two major pharmaceuticals currently available for treatment are Lucentis and Visudyne. Unfortunately, no pharmaceutical or surgical treatment is currently available for the dry form of macular degeneration (which is the more prevalent of these two types of ARMD). In the case of cataracts, although surgery is an accepted treatment, this option is invasive and generates a significant share of medical costs. In fact, over 3 million procedures are performed within the United States each year at an estimated total cost of nearly $10 billion. The benefits of preventing or delaying the onset of cataracts would allow those who are initially developing cataracts an alternative option for managing their sight and forgo the need and costs of surgery.

Both the lens and retinal tissues are subjected to significant amounts of oxidative stress requiring elevated levels of cellular antioxidants to neutralize photochemically-generated reactive oxygen species (ROS). In the lens, glutathione (GSH) is the primary cellular antioxidant which plays a critical role in protecting the lens from oxidative damage. Studies have shown that the lens contains approximately twice the amount of GSH that is found within any other tissue sample, an abundance that indicates that high antioxidant activity is required in a healthy lens [5]. During development, the lens has been determined to contain a relatively high concentration of antioxidant enzymes as well as GSH. However, upon aging, the lens ability to synthesize GSH appears to gradually diminish and the levels of GSH fall as a function of time. This decline in antioxidant activity within the lens leads to increased incidences of oxidative damage, which eventually results in the formation of cataracts.

The macula, the site in the retina on which light is focused, has a high metabolic activity and contains high levels of polyunsaturated fats [6]. Several studies have shown that oxidative stress significantly contributes to the degeneration of the macula [7-13]. Oxidative stress levels tend to be high due to the amount of ultraviolet radiation to which the retina is exposed. Levels of antioxidants in retinal tissues decrease with aging, shifting the balance and making the macula vulnerable to progressive oxidative damage. As the retinal pigmented epithelial cells of the macula age, oxidation of lipids and other cellular components results in an accumulation of indigestible lipid photochemical components leading to the formation of drusen deposits over time. In the case of cataractogenesis, oxidative damage results in the cross-linking of crystallin proteins, which are responsible for maintaining transparency of the lens. Since crystallins comprise over 90% of the lens tissue and remain for long periods of time within the cell, accumulation of oxidative damage over time leads to the

"polymerization" of these proteins. Increasing crosslinking and aggregation of these proteins leads to insolubility and fragmentation, ultimately resulting in cataract formation. The important role of ROS and the resulting oxidative damage in the development of various types of cataracts have been established by a number of studies [14-16].

The loss of antioxidants, such as GSH that are critical to eye tissue protection against oxidative stress, cannot be directly replaced. Instead, compounds are required that can easily pass into cells and cause increases in intracellular GSH levels. The clinical research experience with non-pharmacologic antioxidant regimens (e.g., zinc; vitamins A, C, and E; lutein; cryptoxanthins) has been decidedly mixed. It is probable that this approach provides only a small advantage in the prevention and treatment of AREDs. Therefore, a logical approach to enhancing antioxidant protection would be to use pharmacologic doses of highly-active antioxidants that can be delivered to the sites of damage by ROS. Use of a potent antioxidant represents a method for preventing or delaying the onset of AREDs in high risk populations and for slowing or reversing the progression of these diseases in patient. The development of a therapeutic eye drop containing N-acetyl cysteine (NAC) was recently abandoned by Othera Pharmaceuticals following completion of a phase II study. While preliminary results showed a trend toward preservation of visual acuity, the drug failed to significantly improve vision in dry macular degeneration. Although recently published studies have indicated that antioxidants like N-acetyl carnosine and NAC may ameliorate the risk for cataracts, these compounds cannot efficiently penetrate cells, thereby requiring fairly high doses which increase the incidence of undesirable side effects. Several other types of eye drops have been studied for counteracting oxidative stress, but most of these agents had significant drawbacks for clinical use, such as a short half-life, adverse side-effects, thermostability, or poor bioavailability.

A potential candidate, that possesses far more desirable characteristics for development as an ophthalmologic agent to address oxidative stress damage, is the low molecular weight thiol antioxidant, N-acetylcysteine amide (NACA). NACA has been found to possibly possess the characteristics required to restore the redox balance within the brain. It's characteristics as a drug have been shown to be superior to those of N-acetylcysteine (NAC), a FDA-approved drug, by replacing the carboxylic functional group of NAC with an amide functional group. This modification allows NACA to be more lipophilic and enhances its ability to penetrate cellular membranes.

The development of a useful antioxidant eye drop would significantly improve patient health and the clinical care of AREDs. Slowing the onset of cataract formation or arresting the progression of the disease in its early stages, would mean that surgery could be completely avoided in many cases. For dry macular degeneration, this would represent a first-class treatment for over 8 million Americans who currently have no useful therapy available. Finally, a successful outcome to this research would provide additional momentum for further study of antioxidant-based approaches to treating degenerative eye conditions. For example, in the case of cataract care, treatment broadly targets immune or inflammatory pathways, and emphasis would be shifted toward pharmacology rather than surgical intervention, which is better for patients. Our present and future studies may eventually help ophthalmologists to both prevent cataract formation in high-risk populations and to non-surgically treat early stage cataracts.

2. REVIEW OF LITERATURE

2.1. EYE RELATED DISORDERS

2.1.1 Macular Degeneration. Macular degeneration is a type of retinal degenerative eye-related disorder which is the leading cause of blindness and visual impairment of individuals 50 years and older [17]. This particular disorder is characterized by the formation of deposits of a whitish-yellow substance called drusen between the retinal pigmented epithelial cells and blood vessels [18]. This accumulation of drusen leads to a distortion in the shape of the retina causing visual impairment. Macular degeneration is further classified into dry and wet forms in order of increasing severity.

The dry form of macular degeneration is generally identified by the initial development of the symptom of blurred vision. This visual impairment occurs once there is a significant change in the topography of the retina due to the excessive deposits of drusen. This accumulation of drusen prevents an effective exchange of nutrients and waste with the blood and can eventually lead to an atrophy of the retinal pigmented epithelial cells and a decrease in the number of photoreceptors located in the macula [18]. No medical or surgical treatment is currently available for this condition; however, vitamin supplements with high doses of antioxidants have been suggested by the National Eye Institute to slow the progression of dry macular degeneration and, in some patients, improve visual acuity [19].

Wet macular degeneration, also known as neovascular macular degeneration, progresses from the dry form when abnormal blood vessels begin to develop within the layer of retinal epithelial cells. This causes hemorrhagic fluid to build up and places a strain between the retina and choroid. Eventually, this strain leads to detachment of the retinal pigmented epithelial cells from the choroid, which then further disrupts the arrangement of

photoreceptors leading to a visual impairment known as metamorphosia. This particular impairment in vision is the first sign of symptoms used in diagnosing wet macular degeneration. If left untreated at this stage, patients will become blind within 1-3 years. The differences in wet and dry macular degeneration are depicted in Figure 2.1.

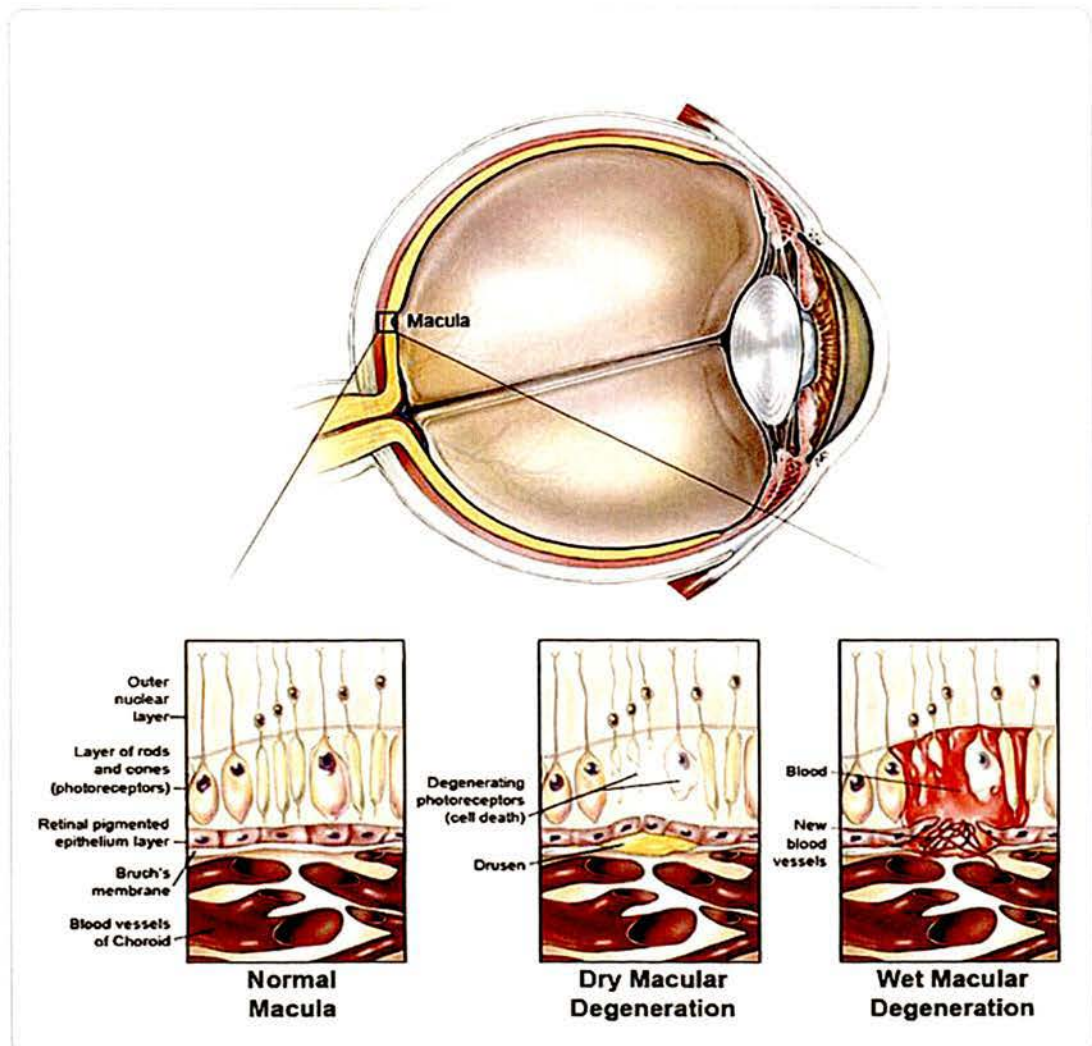


Figure 2.1. Progression of Macular Degeneration. Various retina cell types are shown in the region of the macula. Dry macular degeneration develops as photoreceptors become degraded and drusen begins to accumulate. Wet macular degeneration forms are the result o blood vessel growth into the retina pigmented epithelium.
(Illustration: American Health Association Foundation. www.ahaf.org)

Macular degenerative lesions seem to manifest at drusen formation sites underneath the retina pigmented epithelium. Drusen is a collection of colloid material (lipids, phospholipids, and collagen) excrescences that accumulate in the retina. This accumulation is associated with the progressive degeneration of the retinal pigment epithelium and photoreceptors [20]. Drusen deposits, however, disturb oxygen metabolism and cause the degeneration of photoreceptors. This damage to the photoreceptors in the retina is responsible for the visual impairment that results. Further, soft drusen may also aggregate into clusters and cause macular degeneration which can induce the detachment of the retina pigmented epithelium layer. As the process progresses, new blood vessels grow into these cells, leading to fluid buildup. The early stages of macular degeneration can be defined as the presence of drusen and development of retinal pigmentary abnormalities. Later stages of this age-related eye disorder develop into dry and neovascular macular degeneration. The pathology behind why these drusen deposits develop and lead to macular degeneration is complex and involves the combination of many factors, including oxidative stress, changes in the environment of the lens, and changes in biological activity of the retina pigmented epithelium [21].

The state of aggregation of the polymer melanin may determine its propensity to act either as an antioxidant or as a pro-oxidant. Age-related alterations in its state of aggregation are suggested to alter the degree of polymerization so as to confer increased pro-oxidant propensity to the melanin polymer. Degradative processes of melanosomes and lysosomes in the retinal pigment epithelium (RPE) appear to be intimately connected so that they may involve an exchange of content between these two organelles. An increased pro-oxidant environment inside lysosomes has been associated with preventing the digestion of cellular components including photoreceptor outer rod segments partly by altering the function of

lysosomal hydrolases. It is speculated that age-related accumulation of low-molecular-weight phototoxic pro-oxidant melanin oligomers within lysosomes in the RPE may be partly responsible for decreasing the digestive rate of incorporated cellular components (including photoreceptor outer rod segments) which may lead to drusen formation [22].

2.1.2. Cataractogenesis. Cataract formation is a type of eye-related disorder that occurs when the lens develops a cloudy site which affects the quality of vision. The lens is a clear part of the eye that enables light to be focused upon the retina, the light-sensitive tissue at the back of the eye. In a normal eye, light passes through the transparent lens to the retina. Once light reaches the retina, it is converted into nerve signals via photochemical receptors which are sent to the brain. In order for the retina to transmit a sharp image, the lens must be clear. If the lens is cloudy light will be scattered before arriving at the retina, and the image produced will appear blurred.

Elongated fiber cells that are filled with stable long-lived proteins comprise the majority of the structure of the lens. This region of cells is responsible for the lens clarity and its ability to focus light on the retina. Although the lens has protective mechanisms to preserve its function throughout the life span of an individual, these mechanisms begin to deteriorate during aging, allowing an accumulation of modified proteins. This then leads to gradual aggregation of the lens proteins, with a concomitant loss in the optical quality of the lens, and ultimately leads to opacity, or a cataract [23-30].

The lens derives all of its nutrients and oxygen from the aqueous humor and vitreous body. During development, the epithelial cells begin to differentiate, elongating to become fiber cells, during which time they lose the organelles and begin synthesizing large quantities of the structural proteins called crystallins. This process continues throughout life, though at

a slower pace, with the newer fiber cells pushing the older fibers to the center region of the lens (Figure 2.2.). Lens development is not complete without the removal of all potential light-scattering organelles from the fiber cells, which is accomplished in a programmed process that involves proteases [31,32].

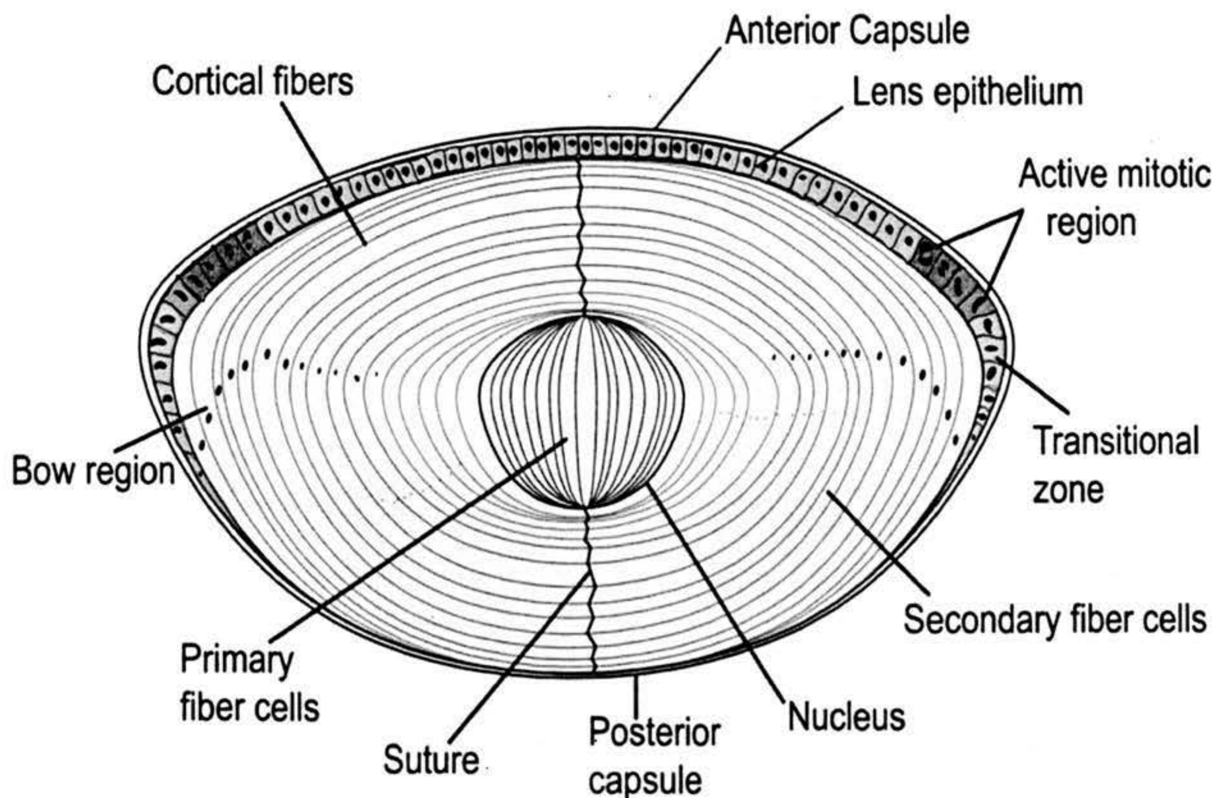


Figure 2.2. Lens Structure. The lens is a complicated structure that can be divided into different regions. This drawing shows a cross section of the entire structure of a lens that has been divided into various regions. A single layer of epithelial cells surrounds the outside of the lens. The bow region is where cells differentiate, lose their organelles, and mature into fiber cells. The elongated fiber cells are in contact with both the epithelial layer as well as the nuclear capsule of the lens.
(Illistration: Sharma KK, Santhoshkumar P. *Biochimica et Biophysica Acta* 1790:1095-108 2009.)

The lens epithelial cells possess the remarkable feature of dividing and differentiating into new fiber cells throughout life but this process diminishes with age. The lens epithelium is the major site of transport, metabolism and detoxification, since the lens fiber cells derive the bulk of their nutrients through the epithelial cells. An age-related decrease in epithelial density has been documented [33]. The epithelial cells are known to provide the lens with the first line of defense against oxidative insults [30,34]. Because the integrity of the epithelial layer is critical to normal lens physiology, epithelial cell death by apoptosis or any abnormalities in the cytoskeleton proteins in these cells are detrimental to the underlying fiber cells.

Oxidation is considered to be a major physiological challenge to lens proteins, and recent reviews have discussed the role of oxidation in the formation of cataracts [35-38]. The lens appears to be subjected to increasing oxidative stress with aging because of the progressive loss of the antioxidant system as the individual ages. The decline in the antioxidant activities of the lens is evident by decreased glutathione levels, reduced efficiency of enzymes associated with glutathione, and the accumulation of proteins with thiol-containing groups [26,27,30,39-41]. While α-crystallin has only two cysteine residues susceptible to oxidation, β-crystallin has several cysteine residues that are susceptible to oxidation, making intra-disulfide linkages more probable. Although tryptophan and histidine are susceptible to oxidation, their oxidation in aging lenses is minimal compared to that of cysteine, yet evidence has been shown that these amino acids are oxidized in advanced cataract development [37]. Investigators have generated protein aggregates *in vitro*, similar to those found in aged lenses, by oxidation of thiol groups in both α-cystallins and, β-crystallins forming disulphide linkages [42]. Free radicals, such as hydroxyl radical, superoxide, and singlet oxygen, are the likely oxidizing species *in vivo* [43–46]. Oxidation of α-crystallins has

also been shown to lead to structural changes and loss of chaperone activity [47]. The loss of chaperone activity in α-crystallin proteins, due to oxidation of methionine, was demonstrated recently and was found to be reversible by methionine sulfoxide reductase [48,49]. The crystallin proteins of the lens have several phosphorylation sites that have been shown to activate the chaperone activity of α-crystallin proteins [50-52]. There is no evidence of age-related changes in the extent of phosphorylation of crystallin proteins, but one study demonstrated that a sub unit of α-crystallin protein from a cataract lens was not phosphorylated [53]. The significance of this absence of phosphorylated α-crystallin is yet to be determined. Methylation of cysteine residues in crystallins has also been reported in several studies and it has been hypothesized that this methylation of cysteine might be a defensive mechanism in the lens to prevent disulphide formation and aggregation of crystallin proteins [40,54-58].

2.2. OXIDATIVE STRESS

Alterations in the glutathione metabolism within the lens and retina, as well as increased oxidative stress have been implicated in the pathogenesis of eye-related disorders [59]. The eye becomes vulnerable to oxidative stress damage after aging due to the decreased levels of antioxidant required to protect vital biochemical functions within the eye. This damage is generated by a variety of ROS (such as superoxide radical, hydrogen peroxide and hydroxyl radical) through the oxidation of various proteins and polyunsaturated fatty acids (PUFAs) [60,61]. The eye is also highly susceptible to free radical damage to both proteins and lipids, due to the relatively high levels of ultraviolet photons that enter the eye tissue and the relatively low amount of compounds to absorb this energy.

2.2.1. Reactive Oxygen Species. Highly reactive biochemical compounds containing oxygen are collectively known as reactive oxygen species (ROS). There are numerous types of ROS; however, those which are also free radicals are the most reactive and damaging species. Reactive oxygen species produced as a function of normal cellular metabolism. The superoxide radical ($O2\cdot^-$), hydroxyl radical ($\cdot OH$), singlet oxygen (O_2), and hydrogen peroxide (H_2O_2) are the most commonly formed ROS in a cell. These species are highly reactive due to the presence of unpaired valence electrons. Reactive oxygen species are created as a natural byproduct in the metabolism of oxygen and play important roles in cell signaling [62]. However, during times of environmental stress, such as UV exposure, heat, mitochondria dysfunction, or high oxygen concentration, ROS levels can increase dramatically. This can lead to significant damage to various cell structures and functions.

2.2.2. Lipid Peroxidation. Oxidative damage to lipids is usually mediated through lipid peroxidation. These damaged lipid byproducts are formed via a free radical chain reaction that occurs within the cellular environment. This chain reaction is responsible for the formation of a wide range of products, including aldehydes, ketones, and cyclic peroxide radicals inside cells [63]. The mechanism of lipid peroxidation is shown in Figure 2.3.

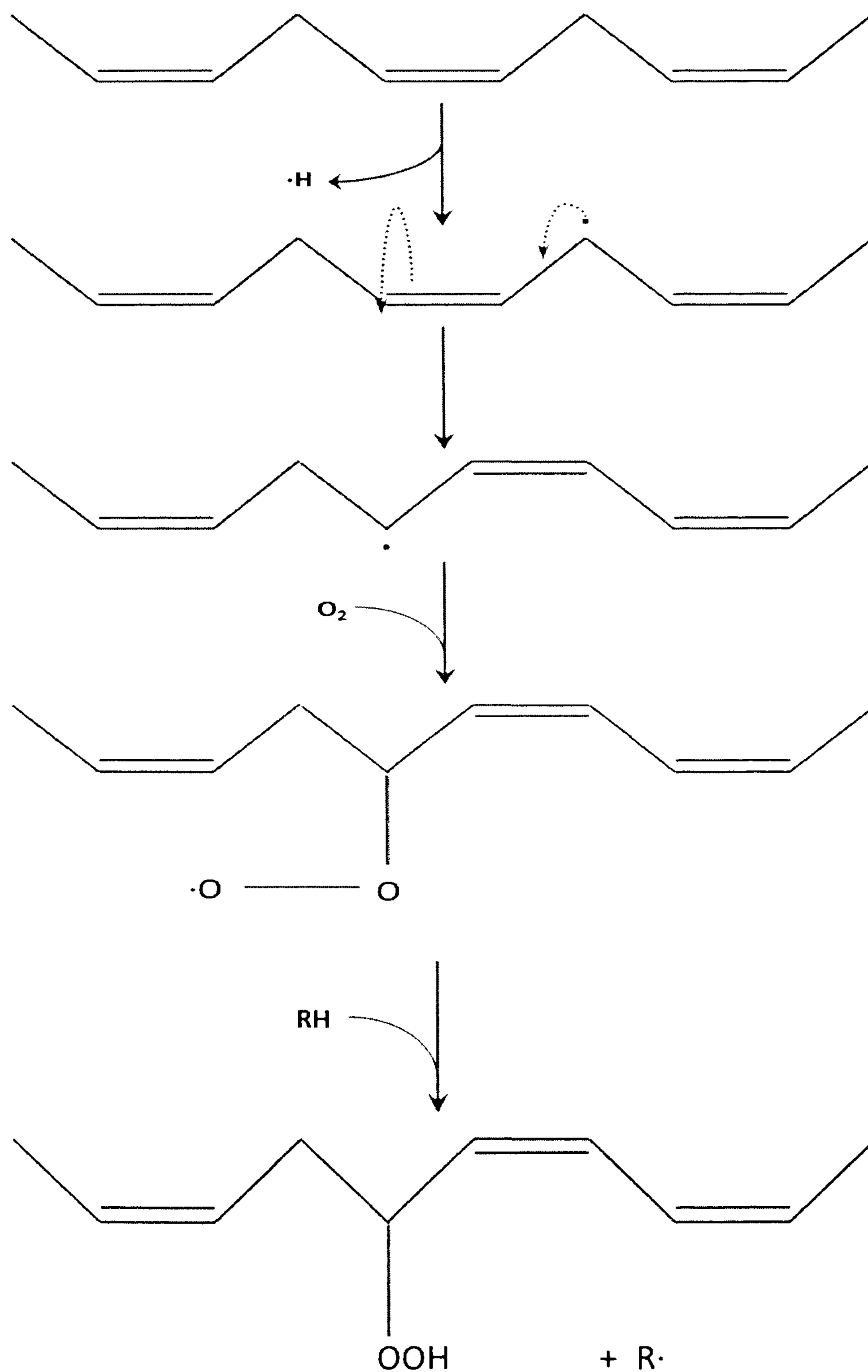


Figure 2.3. Mechanism of Lipid Peroxidation. Reactive species can initiate hydrogen abstraction with PUFAs. This generally creates a more stable radical which can be later oxidized to form organic peroxide as continuing this chain reaction.

The lipid peroxidation chain reaction is initiated by forming a lipid radical through hydrogen abstraction from a methylene carbon on a polyunsaturated fatty acid (PUFA) by a hydroxyl radical. Due to the presence of many double bonds between carbon atoms, the abstraction of the hydrogen atom is easier and the resulting molecule is more stable. The carbon-centered lipid radical is formed which rearranges and readily reacts with molecular oxygen in an aerobic environment to form a peroxyl radical. The peroxyl radicals formed abstract hydrogen from the side chains of neighboring PUFAs and combine with the abstracted hydrogen to form hydroperoxides. This can then lead to the propagation of the chain reaction and attack membrane proteins [64]. Lipid peroxidation causes injury to cells and intracellular membranes which can lead to cell destruction and, subsequently, cell death [65]. The lipid hydroperoxides decompose in the presence of metals, such as iron or copper, to form toxic aldehydes such as 4-hydroxy-2-nonenal (4-HNE), and malondialdehyde (MDA) [66-68].

2.2.3. Protein Oxidation. Protein oxidation occurs when there is a covalent modification of proteins. This modification can be induced either directly by reactive oxygen species or indirectly by a reaction with secondary byproducts of oxidative stress [69]. The damage caused to proteins due to oxidation can result in the inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, alteration of transport properties, altered gene regulation and expression, and modulation of cell signaling [70]. Oxidative changes can lead to both backbone fragmentation and aliphatic-side chain oxidation. Of all the amino acid residues within proteins, cysteine residues are the easiest oxidized and tend to follow one of two oxidation pathways shown in Figure 2.4.

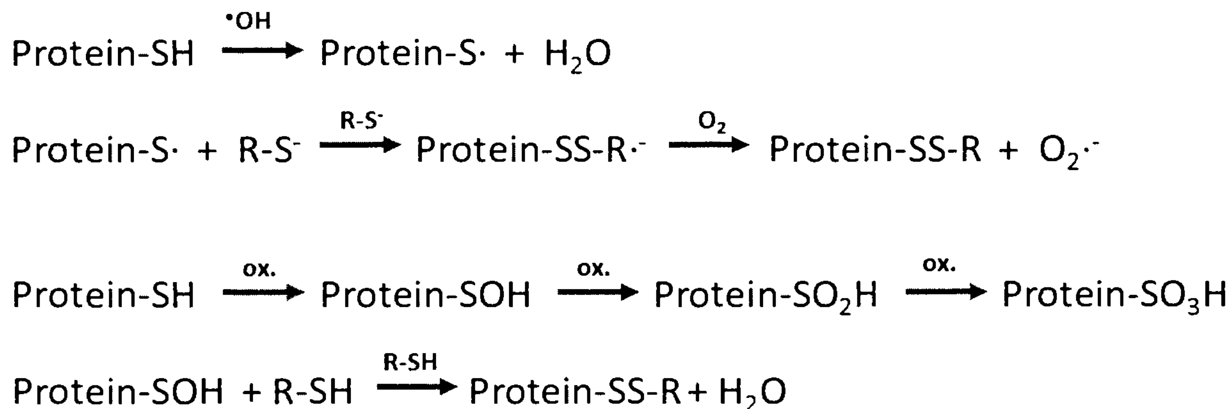


Figure 2.4. Mechanism of Protein Oxidation on Cysteine Residues. The first pathway depicts a cysteine residue forming a thiol radical which then reacts with another thiol group to form a disulfide bond. The second pathway depicts the cysteine residue being oxidized directly and then the formation of a disulfide bond through a thiol exchange mechanism.

2.3. ANTIOXIDANT DEFENSE

2.3.1. Glutathione. Glutathione (Figure 2.5.) is a tripeptide containing the amino acids glutamate, cysteine, and glycine. This compound also has an unusual γ-linkage between glutamate and cysteine which prevents it from being hydrolyzed easily giving rise to its high stability within the cell. The thiol group on the cysteine residue makes up the functional component of this antioxidant which allows for its potent cellular protection against oxidative stress.

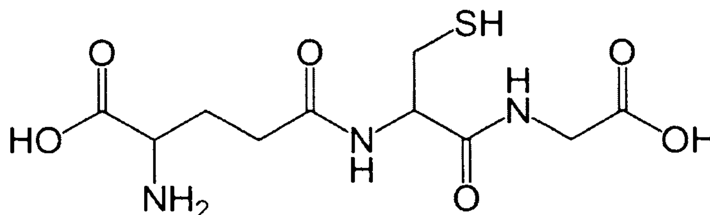


Figure 2.5. Structure of Glutathione.

GSH is synthesized by the action of the two enzymatic processes depicted in Figure 2.6. First, the amino acids of glutamate and cysteine are linked together through a gamma peptide bond by γ-glutamylcysteine synthetase (γ-GCS) to form γ-glutamylcysteine. Then, γ-glutamylcysteine is further reacted with glycine to form glutathione by the enzyme glutathione synthetase (GS).

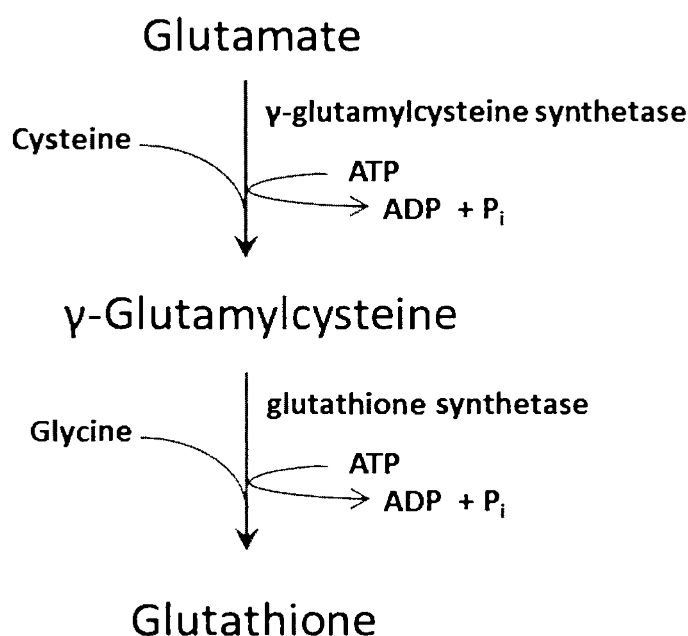


Figure 2.6. Glutathione Synthesis. Glutathione is synthesized as depicted above by the consecutive action of the enzymes γ-glutamylcysteine synthetase and glutathione synthetase.

Reduced glutathione is the most abundant thiol compound within the cell. This hydroxyl radical and singlet oxygen scavenger participates in a wide range of cellular functions and can react non-enzymatically with oxidative compounds or support glutathione peroxidase (GPx) activity in the clearance of hydrogen and organic peroxides to maintain the redox status of the cell. GSH is also closely linked to amino acid transport into the cell. More

importantly, in the case eye related disorders, GSH protects the integrity of crystalline proteins found within the lens, thereby maintaining the clarity and functionality of this ocular component.

2.3.2. Antioxidant Enzymes. The antioxidant enzyme system within the eye consists of several enzymes which also aid in maintaining the redox status of the cells. The most important of these are superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase. Superoxide dismutase acts on a superoxide radical by converting its substrate into a less reactive species, hydrogen peroxide. Glutathione peroxidase is responsible for decomposing hydrogen peroxide and other organic peroxides. Glutathione reductase allows for glutathione which has been oxidized to be regenerated into its reduced form. Catalase focuses on decomposing hydrogen peroxide to water and oxygen.

2.3.3. Crystallin Proteins. Crystallin proteins comprise over 90% of a lens cell. Nuclear magnetic resonance studies show that the C-terminal regions of α-crystallins are more flexible than the N-terminal regions [71]. The α-crystallin molecule is a dynamic oligomer, with the subunits dissociating and reassociating constantly. This subunit exchange property is also implicated in the α-crystallin chaperone activity. Both *in vivo* and *in vitro* studies that were conducted to understand the molecular basis of cataract-causing crystallin mutations and age-related cataract showed that loss of chaperone activity and increased protein aggregation are responsible for the lens pathology [72-75].

The primary structure of the alpha crystallins is shown in Figure 2.7. A primary function of crystallins is to focus light on the retina by maintaining the necessary refractive characteristics and clarity of the lens. Lens proteins undergo very little turnover, but they do

undergo various changes during aging and cataractogenesis, including increased crystallin proteolysis, fragmentation and aggregation. Due to the position, availability, and reactivity of cysteine residues on the peripheral of the crystallin proteins, it is likely that oxidation first occurs at these locations. Proper subunit interaction between crystallins is necessary to prevent the formation of light scattering aggregates of crystallins. Oxidative damage may affect the short-range interactions among α- and β-crystallins which are crucial for lens transparency [76]. Also, equilibrium dialysis studies have shown that an age-related decrease occurs in the interactions between α- and β -crystallins [77]. The changes in the interactions may lead to increased aggregation of proteins and localized fluctuations in protein densities sufficient to cause light scattering [78]. The formation of protein aggregates of crystallins, either by this mechanism or other mechanisms, could be the first event in the development of cataracts. Consistent with this hypothesis, decreased chaperone activity, increased crystalline aggregation, light scattering and loss of lens transparency have indeed been demonstrated in aged human lenses [79].

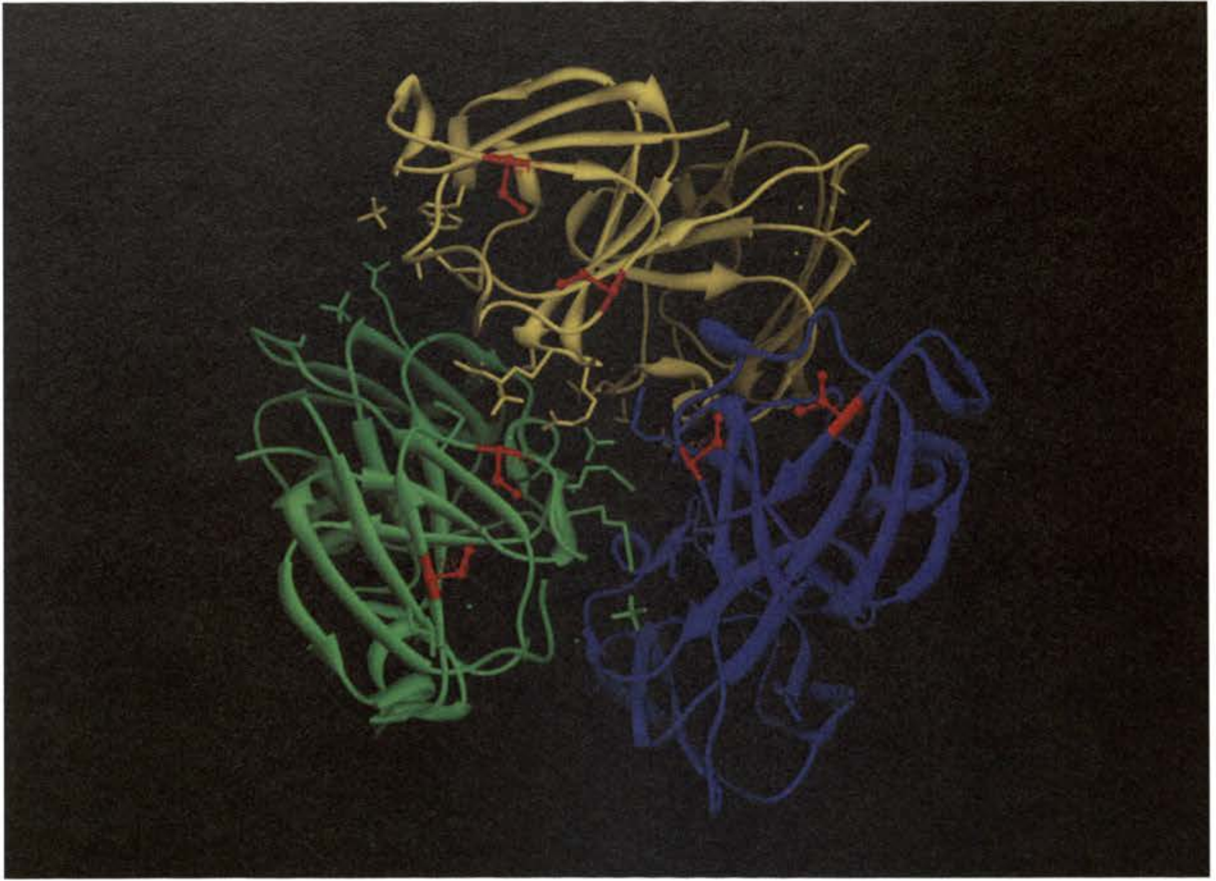


Figure 2.7. Crystallin Protein Structure. Pictured above is the structure of an alpha crystalline protein. The area in red denotes the location of cysteine residues and the arrows designate the location of beta sheets within the protein.

2.4. BLOOD RETINA BARRIER

The blood-retina barrier (BRB) mainly serves as a shield, protecting the eye tissue from toxic substances that can be found in the blood. The barrier, consisting of microvascular endothelial cells that are networked with many tight junctions, is situated directly between the interface of the eye's retina and the blood. The BRB is also responsible for the regulation of osmotic flow, nutrient transportation, and photochemical waste removal [80]. Because of this highly selective membrane, it is important to choose a therapeutic drug that has the ability to cross from the blood into the membrane of the eye.

2.5. PROPERTIES OF N-ACETYLCYSTEINE AMIDE

One logical approach to treating conditions caused by oxidative stress is to supply antioxidants to the affected target site in order to restore the redox balance. However, due the inability of many compounds to cross the BRB, there are very few drugs that can be successfully used to treat neurological disorders. N-acetylcysteine amide (NACA), a low molecular weight thiol antioxidant, is a potential candidate because it may possess the necessary characteristics required to restore the redox balance within the brain. NACA's characteristics as a drug were an improvement over N-acetylcysteine (NAC), a FDA approved drug, because it replaces the carboxylic functional group of NAC with an amide functional group (Figure 2.8.). This modification allows NACA to be more lipophilic and enhances its ability to penetrate cellular membranes. Recent studies have demonstrated that NACA restores significantly more glutathione in plasma and liver tissues than NAC [81]. Administration of this hydrophobic drug would allow passage across the blood-retina barrier and provide an easily oxidizable thiol group to enhance antioxidant properties within the eye tissue. NACA is also an excellent source of sulfhydryl groups that can be enzymatically modified into metabolites that are capable of stimulating glutathione synthesis [82]. The molecule can also promote intracellular detoxification and act directly as a free radical scavenger.

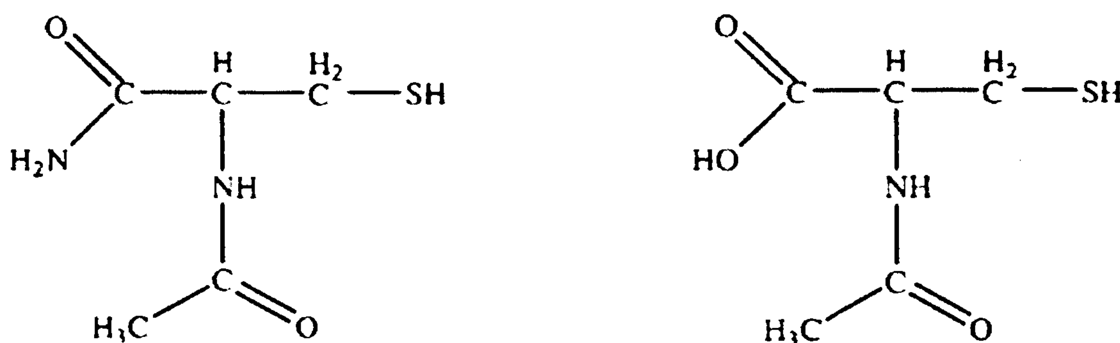


Figure 2.8. Structures of NACA and NAC.

3. METHODS

3.1. HPLC

3.1.1. Measurement of Reduced Thiols. The levels of GSH and cysteine in each of the lens samples were determined using HPLC, according to a method developed within our laboratory [83]. Each of the lens samples was first homogenized in a serine borate buffer (pH 8.4) and then centrifuged at 5000 × g for 10 minutes. Next, 250 μL of diluted supernatant was added to 750 μL of 1 mM N-prenylmaldimide (NPM) in acetonitrile to form a fluorescent thiol derivative (Figure 4.1.). The resulting solution was incubated at room temperature for 5 minutes 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 into 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. (Column Engineering, Ontario, CA, USA). The mobile phase was acetonitrile: water (70:30, v/v) and was adjusted to a pH of 2.5 by the addition of 1.0 mL/L acetic acid and 1.0 mL/L phosphoric acid. The NPM derivatives of GSH and cysteine were eluted from the column isocratically at a flow rate of 1.0 mL/min. For detection of this derivative, the excitation and emission wavelengths were 330 and 376 nm, respectively.

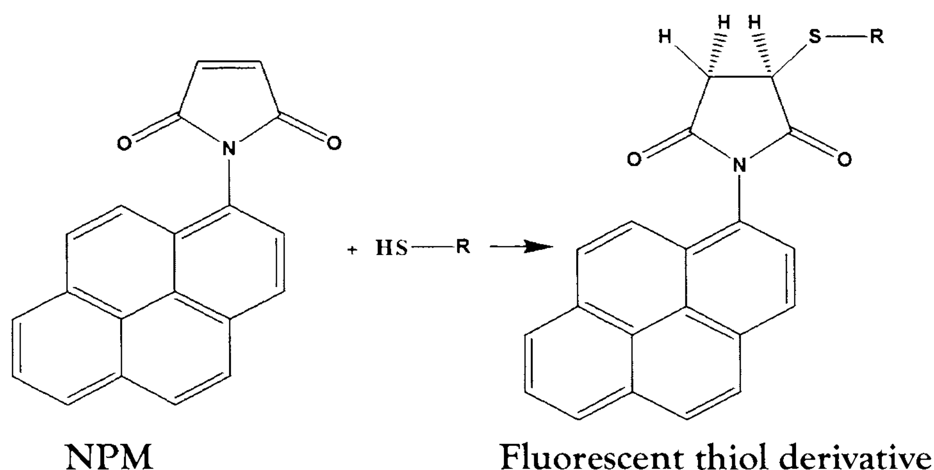


Figure 3.1. NPM Derivatization of Thiol Compounds. N-prenylmaldimide reacts with free thiols in solution to form a fluorescent thiol derivative. This fluorescent compound has an excitation wavelength at 330 nm and emission wavelength at 376 nm.

3.1.2. Measurement of Glutathione Disulfide. The amount of glutathione disulphide (GSSG) in each sample was determined indirectly by reducing the amount of GSSG into two times the amount of GSH, using a method developed in our laboratory [84]. The procedure used was similar to the detection of GSH. However, before the addition of 750 μL of the NPM solution, 125 μL of 500 mM dithiothreitol was first added to 125 μL of diluted supernatant from the centrifuged lens homogenate and then allowed to incubate in a 37 °C water bath for 30 minutes. The rest of the procedure followed that which was used to determine only the reduced form of GSH alone. Data from the original GSH levels and the total GSH levels in each sample could subsequently be used to calculate the levels of GSSG present in each sample.

3.1.3. Measurement of Malondialdehyde. The amount of malondialdehyde (MDA), a by-product of lipid peroxidation, was determined for each sample according to the

method described by Draper et al. [85]. For sample preparation, 350 μL of the lens homogenate was added to 550 μL of 5% TCA and 100 μL of 500 ppm butylated hydroxytoluene in methanol. The resulting solution was then boiled for 30 minutes 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. Again, the contents were heated in a boiling water bath for 30 minutes and then immediately cooled in an ice water bath. The MDA derivative was then transferred into n-butanol by adding 500 μL of the sample mixture into 1 mL of n-butanol and vortexing for 2 minutes. Each sample was then centrifuged to facilitate the separation of the two phases. The resulting organic layers were first filtered through 0.45-μm filters and then injected into the HPLC system (Thermo Electron Corp.), which consisted of a Finnigan Spectra System vacuum membrane degasser, a gradient pump, an autosampler, and a fluorescence detector (Model FL3000). The HPLC column was a Reliasil ODS-1 C_{18} column (5-μm packing material) with 250 × 4.6 mm i.d. (Column Engineering). 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.

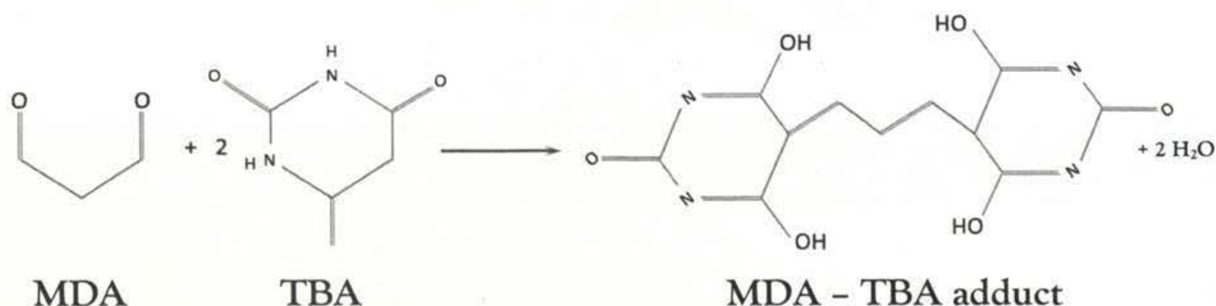


Figure 3.2. TBA Derivatization of MDA. In the presence of TBA, MDA forms a fluorescent compound that has an excitation at 515 nm and emission at 550 nm.

3.2. SPECTROPHOTOMETRIC

3.2.1. Cytotoxicity Determination. Cells were seeded in a 96-well tissue culture plate, at a density of approximately 1.25×10^4 cells/well, for a day. The media was then discarded and the cells were treated with various concentrations of analytes in serum-free media. Calcein AM assay KIT (Biotium, Inc. CA) was used to determine cytotoxicity relative to the control group [86]. The cells were then washed three times with PBS, and 100 μL of 2 M Calcein AM in PBS was added to each well for 30 minutes at 37 °C. Calcein AM is then absorbed into cells and become substrates for esterases which are present in living cells. The resulting fluorescence compounds were measured with an excitation wave-length at 485 nm and an emission wavelength of 530 nm, using a microplate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

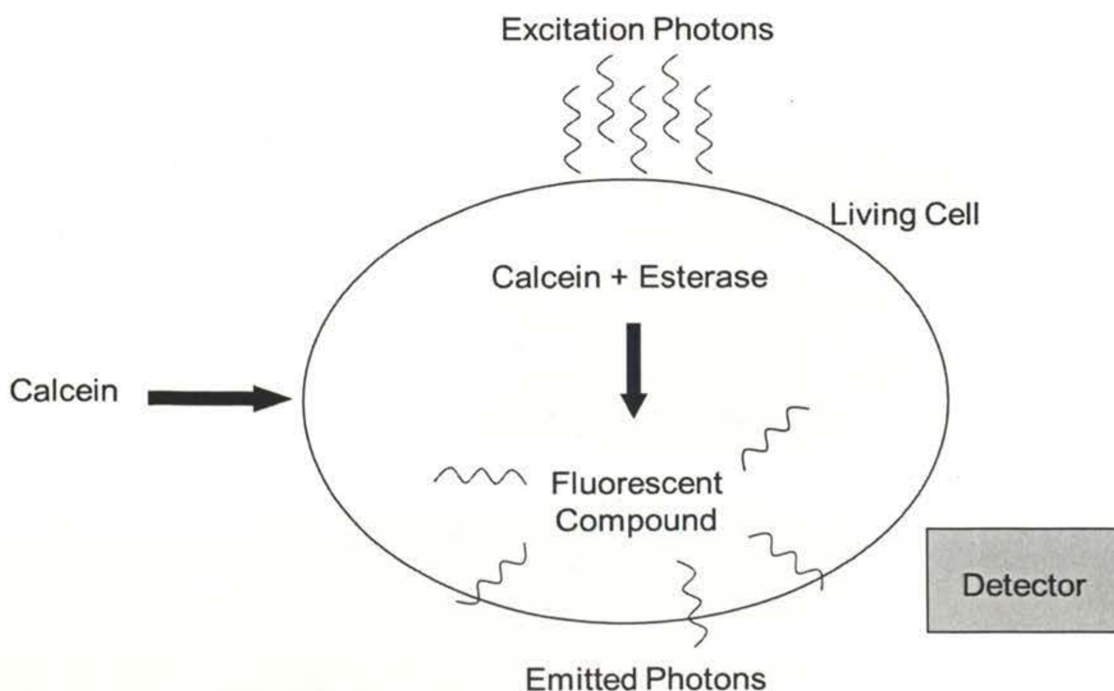


Figure 3.3. Calcein Assay. The action of calcein is depicted above. Once this compound enters into a living cell, it is used as a substrate for esterase which creates a fluorescent compound. Esterases are not present in significant amounts in no longer functioning cells.

3.2.2. Reactive Oxygen Species Determination. Intracellular ROS generation was measured using a well-characterized probe, 2',7'-dichlorofluorescin diacetate ($DCFH_2$-DA) [87]. Cells were seeded at a density of 1.25×10^4 cells/well in a 96-well plate. $DCFH_2$-DA is hydrolyzed by esterases to dichlorofluorescin (DCFH), which is then trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular reactive oxygen species. After dosing with the analyte, the cells were washed twice with PBS and incubated with a solution of 50 μM $DCFH_2$-DA in phenol red free media for 30 minutes. The fluorescence generated by the resulting species was determined at 485 nm excitation and 520 nm emission, using a microplate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

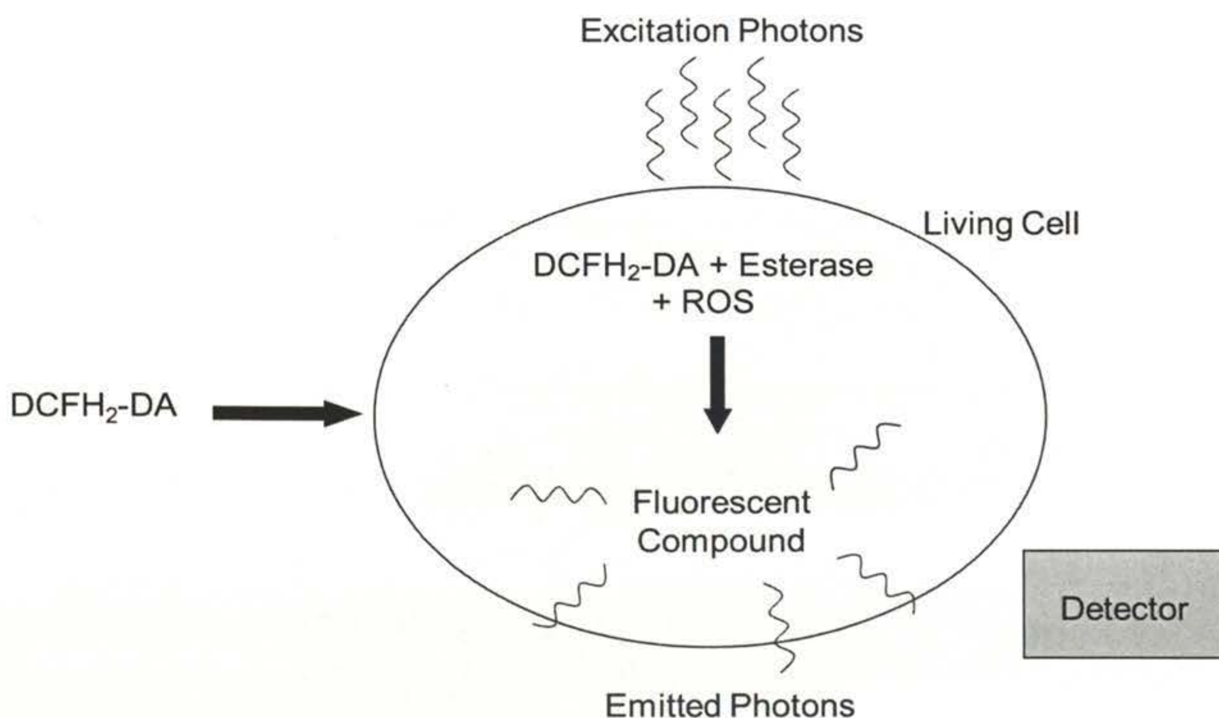


Figure 3.4. DCFH-DA ROS Assay. In this reactive oxygen species assay $DCFH_2$-DA is converted into DCFH in the presence of esterase. DCFH then forms a fluorescent compound upon interacting with ROS present inside the cell.

3.2.3. Catalase Activity. Catalase activity was measured according to the method described by Aebi [88]. Briefly the activity of catalase was measured spectrophotometrically at a wavelength of 240 nm in samples, following the exponential disappearance of H_2O_2 (10 mM). The catalase activity was calculated from the equation $A_{60}=A_{initial}- kt$, where k represents the rate constant, $A_{initial}$ is the initial absorbance, and A_{60} is the absorbance after 60 seconds have passed.

3.2.4. Glutathione Peroxidase Activity. Glutathione peroxidase (GPx) protects mammals against oxidative damage by catalyzing the reduction of a variety of ROOHs or H_2O_2 using GSH as the reducing substance. The GPx-340 assay (Oxis International, Beverly Hills, CA, USA) is an indirect measure of the activity of GPx. Oxidized glutathione, produced upon reduction of an organic peroxide by GPx, is recycled to its reduced state by the enzyme glutathione reductase (GR). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (A_{340}), providing a spectrophotometric means for monitoring GPx enzyme activity. The molar extinction coefficient for NADPH is 6220 M^{-1} cm^{-1} at 340 nm. To measure the activity of GPx within the lens, homogenate was added to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction was initiated by adding the substrate, tert-butyl hydrogen peroxide, and the A_{340} was recorded. The rate of decrease in the A_{340} was directly proportional to the GPx activity in the sample.

3.2.5. Glutathione Reductase Activity. Glutathione reductase 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 within the lens 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.

3.2.6. Protein Carbonyl Determination. The amounts of protein carbonyls in each sample were determined using a modified method by Levine et al. [89]. Each of the lens samples was homogenized in a serine borate buffer (pH 7.4) and then centrifuged at 10,000 g for 15 minutes. Next, 200 μL of supernatant containing approximately 1.5 mg of protein was added to 800 μL of 10 mM 2,4-dinitrophenylhydrazine dissolved in 2 M HCl. In parallel, 200 μL of supernatant was added to 800 μL of 2.0 M HCl to act as a control. The resulting solutions were incubated at room temperature in the dark for 1 hour, with vortexing every 10 minutes. Subsequently, 1 mL of 20% trichloroacetic acid (TCA) was added to each solution, vortexed, and then placed on ice for 5 minutes. The solutions were then centrifuged for 10 minutes at 10,000 g. The resulting pellet was washed three times with an ethanol and ethyl acetate (1:1) mixture. The samples were then left to dry for 10 minutes, after which 800 μL of 6 M guanidine solution (prepared in 20 mM potassium phosphate and adjusted to a pH of 2.3 using trifluoroacetic acid) was added. The solutions were vortexed to resuspend the proteins. The solutions were centrifuged at 10,000 g for 10 minutes and the supernatant was analyzed by spectrometry. Absorption was measured at a wavelength of 370 nm against the sample blank and the protein carbonyl content was determined using the associated molar absorption coefficient (22,000 M^{-1} cm^{-1}).

3.2.7. Total Protein Determination: Protein levels of the tissue samples were measured using the Bradford method [90]. Concentrated Coomassie blue (Bio-Rad, Hercules, CA, USA) was diluted 1:5 (v/v) with distilled water. Fifty microliters of diluted lens homogenate was then added to 1.5 mL of this diluted dye, the solution was then vortexed and allowed to stand at room temperature for 20 minutes. The absorbance was then measured at 595 nm using a UV spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). Bovine serum albumin was used as the protein standard.

3.3. MEMBRANE INTEGRITY

3.3.1. Transepithelial Electrical Resistance (TEER) Assay. Cells were seeded at a density of 1.5×10^5 cells/well onto collagen-coated inserts with a pore size of 0.4 μm and allowed to form a monolayer. Trans-endothelial electric resistance (TEER) measurement by EVOM voltohmmeter (World Precision Instrument, Sarasota, FL, USA) assessed the tightness of the ARPE monolayer [91]. Following the treatment protocol for the cell monolayer, the media was replaced with 150 μL of fresh medium. The insert containing the cell monolayer was then transferred to a fresh plate containing 500 μL of serum-free medium. The TEER reading was recorded immediately and TEER values were calculated as: resistance (Ω) $\times$ 0.32 cm^2 (surface area of insert). Thus, resistance is proportional to the effective membrane.

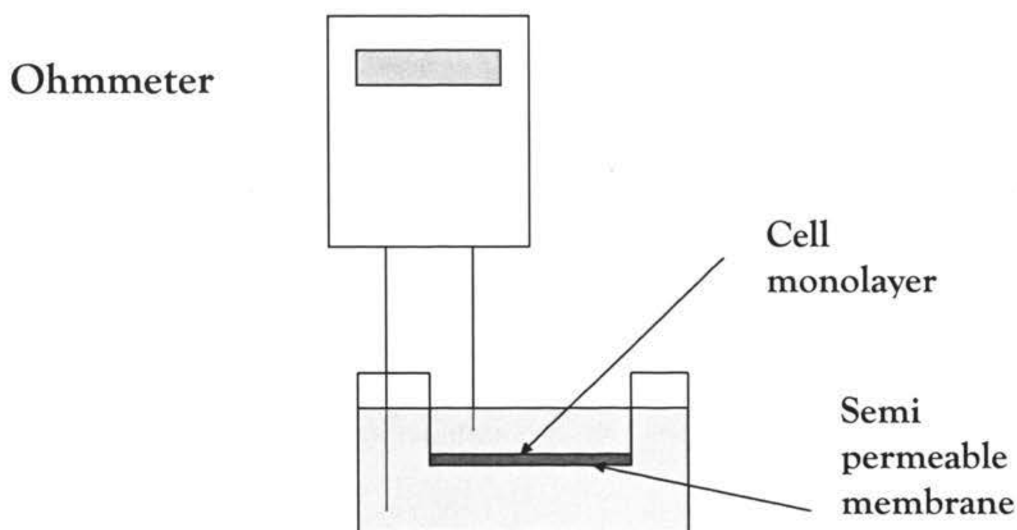


Figure 3.5. TEER Assay. Depicted above is a diagram for the setup of testing the transepithelial electrical resistance across a cell monolayer. This is carried out in cell plates containing semipermeable inserts.

3.3.2. Dextran Permeability Assay. Cells were seeded at a density of 1.5×10^5 cells/well onto collagen-coated inserts with a pore size of 0.4 μm and allowed to form a monolayer. Following the treatment protocol for the cell monolayer, the media was replaced with 150 μL of fluorescein isothiocyanate labeled dextran. The insert containing the cell monolayer was then transferred to a fresh plate containing 500 μL of serum-free medium. The cell plates were then incubated for 10 minutes at 37 °C. Then, 100 μL of the plate well solution was then removed and transferred into a 96-well plate to be analyzed. Fluorescence was determined at 485 nm excitation and 520 nm emission, using a microplate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

3.4. CATARACT EVALUATION

The degree of cataract formation for each of the rat pup lenses was determined visually utilizing a grading system similar to those used by practicing ophthalmologists. The scale used to grade the opacity of each lens was defined as follows: clear, grade 0; slight opacity, grade 1; partial nuclear opacity, grade 2; and complete nuclear opacity, grade 3. To view the lens, a state of mydriasis was generated using a chemical dilation agent. All rat pups received a drop of a 2.5% phenylephrine hydrochloride tropicamide ophthalmic solution in each eye and were placed in a dark room for 1 hour before the examination. The lenses of each rat were then observed using a slit-lamp microscope at 10× magnification. The degree of opacity in each lens was determined and verified by certified ophthalmologists and then documented using a digital camera in macro mode.

3.5. STATISTICAL ANALYSIS

Group comparisons were performed using the one-way analysis of variance and the Tukey post hoc test. Statistical analyses were made using GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at a p value of ≤ 0.05. In the figures, "*" represents a significant difference in comparison with the control group, and "**" represents a significant difference in comparison with the METH-only or BSO-only group.

4. PROTECTIVE EFFECTS OF N-ACETYLCYSTEINE AMIDE ON ARPE-19 CELLS DURING EXPOSURE TO METHAMPHETAMINE

Methamphetamine (METH) abuse is implicated in a number of serious ocular pathologies, including corneal ulceration, retinal vasculitis, episcleritis, scleritis, panophthalmitis, endophthalmitis, retinopathy, and macular degeneration [92-94]. It has been reported that METH induces oxidative stress in the retina and adversely affects the dopaminergic system of the rat retina [95], particularly during central nervous system (CNS) development. Under physiological conditions, high levels of antioxidant enzymes and small antioxidant molecules, particularly glutathione (GSH), in the Muller (glial) cells protects retinal pigment epithelium (RPE) cells against oxidative stress. GSH in the Muller cells is depleted under oxidative stress and, since GSH cannot be transported directly into the cells, the need arises for permeable compounds that can increase intracellular GSH levels. One such compound, known to increase intracellular GSH levels in cells, is the low molecular weight thiol antioxidant, N-acetylcysteine amide. Previous work has demonstrated that NACA restored the levels of GSH, and scavenged the ROS produced in human brain endothelial cells (HBMVEC), after treatment with METH [91].

Even though the adverse effects of METH on the brain are linked to oxidative stress, little is known about its effect on the retina. Considering the ability of NACA to protect cells from oxidative stress [81,96,97], the effectiveness of this antioxidant was evaluated as a treatment option for METH-induced oxidative damage to RPE cells. Understanding NACA's protective role against METH-induced oxidative damage to RPE cells would help develop NACA as a potential therapeutic agent for treating RPE cells against numerous oxidative stress related ocular diseases.

4.1. EXPERIMENTAL DESIGN

The human adult retina pigmented epithelial cell line, ARPE-19, was used as a model for the blood-retina barrier. These ARPE-19 cells were cultured in a one-to-one ratio of DMEM:F-12 culture medium, which was supplemented with 10% (v/v) FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were maintained in a 37 °C incubator and supplied with 95% air and 5% CO_2. Only cells between passage numbers 26 and 32 were used for the experiments reported within.

In order to determine the minimum toxic dose of methamphetamine and maximum non-toxic dose of N-acetylcysteine amide to use for this study, a cell viability study for each analyte was performed. The ARPE-19 cells were seeded in a 96-well tissue culture plate, at a density of approximately 1.25×10^4 cells/well, and allowed to attach and grow for a period of 24 hours. The media was then discarded and the cells were treated with various concentrations of METH and NACA in serum-free media for 24 hours and 2 hours, respectively. Protective effects of NACA were then studied by pre-incubating cells with NACA for 2 hours, followed by treatment with 500 μM of METH for 24 hours.

Oxidative stress parameters, including cytotoxicity, reactive oxygen species, glutathione, malondialdehyde, activities of glutathione peroxidase and catalase, dextran permeability, and transepithelial electrical resistance were measured after the cells were treated, as described below. After seeding the cells, flasks were divided into the following four groups: 1) control, 2) NACA-only, 3) METH-only, and 4) METH+NACA. In groups with NACA pretreatment, media containing 1 mM NACA was added and incubated for a period of 2 hours. After pretreatment, the media in the control and NACA-only groups were replaced with plain media, while the remaining two groups received media containing

METH for 24 hours. The cell pellets obtained were then further processed for appropriate assays.

4.2. RESULTS

4.2.1. Effects of NACA and METH on Cytotoxicity Studies. ARPE-19 cells were incubated with 500 μM, 1.0 mM, and 2.0 mM concentration of NACA for a period of 2 hours (Figure 4.1.). After this time, Calcein AM was added and fluorescence levels were measured. Slight cytotoxic effects were noticed at a concentration of 2 mM. Therefore 1.0 mM was selected to be the maximum non-toxic dose of NACA to be used for treating the cells for future experiments. The cytotoxic effect was also studied for ARPE-19 cells using METH at concentrations of 200 μM, 500 μM, 750 μM, 1.0 mM, and 2 mM for a period of 24 hours (Figure 4.2.). A dose dependent response in the cell viability was observed as the concentration of METH increased. The results indicated that a concentration of 500 μM METH was minimum amount needed to produce a cytotoxic effect on the retinal cell line. Figure 4.3. indicates that pretreatment with 1 mM of NACA successfully protected the ARPE-19 cells from METH-induced death, thereby increasing viability to ~92% of that of control levels.

4.2.2. Effects of NACA and METH on Reactive Oxygen Species. Reactive oxygen species were measured by monitoring the change in fluorescence produced during the conversion of $DCFH_2$-DA to DCF. The retinal cells were pretreated with NACA, then introduced to $DCFH_2$-DA, then exposed to METH. Treatment with METH increased ROS production within the ARPE-19 cells by about 30% (Figure 4.4.). However, pretreatment with NACA significantly decreased ROS production, although NACA alone did not significantly alter ROS production, when compared to the control group.

4.2.3. Effects of NACA and METH on Intracellular Glutathione Levels. Glutathione levels give a good reflection of the oxidative status of the cellular environment. METH decreased GSH levels in APRE cells by approximately 20% when compared with the control level (Figure 4.5.). However, pretreatment with 1.0 mM of NACA increased the GSH levels in METH-treated cells to levels nearly that of the control, indicating that NACA protects these cells. Treatment with 1.0 mM of NACA alone did not significantly impact the levels of intracellular GSH, as compared to the control group.

4.2.4. Effects of NACA and METH on Malondialdehyde Levels. Lipid peroxidation can be estimated by measuring the levels of malondialdehyde (MDA), a stable by-product of lipid peroxidation. METH-treated cells had significantly higher levels (about four fold) of MDA, as compared to those of the control (Figure 4.6.). Pretreatment with NACA significantly lowered MDA levels to those of the control group. Treatment with 1 mM of NACA only did not significantly alter MDA levels, as compared to those of the control group.

4.2.5. Effects of NACA and METH on Antioxidant Enzyme Activities. Treatment of cells with METH decreased catalase activity by about 30%, as compared to the control group (Table 4.1.), although it returned to the control level upon pretreatment with NACA. However, treatment with 1 mM of NACA did not significantly alter catalase activity as compared to the control group. Table 1 shows GPx activity in METH-treated cells. METH treatment markedly decreased GPx activity by about 30% as compared to the control group. Pretreatment with NACA helped accelerate the activity of GPx in the METH-treated cells to about 90% of control activity. NACA alone did not significantly alter the results.

4.2.6. Effects of NACA and METH on Membrane Integrity. Dextran permeability and TEER are both indicators of the integrity of the BRB in which a monolayer of ARPE-19 cells makes a good model. METH treatment increased permeability by approximately 15%, as compared to controls (Figure 4.7.). Pretreatment with NACA significantly reduced this permeability to levels similar to that of the control group. TEER results (Figure 4.8.) also indicated the protective effect of NACA in ARPE-19 cells. Cells exposed to METH resulted in a 10% decrease in TEER, as compared to the control, while pretreatment with NACA gave resistance values only slightly less than control levels. Treatment with NACA alone did not significantly alter TEER, as compared to that of the control.

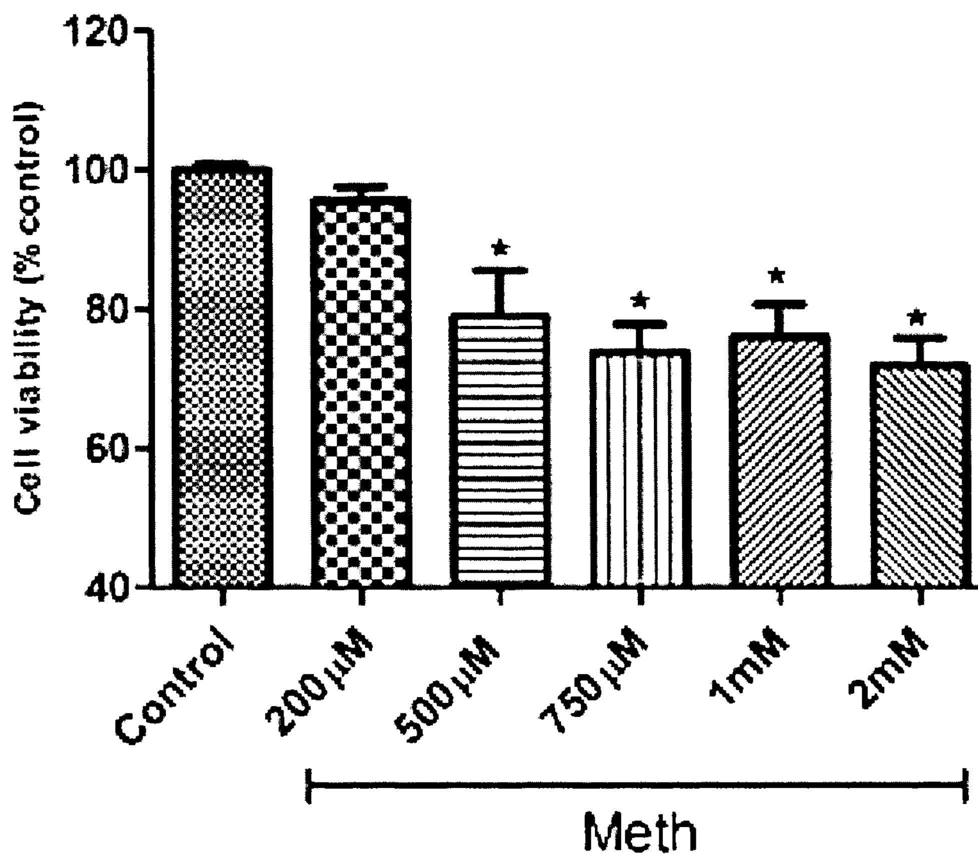


Figure 4.1. Cytotoxicity of METH in ARPE-19 Cells. Cell viability after varying treatments with METH for 24 hours. A concentration of 500 μM was found to be the minimum toxic dose of METH for ARPE-19 cells and was used as the optimum dose for all experiments. Values are represented as mean ± SD. (*) refers to significant differences from the control with $p \leq 0.05$. The graph is representative of at least three independent experiments.

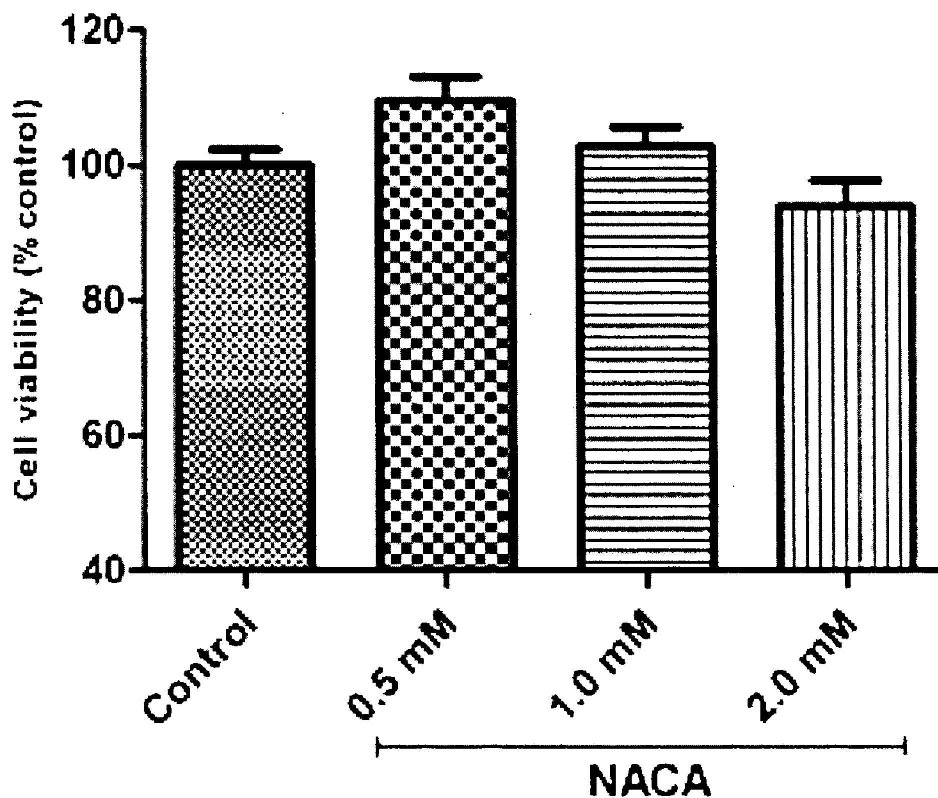


Figure 4.2. Cytotoxicity of NACA in ARPE-19 Cells. ARPE-19 cells were treated with various concentrations of NACA (0.5 mM, 1.0 mM, and 2.0 mM). After 2 hours of treatment, cell viability was quantified by Calcein AM. A concentration of 1 mM NACA was determined to be the maximum non-toxic dose which was used for the rest of the experiments in this study. Values are represented as mean ± SD. (*) refers to significant differences from the control with $p \leq 0.05$. The graph is representative of at least three independent experiments.

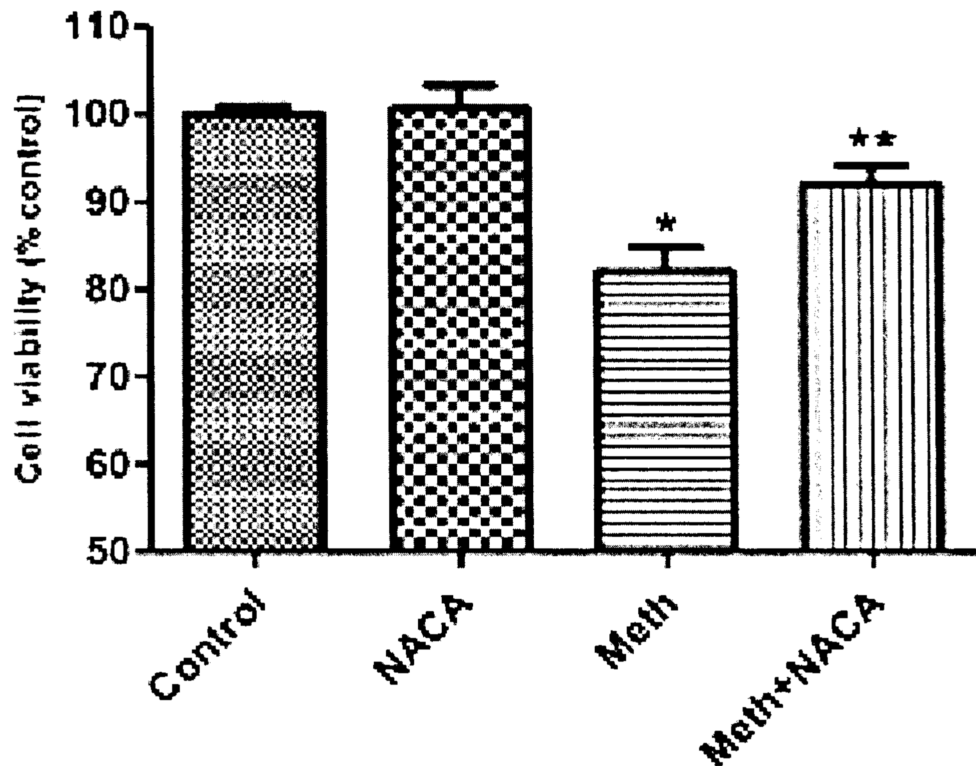


Figure 4.3. Cell Viability of ARPE-19 Cells after Treatment with METH and NACA. Cell viability was quantified by Calcein AM assay 24 hours after exposure to METH, following a 2-hour pretreatment with NACA. Treatment with METH (500 μM) alone was seen to significantly decrease cell viability. NACA (1.0 mM) was seen to protect against some METH-induced cell toxicity. * $p \leq 0.05$ compared to the control group, and ** $p \leq 0.05$ compared to the corresponding value of the METH group. The results are representative of at least three independent experiments.

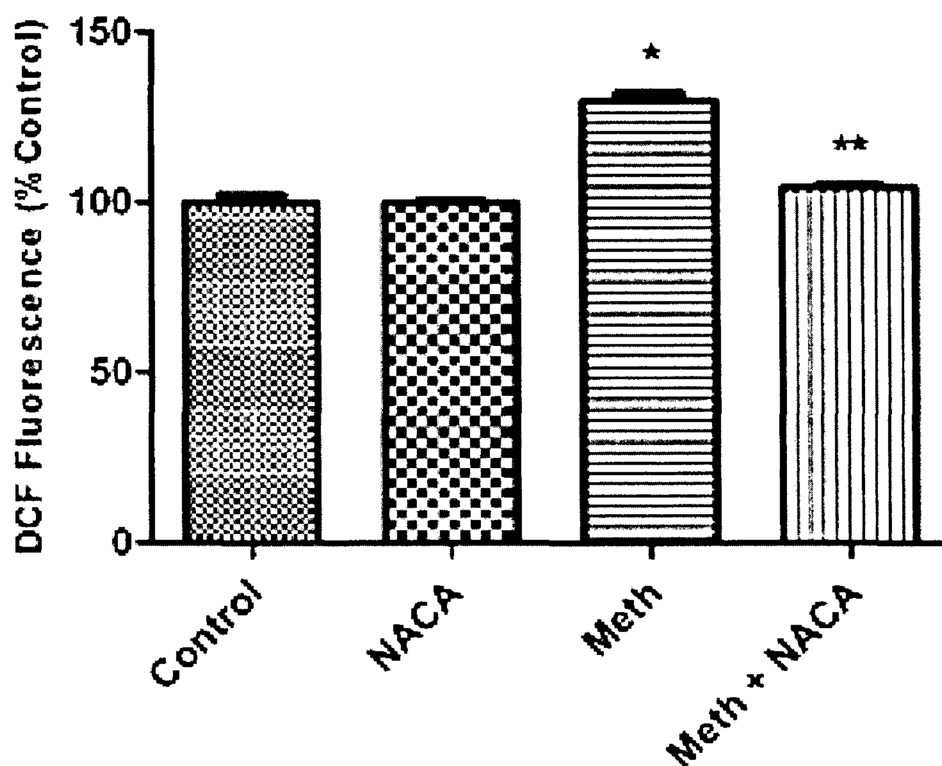


Figure 4.4. ROS Levels in ARPE-19 cells after Treatment with METH and NACA. ROS levels were measured after 4 hours of treatment for control, NACA, METH, and METH+NACA groups. The ROS level in the NACA-only group was very similar to that of the control group. However, a concentration of 500 μM METH significantly decreased the ROS level (* $p \leq 0.05$). Pretreatment with 1.0 mM NACA returned the ROS level to near control level (** $p \leq 0.05$). The results are representative of at least three independent experiments.

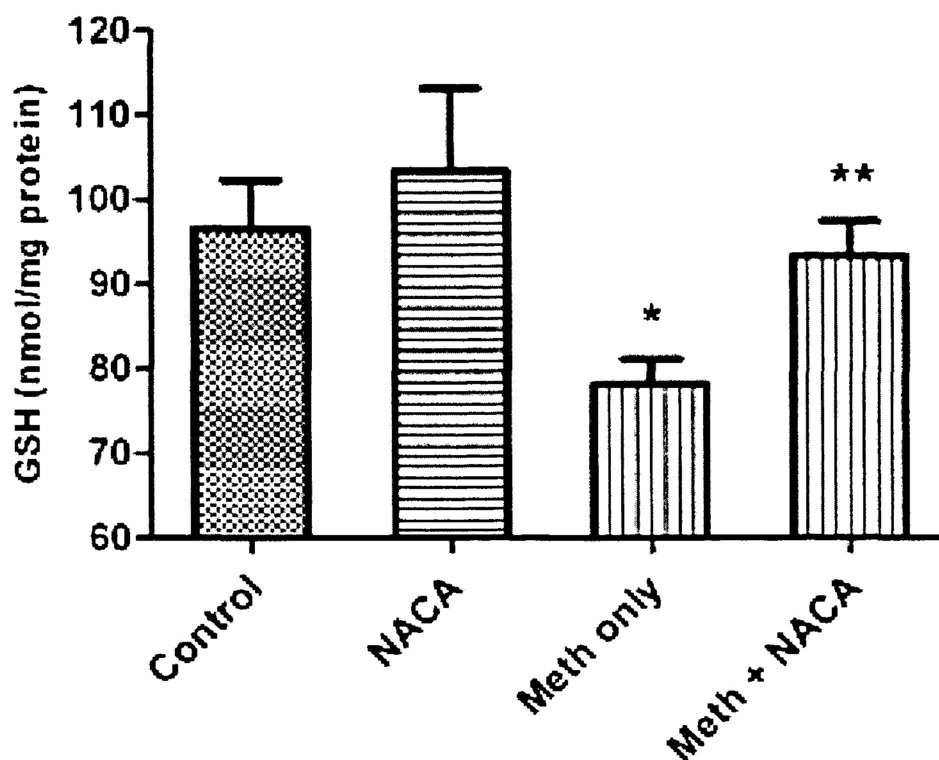


Figure 4.5. Intracellular GSH Levels in ARPE-19 Cells after Treatment with METH and NACA. GSH levels were measured after 24 hours of treatment for control, NACA, METH, and METH+NACA groups. The GSH level in the NACA-only group is similar to that of the control. Exposure to METH (500 μM) significantly decreased intracellular GSH (* $p \leq 0.05$). Pretreatment with NACA (1.0 mM), 2 hours before the addition of METH, prevented such a dramatic decrease (** $p \leq 0.05$). At least three independent experiments were performed.

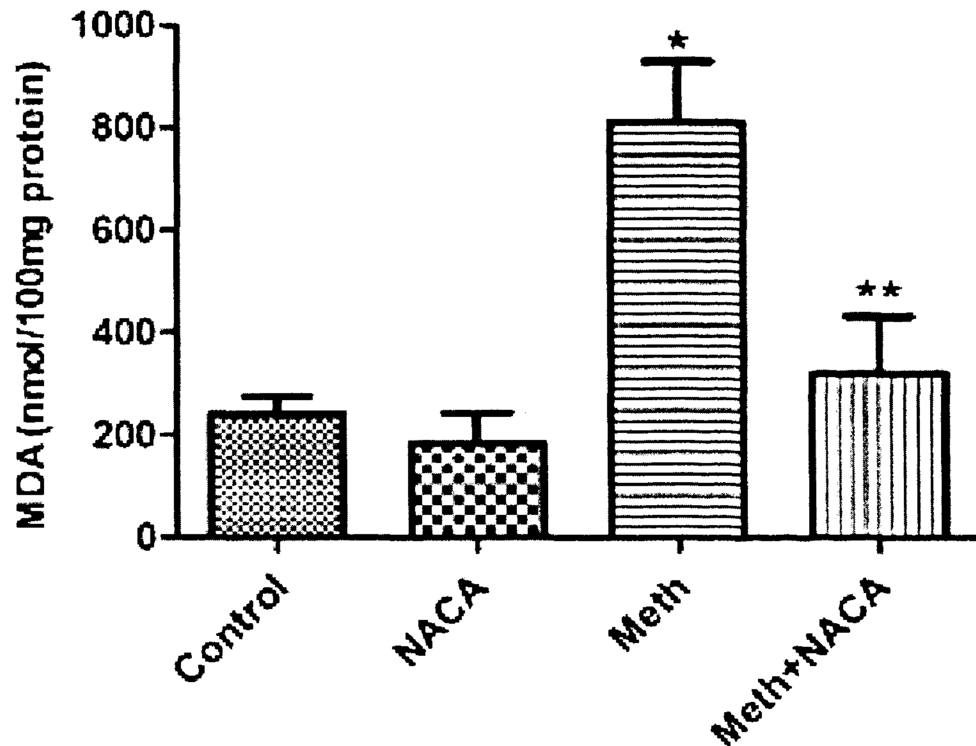


Figure 4.6. MDA Levels in ARPE-19 Cells after Treatment with METH and NACA. It was found that after 24 hours the MDA levels significantly increased. MDA levels in the NACA-only group were very similar to that of the control group. METH (500 μM) induced a significant increase in the MDA level. Pretreatment with 1.0 mM of NACA decreased lipid peroxidation significantly below that of METH alone. Values represent mean ± SD. '*' refers to significant differences from the control with $p \leq 0.05$ and '**' refers to the significant differences from the METH-only group. The graph is representative of three independent experiments.

Group	CAT (mU/mg protein)	GPx (mU/mg protein)
Control	19.5 ± 3.1	56.6 ± 9.8
NACA only	20.1 ± 5.2	59.5 ± 8.4
Meth only	14.1 ± 0.6*	39.2 ± 1.7*
Meth + NACA	19.1 ± 7.8	51.0 ± 7.4**

Table 4.1. Catalase and GPx Activity in ARPE-19 Cells after Treatment with Meth and NACA. CAT and GPx activity in METH-treated cells were decreased significantly during METH exposure. Pretreatment with NACA helped accelerate the activity of both CAT and GPx activity to METH-treated cells to values similar to that of control activity. NACA alone did not significantly alter the results compared to the control. Values represent mean ± SD. '*' refers to significant differences from the control with $p \leq 0.05$ and '**' refers to the significant differences from the METH-only group. The values are representative of three independent experiments.

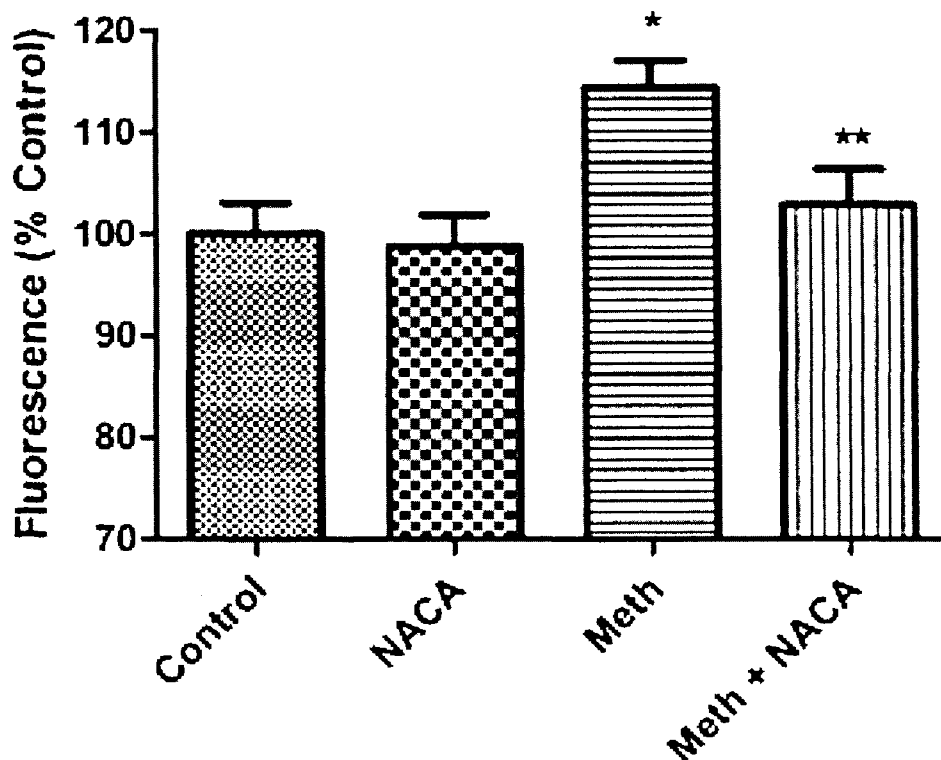


Figure 4.7. Protective Effects of NACA on Dextran Permeability for ARPE-19 Cells Treated with METH. Cells were seeded onto a collagen-coated insert with a pore size of 0.4 μm at a density of 1.5×10^5/well, and allowed to culture until a monolayer formed. The cell monolayer was then treated with 500 μM of METH with or without the 2-hour pretreatment using 1.0 mM of NACA for 24 hours. Significantly higher permeability of dextran across the cell monolayer was observed after treatment with METH, as compared to the control (* $p \leq 0.05$). Pretreatment with NACA, however, was shown to reduce this permeability (** $p \leq 0.05$) to levels similar to control values. At least three independent experiments were performed for resistance measurements.

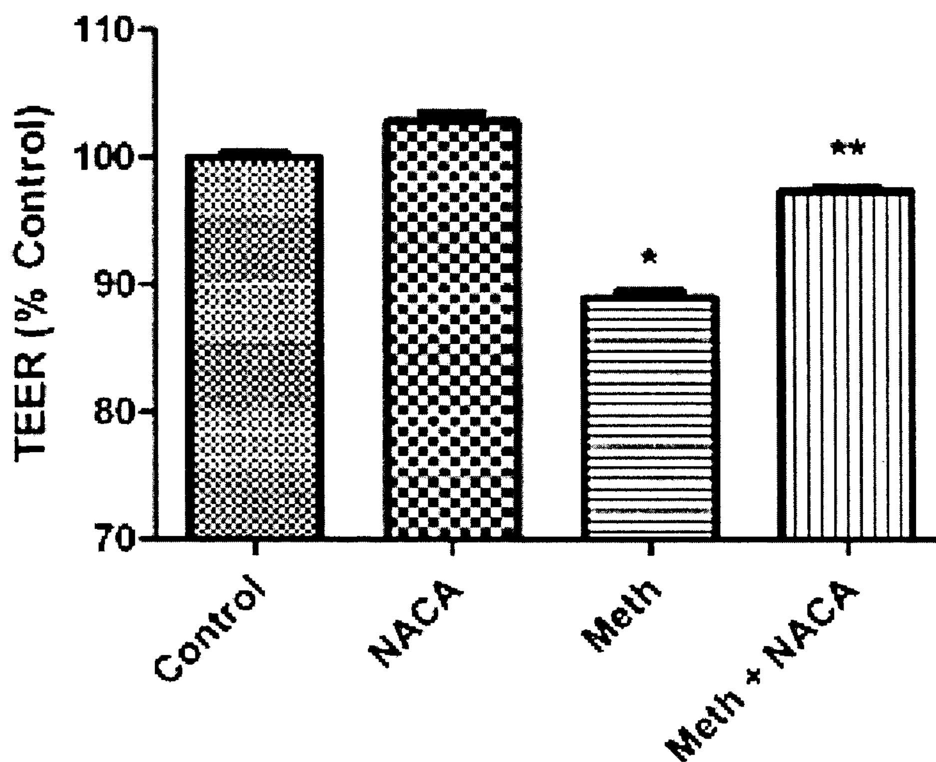


Figure 4.8. Protective Effects of NACA on TEER for ARPE-19 Cells Treated with METH. Cells were seeded onto a collagen-coated insert with a pore size of 0.4 μm at a density of 1.5×10^5/well, and allowed to culture until a monolayer formed. The cell monolayer was then treated with 500 μM of METH with or without the 2-hour pretreatment using 1.0 mM of NACA for 24 hours. Cells treated with only METH resulted in significantly decreased TEER values, as compared with both control (* $p \leq 0.05$) and METH+NACA (** $p \leq 0.05$) groups. At least three independent experiments were performed for resistance measurements.

4.3. DISCUSSION

Oxidative effects of METH on retinal pigmented epithelial cells have not been extensively studied, despite the fact that METH abuse is quite prevalent worldwide, due to the euphoria produced, its wide availability, and relatively low cost. More effective treatments are essential to prevent retinal degeneration induced by increased use of METH.

Herein, we report the in vitro effect of METH on human retinal cells (ARPE-19 cell line) and the role of a novel thiol antioxidant, N-acetylcysteine amide (NACA), in preventing RPE cell death and to maintain RPE redox status. Results indicate that NACA prevented oxidative damage, possibly by scavenging existing ROS, while halting the production of further ROS and lipid peroxidation. In addition, NACA acted by increasing levels of reduced glutathione (GSH), as well as the activity of the detoxification enzyme, glutathione peroxidase (GPx). Significant decreases in cell viability and GSH levels were observed upon METH exposure, indicating oxidative stress. Decreased GSH levels could possibly lead to reduced cell viability. Pretreatment with NACA increased the GSH levels and cell viability in the METH-treated group, indicating that NACA had replenished the GSH levels in these cells. Our previous results showed that increased ROS production in cells depleted the intracellular levels of GSH inside cells [81]. METH-treated cells also had significant increases (~30%) in ROS accumulation, which was abrogated with NACA treatment. These results are also in accordance with our previous study on METH-induced toxicity [91]. The protective effect of NACA was probably mediated by NACA's ability to increase GSH biosynthesis by reducing extracellular cystine to cysteine, and/or by supplying the sulfydryl groups that can stimulate GSH biosynthesis, or by conversion of GSSG to GSH by non-enzymatic thiol disulfide exchange [98].

Lipid peroxidation is thought to play an important role in the pathogenesis of age-related macular degeneration, and is a consequence of increased levels of ROS in the macula [99]. The membranes of outer segments of photoreceptors are rich in poly unsaturated fatty acids (PUFAs), which are highly susceptible to radical damage and peroxidation [100]. Increased levels of lipid peroxidation products can also lead to lysosomal dysfunction in RPE [99]. Therefore, MDA, an important lipid peroxidation by-product, showed a significant increase in the levels when measured in ARPE-19 cells treated with METH. Lipid peroxidation is a key mechanism by which METH and its radicals induce cell death. It has been postulated that radicals attack membrane lipids and initiate a chain of events leading to lipid peroxidation [101-102]. Pretreatment with NACA successfully reversed the increase in MDA levels, indicating that NACA can protect RPE cells from lipid peroxidation. Preventing this is important for improving RPE lysosomal function, as highlighted in a study of the effects of N-acetylcysteine on lysosomal volume and metabolism in RPE cells loaded with regular or oxidized human and porcine rod outer segments [103]. These results are also in line with a previously reported increase in MDA levels [95]. Concomitant reduction of GSH levels (a substrate for glutathione peroxidase) might have hampered the decomposition of lipid peroxides in METH-treated animals. NACA was able to prevent lipid peroxidation by supplying an adequate amount of GSH as a substrate for glutathione peroxidase to effectively decompose lipid peroxides, thus reducing MDA levels.

A significant reduction in the activity of GPx and catalase was observed after METH administration. For GPx this may have been partially due to diminished GSH levels that GPx needs as a substrate. Decrease in GPx activity under oxidative stress has been reported previously [91, 104]. NACA pretreatment reversed catalase activity in the METH+NACA group. This decrease was reported in various tissues that were undergoing oxidative stress

[105]. In contrast, another study reported an increase in catalase activity when RPE cells were challenged with H_2O_2 [106]. On the other hand, Melo et al. were unable to find differences in the catalase activity in the retinas of METH-treated rats [107]. The response of this antioxidant enzyme to oxidative agents could be tissue/organ specific and may be adaptive. A possible protective mechanism of NACA may have been by scavenging free radicals or by providing more GSH, which is a substrate for GPx. However, further investigation is needed to confirm this theory.

In the retina, RPE forms the blood-retinal barrier (BRB) by forming tight junctions that control the exchange of nutrients and metabolites between the retina and the underlying capillaries. It is thought that oxidative stress may induce changes in the BRB, which may contribute to the pathogenesis of retinal degeneration. It is, therefore, important to determine if NACA can protect the junctional integrity of ARPE-19 cells exposed to oxidative stress. NACA prevented a METH-induced decrease in TEER, verifying its ability to protect cellular homeostasis and outer BRB integrity. Such a decrease in TEER was seen in another study, where ARPE-19 cells were exposed to hydrogen peroxide [108]. All of these results collectively indicate that METH induces oxidative stress in retina pigmented epithelial cells and NACA prevents these cells against METH-induced oxidative stress. More studies should be performed to explain the cellular signaling mechanisms in which NACA protects retina pigmented epithelial cells exposed to METH.

5. IN VIVO INHIBITION OF L-BUTHIONINE-(S,R)-SULFOXIMINE-INDUCED CATARACTS BY A NOVEL ANTIOXIDANT, N-ACETYLCYSTEINE AMIDE

L-Buthionine-(S,R)-sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase [109], is commonly used to deplete GSH levels and study the effects of GSH in both *in vitro* and *in vivo* models. The role of GSH in maintaining the transparency of a lens can be investigated and determined by studying an animal model of a BSO-induced cataract. GSH levels were found to decrease by approximately 90% in human lymphoid cells cultured in the presence of BSO [21]. Reduced levels of GSH led to the clouding of the lens in newborn rats due to uninhibited oxidation of cytosolic proteins and membrane lipids [14,110-111]. BSO was shown to induce an age-dependent cataract in animals [109,112-114]. BSO also indirectly reduced the levels of glutathione peroxidase, glutathione reductase, and glutathione S-transferase by inhibiting GSH formation in these animal models. BSO or selenite-induced cataracts were prevented or reduced in frequency in vivo by using well-known antioxidants such as esters of GSH [109,113], lipoic acid [114], acetyl-L-carnitine [115], melatonin [116-117], ascorbate [112], and N-acetylcysteine (NAC) [118]. BSO also induced lipid peroxidation, as it increased malondialdehyde (MDA) levels [119-120].

Antioxidants are becoming more popular for use in preventing oxidative stress-related disorders. Thiol antioxidants, such as cysteine, glutathione, and NAC, have been shown to provide some protection against various oxidative stress-related disorders [81,96-97]. However, N-acetylcysteine amide (NACA) (Fig. 1A), a modified form of NAC (Fig. 1B), was found to be more effective because of its ability to permeate cell membranes [97,121]. This allows NACA to be administered at lower doses than NAC [122].

In this study, the effects of N-acetylcysteine amide (NACA), a free radical scavenger, on cataract development were evaluated in Wistar rat pups. Cataract formation was induced in these animals with an intraperitoneal injection of BSO. To evaluate NACA's ability in preventing cataract formation, this compound was administered prior to injection of BSO and treatments were given daily until the conclusion of the experiment. The findings within demonstrate that NACA inhibits cataract formation significantly (80%) in Wistar rats treated with BSO.

5.1. EXPERIMENTAL DESIGN

Lactating female Wistar rats with 2-day-old 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-hour light and dark cycle. The animals had unlimited access to rodent chow and water and were utilized after 1 day 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 rats were divided into four groups, (1) control, (2) BSO only, (3) NACA only, and (4) NACA+BSO, so that each group contained one female lactating rat with 10 male pups. All rat pups in the BSO-only and NACA+BSO groups received an i.p. injection of either saline or NACA (250 mg/kg body wt), 30 minutes before an i.p. injection of BSO (4 mmol/kg body wt) once a day for the first 3 days of the experiment. The rat pups in the control and NACA-only groups were given a daily injection of either saline or NACA (250 mg/kg body wt). After the first 3 days of the experiment, the rat pups in all four groups were then given an i.p. injection of saline or NACA (250 mg/kg body wt) once every other day until all of the pups' eyes were open (approximately 15 days). Grading of the cataract

formation was performed with the use of a slit microscope on the last day of injection, along with picture documentation. All rats were anesthetized 24 hours after the last saline or NACA injection by i.p. injection with a 40% urethane solution (0.1 ml/10 g body wt). All rat pups were massed at the beginning and the end of the study. After sacrifice, their lenses were harvested, rinsed with PBS solution, and then immediately placed on dry ice. Samples were stored at a temperature of -80 °C for further analysis. Their lenses were then analyzed for selective oxidative stress parameters, including glutathione (reduced and oxidized), protein carbonyls, catalase, glutathione peroxidase, glutathione reductase, and malondialdehyde.

5.2. RESULTS

5.2.1. Effects of BSO and NACA on Cataract Formation in the Lens. An injection of 4 mmol/kg body wt of BSO, administered once daily for 3 days, 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 also found that all rats within the BSO-only group developed cataracts. Of the lenses examined within this group, 60% were classified as grade 2 and the other 40% were classified as grade 3. However, in the rats treated with NACA in conjunction with BSO, it was found that only 20% of the lenses had varying degrees of opacity. The majority of lenses were of a clarity similar to that seen in the control group. Lenses observed within the control and NACA-only groups were completely clear. The grading of the lenses in all groups can be found tabulated in Table 5.1. Slit-lamp photos of the lenticular opacities observed for each group are shown in Figures 5.1.

5.2.2. Effects of BSO and NACA on GSH and GSSG Levels in the Lens. Glutathione levels within the cell give a good indication as to the redox status of the lens

itself. GSH levels measured in the lenses of rat pups within the BSO-only group were significantly lower than levels found in lenses obtained from the control group. The levels of GSH found in the lenses of the NACA-only treated rat pups were very similar to those found in the control group. The rat pups that received injections of NACA while being exposed to BSO were found to have significantly higher levels of GSH in the lenses (approximately 50%) compared with the BSO-only group; however, these GSH levels did not quite reach those found in the control group (Figure 5.2.).

Glutathione disulphide (GSSG) levels in the lenses of the BSO-only group were found to have significantly increased by approximately four times the GSSG levels in the control group. The amounts of GSSG found in lenses with NACA treatment during exposure to BSO were significantly lower than levels found in the BSO-only group. These were approximately two times higher than the levels determined for the control group. Treatment with NACA alone was also found to contribute to an estimated 50% increase in the GSSG levels, compared with those of the control group. A graph with these results is shown in Figure 5.3.

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 next highest ratio was found in the NACA-only group, which had a value of about 65% of that of the control group. The NACA+BSO group was found to have a ratio of GSH to GSSG of approximately 30% of the control group value. Finally, the BSO-only group was found to have a GSH to GSSG ratio of about 10% of the value calculated for the control group (Figure 5.4.).

5.2.3. Effects of BSO and NACA on Protein Carbonyl Levels. In order to estimate the amount of protein oxidation present in the lens, protein carbonyl levels were determined. Animals in the BSO-only group had a fourfold increase in protein carbonyl levels within the lens compared to the control group. Levels of protein carbonyls in the NACA group were not significantly different from control levels. However, when animals were pretreated with NACA before exposure to BSO, protein carbonyl levels within the lens were significantly lower than those in the BSO-treated group (Figure 5.5.).

5.2.4. Effects of BSO and NACA on GR, GPx, and Catalase Levels in the Lens. The activity of three major antioxidant enzymes: glutathione reductase (GR), glutathione peroxidase (GPx), and catalase were measured within the lens. Animals injected with BSO only had significantly lower levels of the enzymes GR, GPx, and catalase in their lenses, compared to animals in the control group and the NACA-only treated group. The rat pups that were treated with NACA before exposure to BSO had significant increases in these enzyme levels, compared to the BSO-only animals, returning them to levels similar to those of the control group (Table 5.2.). No significant differences between GR, GPx, and catalase activities for the control and NACA-only groups were determined.

5.2.5. Effects of BSO and NACA on Lipid Peroxidation in the Lens. The levels of malondialdehyde (MDA) were measured in the lens samples to detect the extent of lipid peroxidation which consistently high in lens containing cataracts. These results indicate that there was a significant twofold increase in MDA levels in the BSO-only group compared to the control group. However, MDA levels in the rat lens of the NACA+BSO group were significantly lower than those in the BSO-only group and approached the control level (Figure 5.6.).

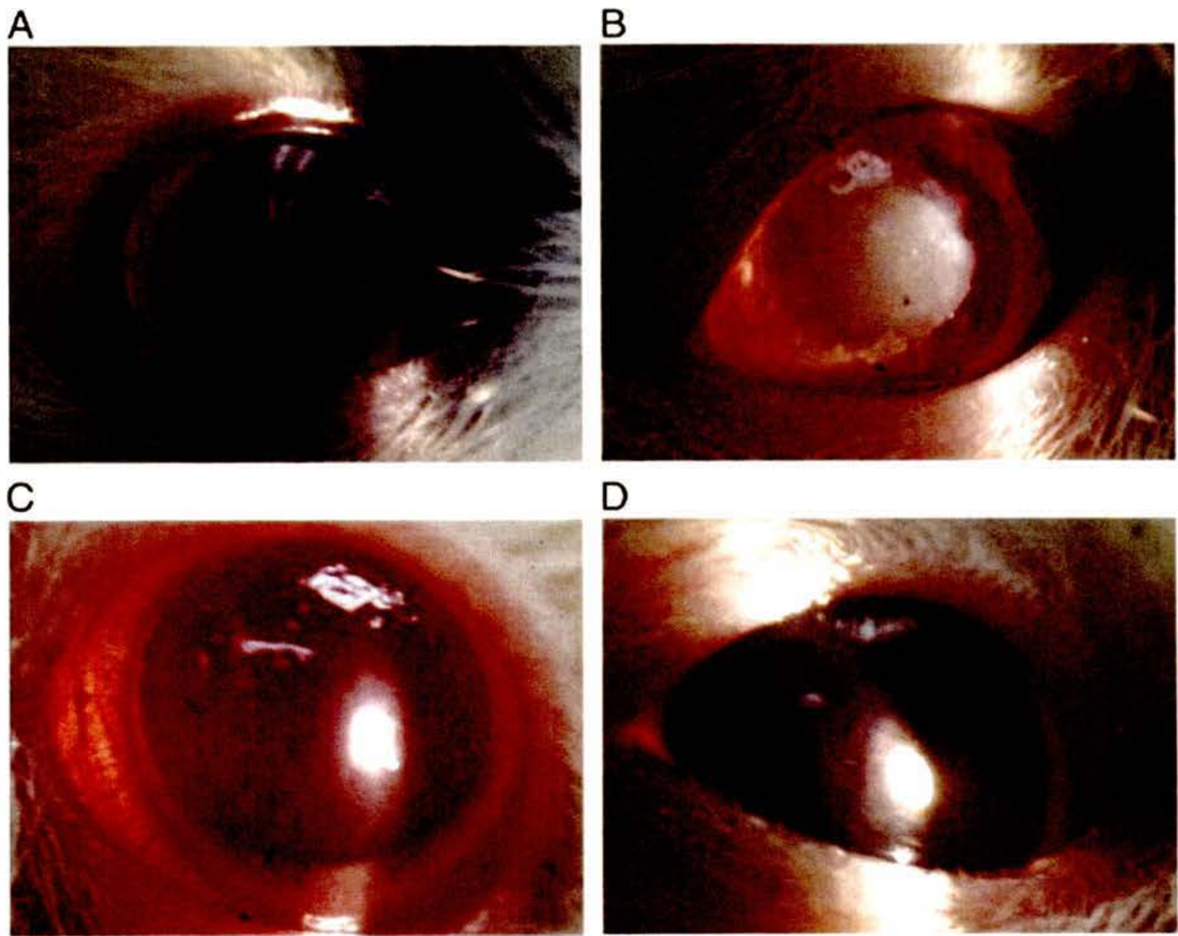


Figure 5.1. Lens Photos. Images of cataract formation utilizing a slit-lamp microscope at 10× original magnification and a digital camera in macro mode are shown. These pictures were taken when the rat pups were 15 days of age, 1 day before sacrifice. A representative picture of the lenses observed for each group is shown. (A) Control lens. The lenses in this group were found to contain no detectable cataracts. (B) BSO-only lens. All lenses in this group developed very distinct cataracts, with most being nearly completely opaque. (C) NACA-only lens. Results similar to those in the control group were obtained, with no detectable signs of cataract formation. (D) NACA+BSO lens. The lens depicted has a grade 1 opacity, which was evident by the amount of scattering light.

Group	Grade 0	Grade 1	Grade 2	Grade 3
Control	20	0	0	0
BSO only	0	0	12	8
NACA only	20	0	0	0
NACA + BSO	16	2	1	1

Table 5.1. Degree of Cataract Formation within the Lenses after i.p. Injections of NACA. The lenses (within their associated groups) that developed varying degrees of opacities are summarized. The degrees of opacity are defined as follows: grade 0, normal transparent lens; grade 1, presence of the scattering of light or initial signs of opacity; grade 2, presence of a partial nuclear opacity; grade 3, presence of complete or nearly complete nuclear opacity.

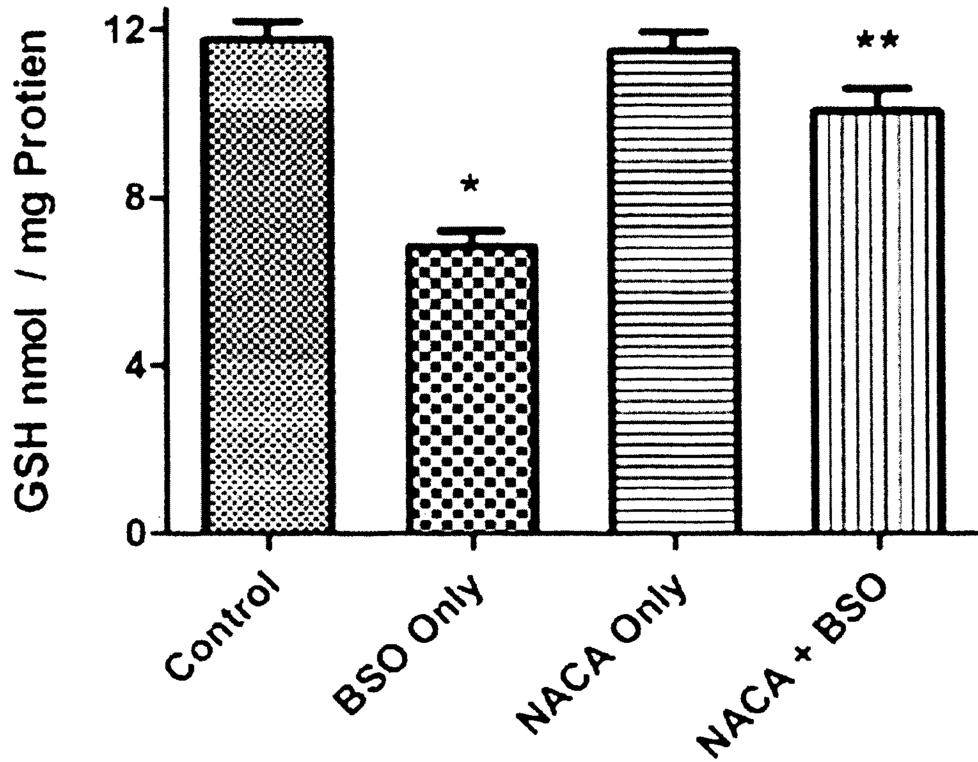


Figure 5.2. Glutathione Levels within the Lens. The GSH level in the NACA-only group was similar to that of the control; exposure to BSO significantly decreased the amount of GSH within the lens. Exposure of rat pups to BSO and treatment with NACA was found to prevent such a dramatic decrease. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

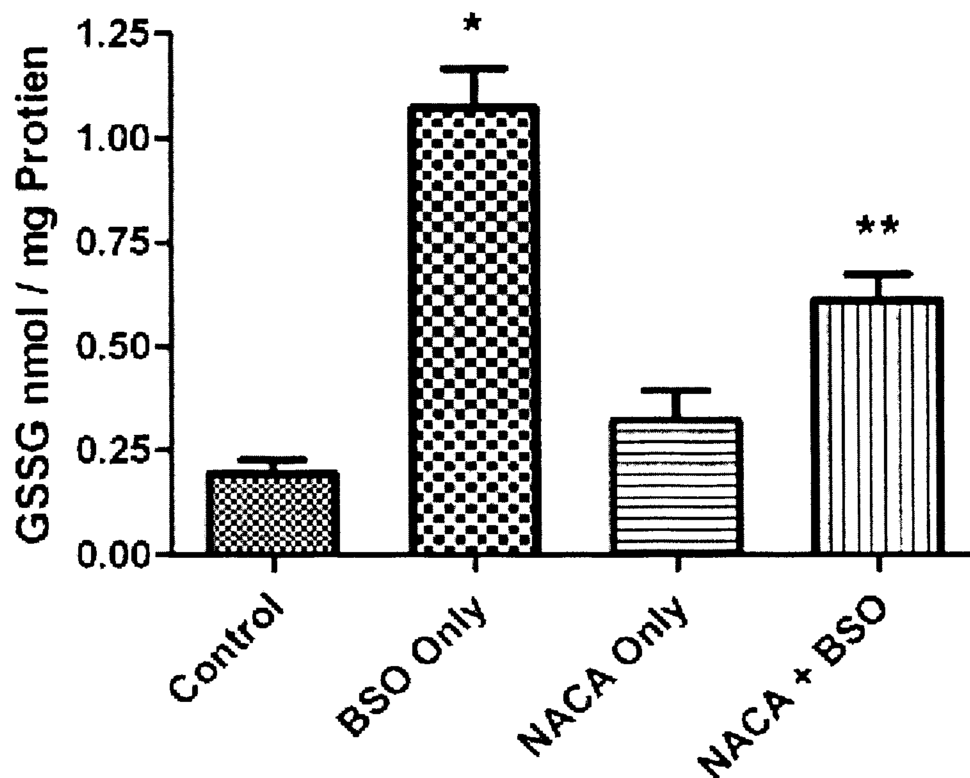


Figure 5.3. Glutathione Disulphide Levels within the Lens. The GSSG level in the control group was significantly higher in the BSO-only group than in the control. This GSSG level was significantly reduced when BSO given pups were treated with NACA. A small increase was also observed in the NACA-only group, but this may have been due to a NACA–GSH interaction. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

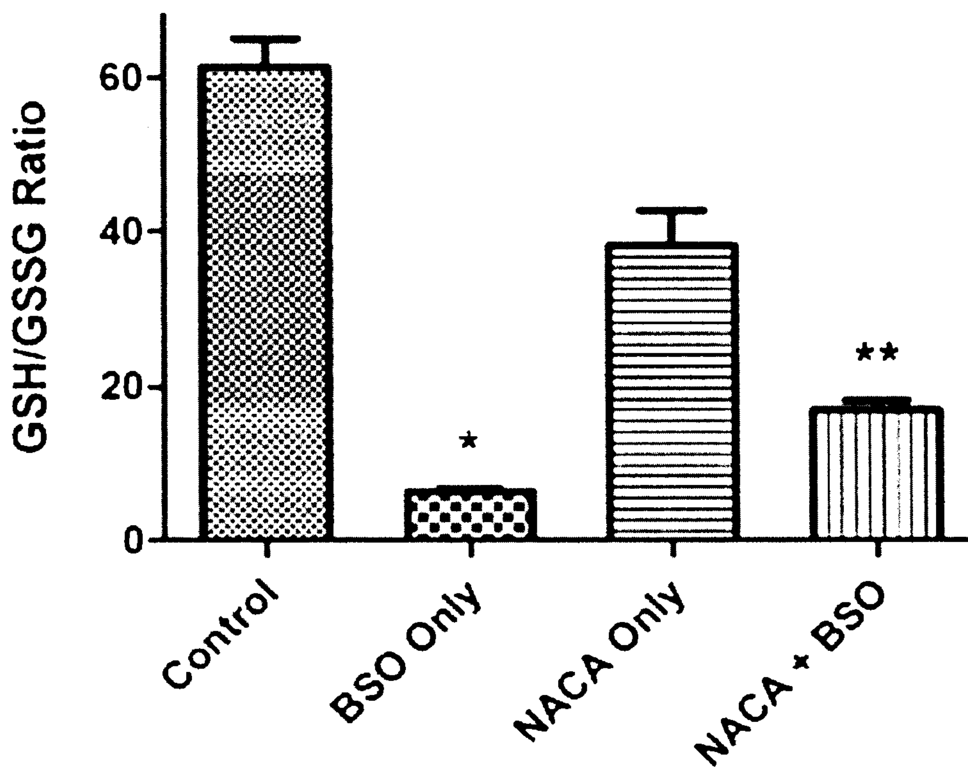


Figure 5.4. GSH/GSSG Ratio within the Lens. A more accurate picture of the redox status within the lens is obtained when looking at the ratio of reduced glutathione (GSH) to glutathione disulphide (GSSG). The higher the value of this ratio the more of a reduced environment is present within the sample. The BSO-only group had a significantly lower ratio than the control group. NACA treatment was found to increase this ration but not to the amount determined within the control group. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

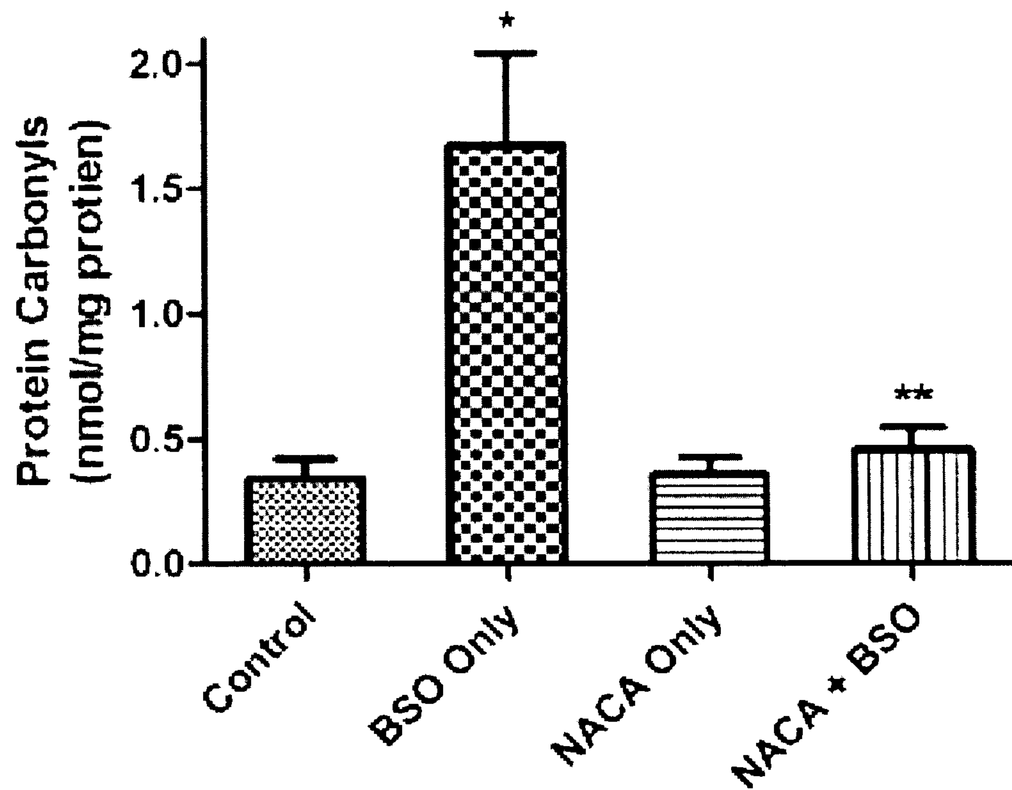


Figure 5.5. Protein Carbonyl Levels within the Lens. Protein carbonyl levels were measured in homogenized lens samples for control, BSO-only, NACA-only, and NACA+BSO groups. The data show that protein oxidation is significantly increased in the lenses of rats exposed to BSO. Pretreatment with NACA significantly reduced the levels of protein carbonyls in rats also treated with BSO to levels similar to that of the control group. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

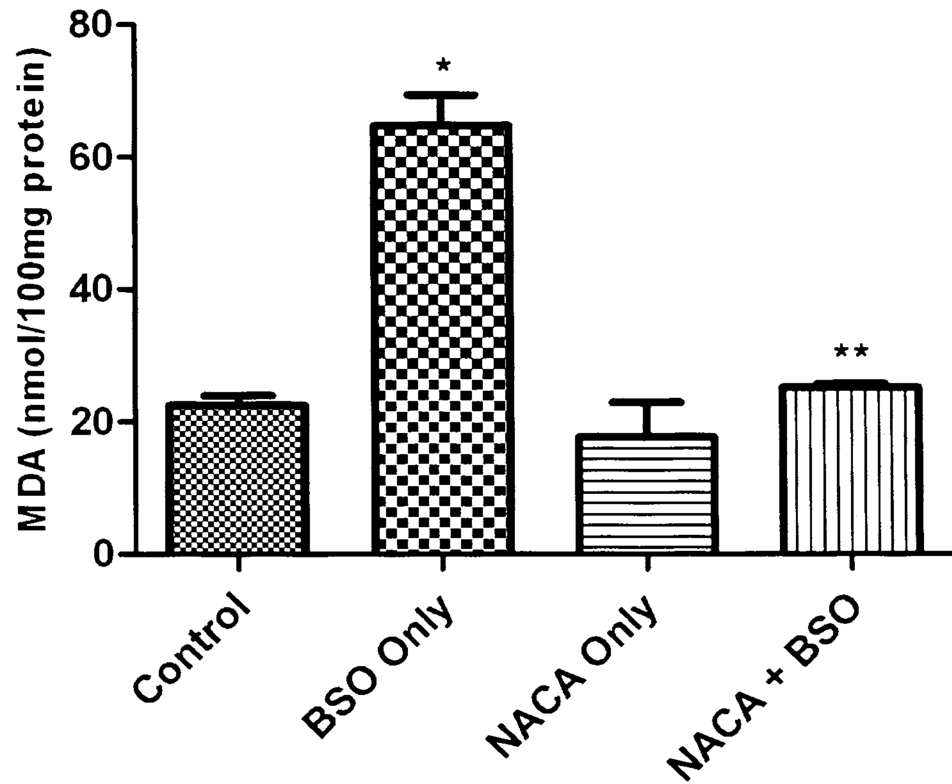


Figure 5.6. Malondialdehyde Levels within the Lens. MDA levels within the lenses of animals in each group where determined upon homogenization of samples. Results indicate that the amount of malondialdehyde in the lenses of the NACA-only group were very similar to those of the control group, while the BSO-only group showed elevated lipid peroxidation due to the significantly larger amount of MDA detected. Treatments with NACA were able to keep this oxidative stress parameter to levels similar to that of the control. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

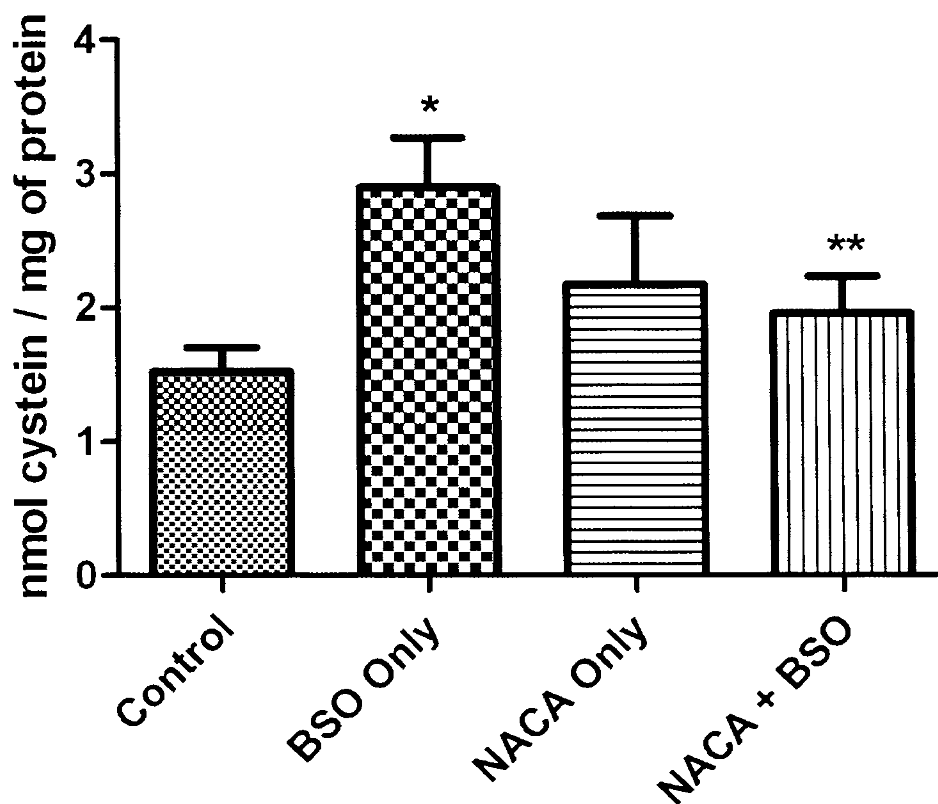


Figure 5.7. Cysteine Levels within the Lens. The amount of cysteine within the lenses of animals in each group where determined simultaneously with glutathione determination. Results indicate that the amount of cysteine in the lenses of the BSO-only group showed elevated cysteine due to this compounds ability to inhibit the activity of gamma glutamylcysteine synthetase. Treatments with NACA were able to minimize the amount of cysteine available bringing this amino acid to levels similar to that of the control. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

Group	GR (mU/mg protein)	Catalase (mU/mg protein)	GPx (mU/mg protein)
Control	2.13 ± 0.18	8.21 ± 0.48	50.0 ± 3.6
BSO only	1.36 ± 0.31*	5.12 ± 0.62*	36.9 ± 4.6*
NACA only	2.08 ± 0.16	7.93 ± 0.55	47.5 ± 3.0
NACA + BSO	1.94 ± 0.22**	7.26 ± 0.39**	43.6 ± 2.4**

Table 5.2. Antioxidant Enzyme Activity of GR, Catalase, and GPx. Glutathione reductase (GR) activity, catalase activity and glutathione peroxidase (GPx) activity within the lenses of animals in each group were determined after homogenization of the sample. Results indicate that the activity of these antioxidant enzymes in the NACA-only group were very similar to those of the control group, while the BSO-only group had a significant reduction in each enzymatic activity. Treatments with NACA were able to maintain the activity of these enzymes while being exposed to the cataract inducing agent. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

5.3. DISCUSSION

Oxidative stress is the result of an imbalance of antioxidants and pro-oxidants. Lens opacity, due to cataract formation, is directly attributed to oxidative processes that occur 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 glutathione, lipid peroxidation, and a decrease in antioxidant enzyme activity [123-125]. Glutathione is an indispensable and primary lenticular antioxidant [126]. There is a wide body of evidence that indicates loss of glutathione occurs because of the oxidation of glutathione to GSSG, because its levels increase drastically once the cataracts develop. In addition, hyperbaric oxygen causes cataracts, as it is characterized by the loss of GSH and protein–SH, as well as protein insolubilization [127]. Therefore, an alternative method for treating or preventing the occurrence of cataracts would be through the use of a potent thiol-exchange compound.

With this background, we have evaluated the effects of a novel antioxidant and a potent thiol exchanger, N-acetylcysteine amide, in the inhibition of cataracts induced by BSO in Wistar rat pups. BSO has been used to induce cataracts in animal models [128]. Results from morphological observations indicate that NACA is able to prevent, or at least significantly reduce, the opacification of the lens within an experimental cataract model. This premise is very evident from our study, in that 80% of the rats treated with BSO and NACA did not develop any opacification of the lens, whereas 100% of the rats treated with BSO alone developed lenticular opacification (Table 5.1). Previous studies have shown that 60% of the rats treated with BSO and antioxidant had reduced lenticular opacification, compared to the BSO-only treated rats [129].

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 [130], as well as playing an important role in antioxidant defense and redox regulation [131]. Studies indicate that loss of GSH will directly affect the activity of the GSH-dependent enzyme glutathione reductase. This enzyme plays an important role in GSH homeostasis: it regenerates GSH from GSSG [132]. Based on the current literature, more than 90% of protein sulfhydryl (protein–SH) groups are lost in the most advanced cataracts [133]. Considering the above two facts, results from this study indicate that treatment with NACA decreases the oxidative damage. GSH levels can be significantly restored in rats to a level 90% of that of the control groups during BSO exposure (Figure 5.5). Supporting these data, it was observed that NACA caused an increase in GR activity in BSO-treated rats (Table 5.2). Increases in GSH levels and the GSH/GSSG ratio (Figure 5.7) may be attributed to the increased activity of GR by preserving the integrity of cell membranes and by stabilizing the sulfhydryl groups of proteins. Further, NACA itself may act as a sulfhydryl group donor for GSH synthesis and thereby decreasing the loss of protein sulfhydryl groups, as well as opacification of the lens.

NACA was shown to have a protective effect on other portions of the lens's antioxidant defenses as well. Decreasing the levels of GSH when reactive oxygen species are present can trigger a cascade of further oxidative damage. Lipid peroxidation has been associated with the formation of cataracts in patients [119,133-135]. The extent of lipid peroxidation was determined in this study by measuring the amount of MDA, a by-product of lipid peroxidation, within the lens. Concomitant reduction of GSH levels contributed to its rapid metabolism as a substrate for glutathione peroxidase to eliminate lipid peroxides from BSO-exposed rats. Unavailability of GSH as a substrate for glutathione peroxidase

stalls the process of lipid peroxide decomposition, thus increasing the levels of MDA. NACA supplied an adequate amount of GSH as a substrate for glutathione peroxidase to effectively decompose lipid peroxides in the rats, reducing MDA levels (Table 5.2).

It is well established that proteins, like lipids and DNA, are oxidized by ROS. Oxidative damage to lens proteins is an important factor for cataract formation [136-138]. Protein carbonyls are commonly used as an indicator of protein oxidation in many tissues [139]. Our results showed that protein carbonyls had been significantly increased in BSO-treated animals and this was reversed by NACA (Fig. 4). Depletion of GSH by BSO initiates a myriad of events. There is an initial increase in free radicals that overwhelms the scavenging ability of the GSH-dependent enzymes (glutathione peroxidase), which leads to oxidation of lipids and proteins. Several studies have indicated a positive correlation between protein carbonyl levels and the genesis of cataract formation [140-142]. 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. Thiol-containing compounds have been shown to inhibit cataract formation in previous studies by reducing protein oxidation in the lens [114,115].

NACA's other antioxidant effect was evident in changes seen in cysteine levels. In the BSO-only group, we observed a significant increase in the amount of cysteine (Table 2). This is an expected result of inhibition of the γ-glutamylcysteine synthetase enzyme by BSO. The NACA-only group had increased levels of cysteine as well, due to deacetylation of NACA itself. Significantly increased levels of this particular amino acid were observed in our previous studies using other tissue samples [81,96]. The NACA+BSO group showed significant decreases in cysteine levels. This may seem contradictory, considering the BSO-only and NACA-only results. However, we believe that cysteine made available by NACA

and BSO is nonenzymatically used for GSH formation. NACA has been shown to increase GSH nonenzymatically [81,143], thereby explaining the decrease in cysteine and the increase in GSH in the NACA+BSO lenses.

Catalase and GPx are two enzymes involved in minimizing ROS and recycling glutathione through an oxidation–reduction cycle. Studies have shown that activities of catalase and GPx were significantly decreased in lenses that were exposed to BSO [114,116]. Our study supported this, because NACA was able to restore both catalase and GPx to those levels seen within the control group (Table 5.2). The possible mechanism for the restored catalase activity in BSO-exposed rats, when treated with NACA, may be the scavenging of free radicals by NACA. However, further investigation is needed to confirm this theory. Increased GPx activity is probably due to higher levels of GSH, which GPx uses as a substrate for its action.

In summary, these results suggest strongly that antioxidants have the ability to protect against or to delay the onset of cataract formation by reducing oxidative damage. Our results suggest that NACA can prevent the formation of cataracts by directly and indirectly maintaining GSH levels 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 and the ability to maintain antioxidant levels within the lens and, possibly, through disulfide-exchange mechanisms. Treatment with NACA may prove to have a major therapeutic role. In future studies, we will focus on the prophylactic role of NACA on induced cataract formation and investigate the development of a topical formulation for the application of this antioxidant.

6. THERAPUTIC EFFECTS OF N-ACETYLCYSTEINE AMIDE EYE DROPS IN REVERSING THE OPACITY OF LENS CONTAINING CATARACTS

The effect of N-acetylcysteine amide (NACA), a free radical scavenger, on cataract development was evaluated in Wistar rat pups. Cataract formation was induced in these animals with an i.p. injection of a glutathione (GSH) synthesis inhibitor, L-buthionine-(S,R)-sulfoximine (BSO). In order to assess whether NACA eye drops would be effective in reversing Grade I and Grade II BSO-induced cataracts, we divided newborn male rat pups into five groups. Each group received a different i.p. injection for 3 days, starting on postpartum day 9: (1) a sham-treated control group received saline; (2) a BSO-only group received BSO at 4 mmol/kg body weight; (3) a NACA-only group received saline; (4) a NACA + Grade I cataract group received BSO at 4 mmol/kg body weight; and (5) a NACA+Grade II cataract group received BSO at 4 mmol/kg body weight. Groups receiving BSO injections were monitored for grade I and II cataract formation. Once cataracts formed, the rat pups received two 10 μL drops of 1.0% NACA/buffer solution into each eye, twice a day for 3 weeks. After completing visual examinations under a slit-lamp microscope, rats were sacrificed and samples were prepared for assessment of antioxidant levels. Our results indicated that NACA eye drops were able to reverse Grade I cataracts completely to Grade 0, whereas for Grade II, 62% were reverted to Grade 0 and 38% to Grade I cataracts. In addition, NACA helped restore levels of GSH and antioxidant enzyme activity. NACA eye drops increased GSH levels significantly in the BSO-treated groups with a higher increase in Grade I cataracts in comparison with Grade II cataracts. Similarly, GSSG levels were markedly reduced in NACA- treated groups with a greater decrease in the case of Grade I cataracts. NACA was also able to significantly restore antioxidant enzymatic activity.

6.1. EXPERIMENTAL DESIGN

Lactating female Wistar rats with 2-day-old 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-hour light and dark cycle. The animals had unlimited access to rodent chow and water and were utilized after 1 day 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 rats were divided into five groups, (1) control, (2) NACA only, (3) BSO only, (4) Grade I + NACA, and (5) Grade II + NACA so that each group contained one female lactating rat with 10 male pups. Once the rat pups had aged to 9 days the animals to develop cataracts were given an i.p. injection of BSO (4 mmol / kg body wt) once a day for three consecutive days. All other rats received an i.p. injection of physiological saline instead of the cataract inducing agent. Upon the opening of the rat pups eyes, cataracts were evaluated in each of the animals. After which animals were separated according to the severity of the cataract formation. Once cataracts formed, the rat pups received two 10 μL drops of 1.0% NACA/buffer solution into each eye, twice a day for 3 weeks. Cataracts were revaluated at this time using a slit-lamp microscope and images recorded with a canon digital camera in macro mode. Animals were then sacrificed and the lens removed, rinsed with PBS solution, and then immediately placed on dry ice. Samples were stored at a temperature of -80 °C for further analysis. Their lenses were then analyzed for selective oxidative stress parameters, including glutathione (reduced and oxidized), protein carbonyls, catalase, glutathione peroxidase, glutathione reductase, and malondialdehyde.

6.2. RESULTS

6.2.1. Effects of BSO and NACA on Cataract Formation in the Lens. An injection of 4 mmol/kg body wt of BSO, administered once daily for 3 days, 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 also found that all rats treated with BSO developed cataracts. Of the lenses examined within these groups, a majority of the cataracts were grade I while only eight animals had grade II cataracts. The eight rats containing grade II cataracts were placed in one group and twelve of the rat pups were used for the second group. After treating with NACA eye drops for a period of three weeks, it was found that all animals which had grade I cataracts showed no signs of the presence of cataracts with the slit-lamp microscope. In the group containing pups with grade II cataracts it was found that more than 60% of lens no longer showed any signs of cataracts and the remaining lenses all showed significant decrease in the severity of the cataract formation by analysis with the slit-lamp microscope. The majority of lenses after a three week eye drop treatment were of a clarity similar to that seen in the control group. Lenses observed within the control and NACA-only groups were completely clear. The grading of the lenses in all groups can be found tabulated in Table 6.1. Slit-lamp photos of the lenticular opacities observed for animals having grade I cataracts and grade II cataracts before and after administration of NACA containing eye drops are shown in Figure 6.1. and Figure 6.2.

6.2.2. Effects of BSO and NACA on GSH and GSSG Levels in the Lens. Glutathione levels within the lens sample give a good indication as to the redox status of the rat pup's eye. The levels of GSH found in the lenses of the NACA-only treated rat pups were very similar to those found in the control group. GSH levels measured in the cataract

containing lenses of rat pups which did not receive treatment with NACA were significantly lower than levels found in normal lenses. Lenses containing grade I cataracts that received NACA eye drops were found to have GSH levels which were not significantly different from normal lenses. For lenses containing grade II cataracts, even though the GSH levels were not restored to the levels as that of normal lenses, the results indicate that the amount of glutathione is significantly higher than lenses with cataracts (Figure 6.3.).

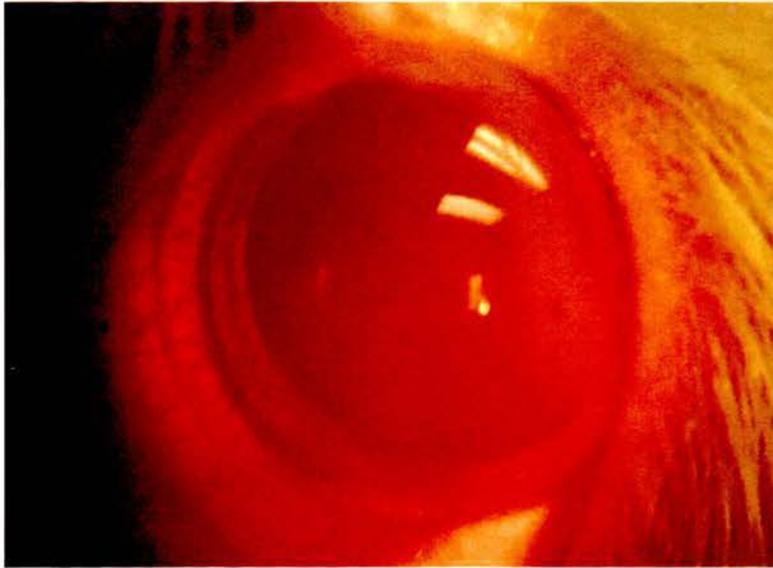
Glutathione disulphide levels in the lenses of the BSO-only group were found to have significantly increased by approximately four times the GSSG levels detected in normal lenses. The levels of GSSG determined in lenses containing grade I and grade II cataracts were significantly reduced upon treatment with NACA eye drops. Lenses which initially had grade I cataracts had levels of glutathione disulphide which were very similar to that of normal lenses. The lenses in the Grade II + NACA group were found to have a significantly lower level of GSSG that lenses containing cataracts which received no treatment. A graph with these results is shown in Figure 6.4.

6.2.3. Effects of BSO and NACA on MDA Levels in the Lens. The levels of malondialdehyde where measured in the lens of animals in each group to detect the extent of lipid peroxidation which consistently high in lens containing cataracts. Results indicate that the amount of MDA in the lenses of the NACA-only group was not significantly different from levels found in the control group. The lenses of the BSO-only group were found to contain elevated levels of MDA than lenses in the rat pups which did not develop cataracts. The lenses containing grade I cataracts were found to have MDA levels very similar to normal lenses after treatment with NACA eye drops. NACA eye drops were also able to reduce MDA levels within grade II cataracts by a significant amount compared to lenses containing cataracts which received no treatment (Figure 6.5.).

6.2.4. Effects of BSO and NACA on GPx and GR Activities in the Lens. Glutathione peroxidase activity within the lenses of animals in each group was determined after homogenization of the sample. The activity of the enzyme after treating normal lenses with NACA was not significantly different from normal lenses which received no NACA eye drops. Animals injected with BSO only had significantly lower levels of GPx in their lenses compared to animals with normal lenses. Results indicate that the presence of NACA eye drops the activity of the enzyme was increased in lens containing cataracts compared with normal lenses (Figure 6.6.).

Glutathione reductase activity was also determined within the lenses of the animals in each group. Results indicate that the activity of this antioxidant enzyme was significantly lower in lenses containing cataracts which were not treated with NACA than that of normal lenses. In the presence of NACA eye drops the activity of the enzyme in lenses containing grade I cataracts was approximately the same as compared with normal lenses. The activity of the enzyme after treating lenses containing grade II cataracts was significantly higher than lenses containing cataracts which received no treatment (Figure 6.7.).

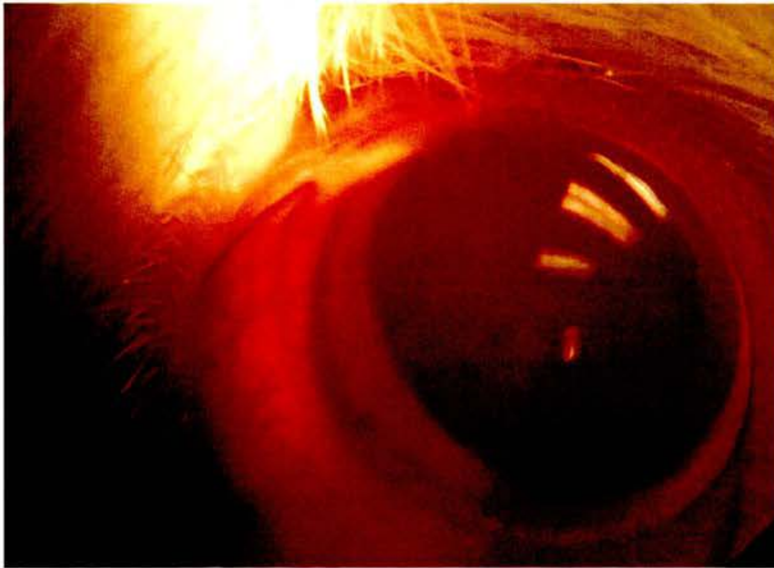
6.2.5. Effects of BSO and NACA on Protein Carbonyl Levels. Protein carbonyl levels were measured in order to estimate the amount of protein oxidation present in the lens. The lenses of animals which had cataracts and received no treatment had a significantly higher amount of protein carbonyl content than those of normal lens. The protein carbonyl levels for lenses which had grade I cataracts prior to NACA eye drop treatment showed levels which were similar to normal levels after the treatment period. Lenses which containing grade II cataracts had protein carbonyl levels significantly lower than the BSO only group, although they did not reach the levels found within normal lenses (Figure 6.8.).



Grade I

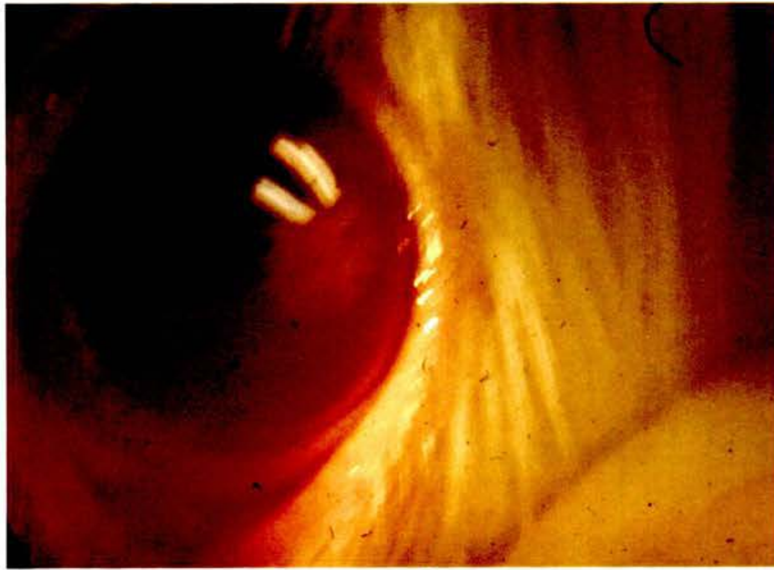
NACA

Eye drops

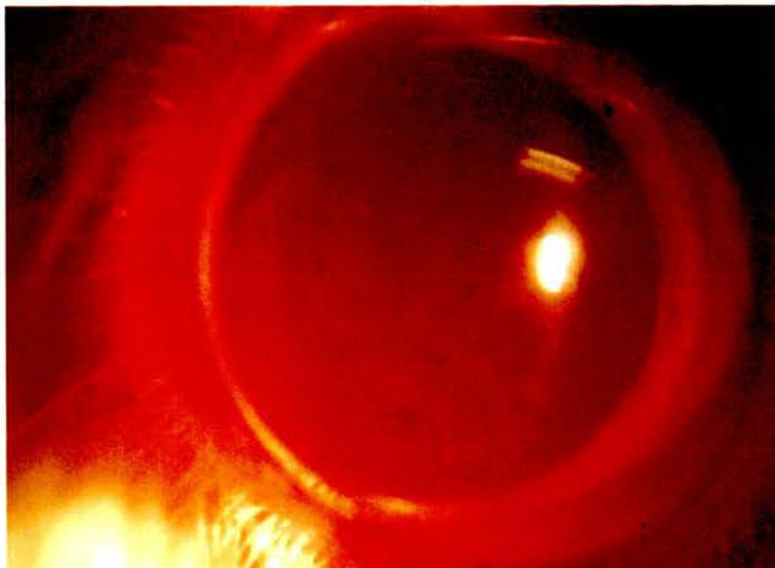


Grade 0

Figure 6.1. Image of Grade I Cataract after Administration of NACA Eye Drops. Displayed above are slit-lamp viewings of the lens of an animal in the Grade I + NACA group. The top picture shows the lens with a grade I cataract after administration of BSO. The bottom picture shows the lens with no signs of cataract formation after the administration of NACA eye drops for three weeks.



Grade II



Grade I

Figure 6.2. Image of Grade II Cataract after Administration of NACA Eye Drops. Displayed above are slit-lamp viewings of the lens of an animal in the Grade II + NACA group. The top picture shows the lens with a grade II cataract after administration of BSO. The bottom picture shows the lens with a significant decrease in the cataract opacity after the administration of NACA eye drops for three weeks. The scattering of light, however, suggests the presence of cataracts but within the less severe grade I classification.

Group	Grade 0	Grade I	Grade II	Grade III
BSO only (n=10)	0	11	9	0
NACA only (n=10)	20	0	0	0
Grade I + NACA (n=12)	24	0	0	0
Grade II + NACA (n=8)	10	6	0	0

Table 6.1. Degree of Cataract Formation within the Lenses after Treatment with NACA Eye Drops. The lenses (within their associated groups) that developed varying degrees of opacities are summarized. The degrees of opacity are defined as follows: grade 0, normal transparent lens; grade 1, presence of the scattering of light or initial signs of opacity; grade 2, presence of a partial nuclear opacity; grade 3, presence of complete or nearly complete nuclear opacity. 'n' represents the number of animals utilized in each group.

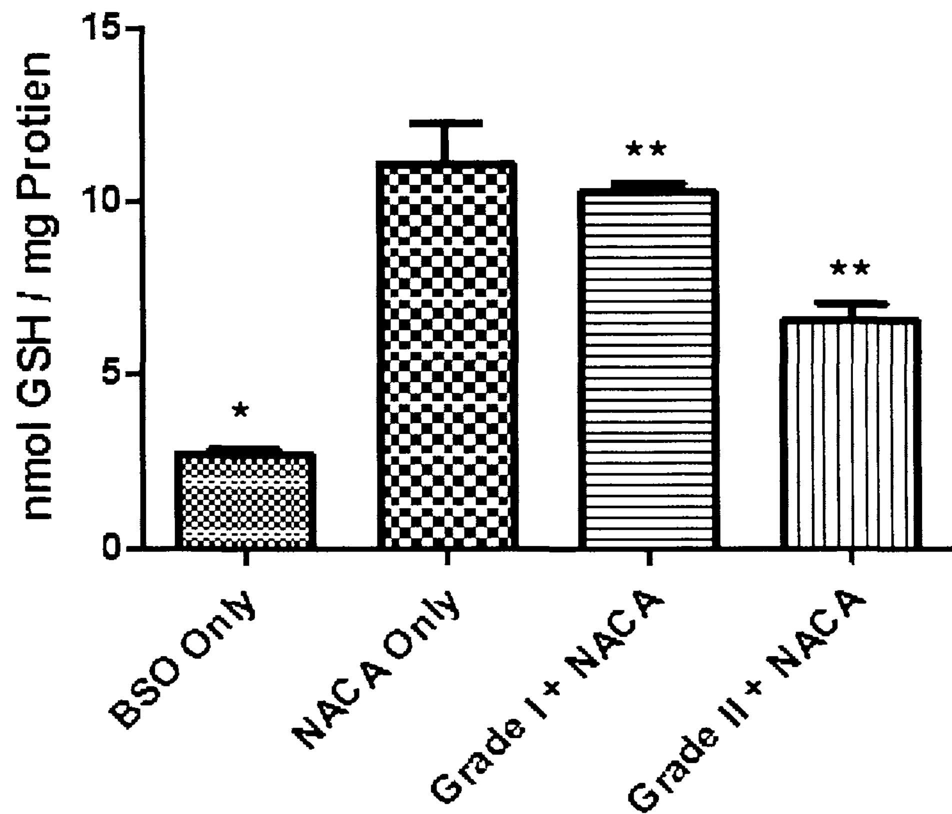


Figure 6.3. Glutathione Levels within the Lens. The GSH level in the NACA-only group was similar to that of the control; the control group is not pictured. Results indicate that eye drop treatment of NACA was able to restore levels of GSH of lenses containing grade I cataracts to levels similar as that of normal lenses. For lenses containing grade II cataracts, even though the GSH levels were not restored to the levels as that of normal lenses, the results indicate that the amount of glutathione is significantly higher than lenses with cataracts. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

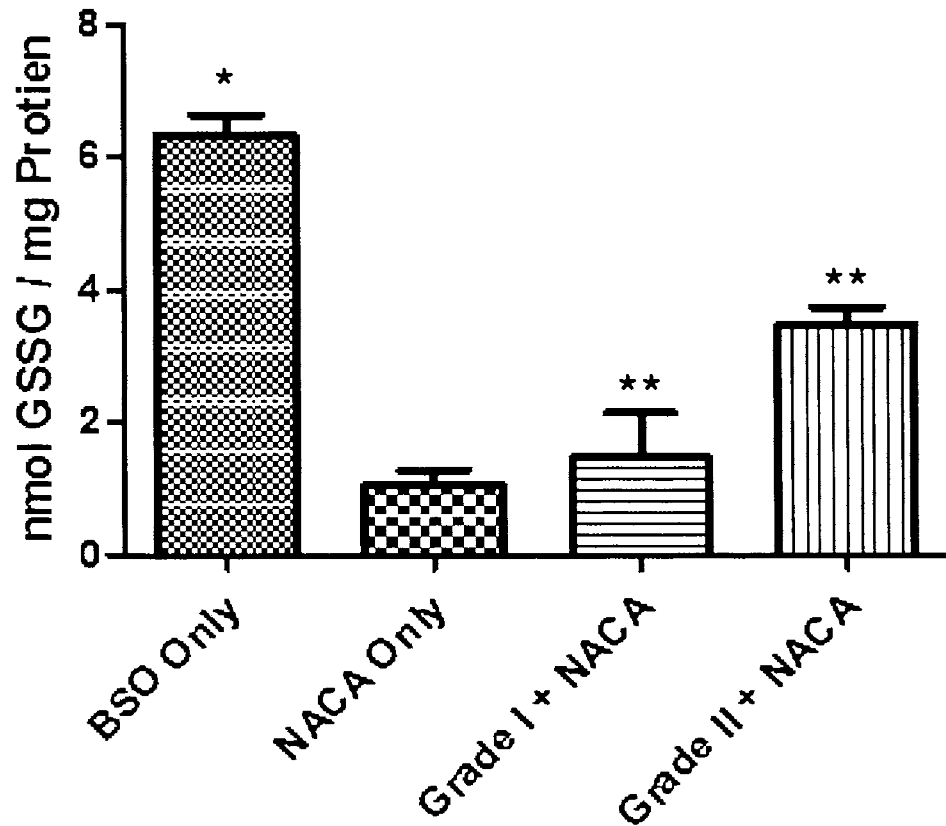


Figure 6.4. Glutathione Disulphide Levels within the Lens. The GSSG levels in the BSO only group was significantly higher than those found within normal lenses. This GSSG level was significantly reduced when pups containing grade I and grade II cataracts were treated with NACA eye drops. Lenses which initially had grade I cataracts had levels of GSSG very similar to that of the control upon NACA eye drop treatment. Lenses which initially had grade II cataracts contained significantly lower amounts of GSSG upon treatment with NACA eye drops. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

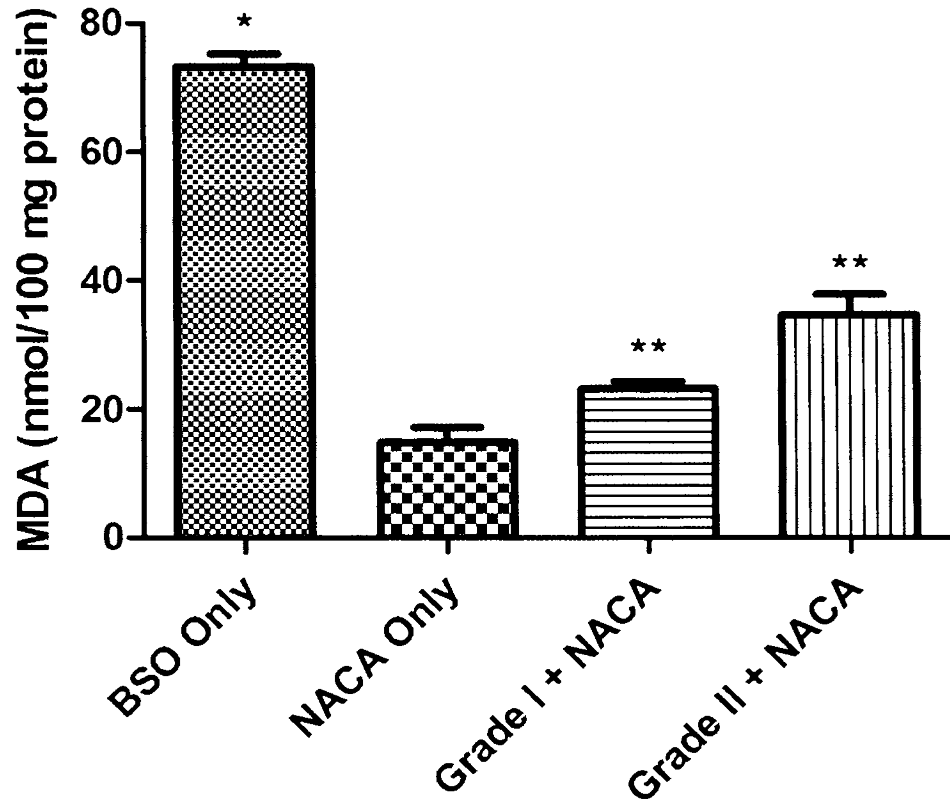


Figure 6.5. Malondialdehyde Levels within the Lens. Malondialdehyde (MDA) levels within the lenses of animals in each group where determined upon homogenization of samples. Results indicate that the amount of MDA in the lenses of the NACA-only group were very similar to those of the control group, while the BSO-only group showed elevated lipid peroxidation due to the significantly larger amount of MDA detected. Treatments with NACA were able to keep this oxidative stress parameter to levels similar to that of the control. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

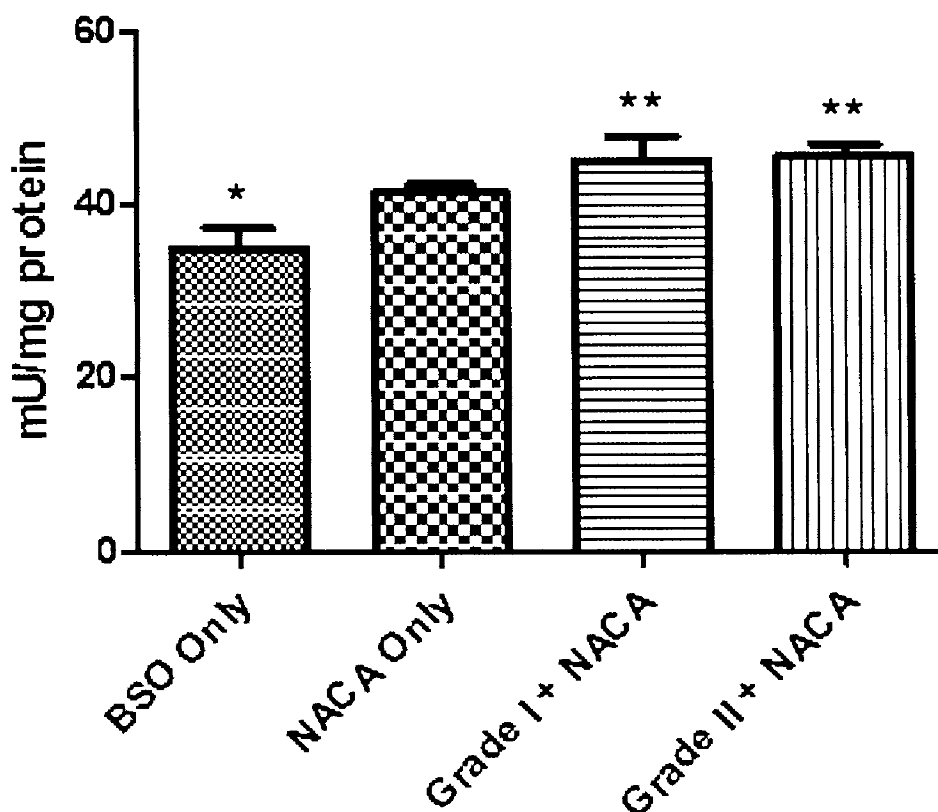


Figure 6.6. Antioxidant Enzyme Activity of Glutathione Peroxidase. Glutathione peroxidase activity within the lenses of animals in each group were determined after homogenation of the sample. Results indicate that the activity of this antioxidant enzyme was lower in lenses containing cataracts which were not treated with NACA than that of normal lenses. However in the presence of NACA eyedrops the activity of the enzyme was increased in lens containing cataracts compared with normal lenses. The activity of the enzyme after treating normal lenses with NACA was not significantly different from normal lenses which received no NACA eye drops. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

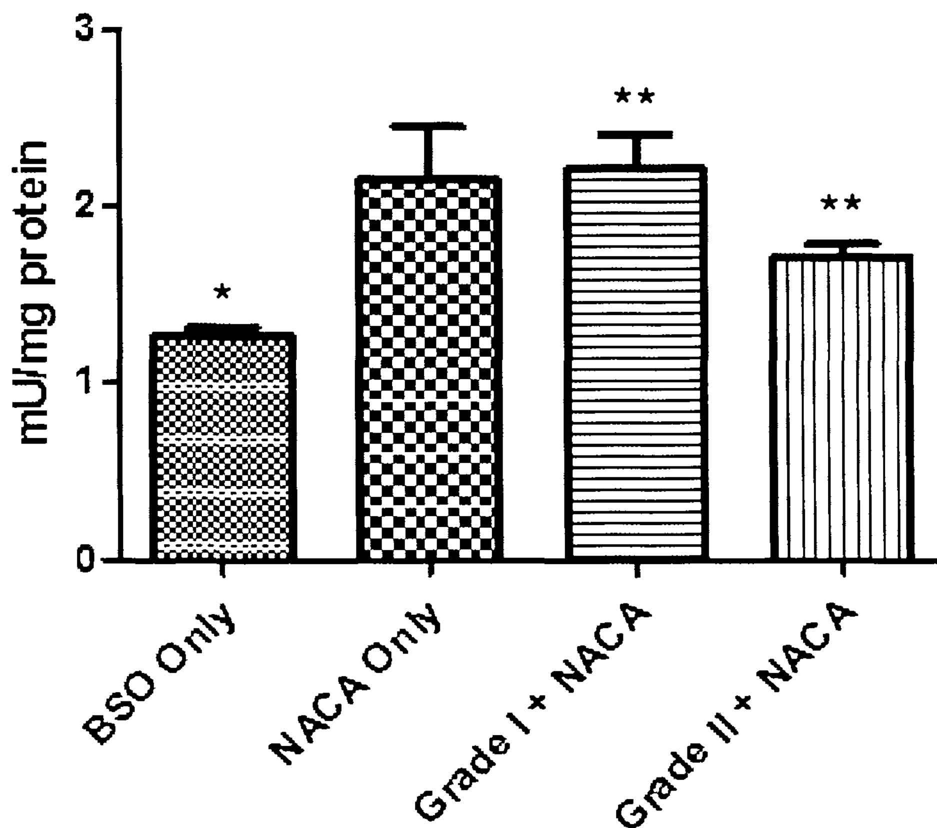


Figure 6.7. Antioxidant Enzyme Activity of Glutathione Reductase. Glutathione reductase activity within the lenses of the animals in each group was determined after homogenization of the samples. Results indicate that the activity of this antioxidant enzyme was significantly lower in lenses containing cataracts which were not treated with NACA than that of normal lenses. In the presence of NACA eye drops the activity of the enzyme in lenses containing grade I cataracts was approximately the same as compared with normal lenses. The activity of the enzyme after treating lenses containing grade II cataracts was significantly higher than lenses containing cataracts which received no treatment. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

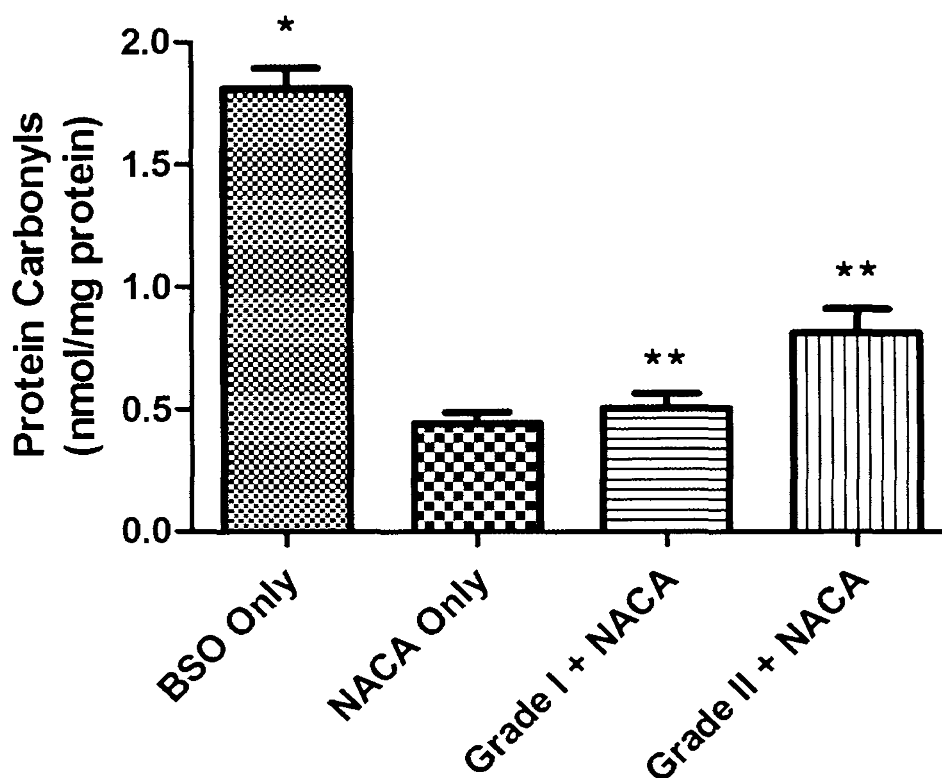


Figure 6.8. Protein Carbonyl Levels within the Lens. The lenses of animals which had cataracts and received no treatment had a significantly higher amount of protein carbonyl content than those of normal lenses. The protein carbonyl levels for lenses which had grade I cataracts prior to NACA eye drop treatment showed levels which were similar to normal levels after the treatment period. Lenses which contained grade II cataracts had protein carbonyl levels which were significantly lower than the BSO only group, although levels did not reach those found within lenses of the control group. '*' refers to significant differences from the control and '**' refers to the significant differences from the BSO only group where $p \leq 0.05$.

6.3. DISCUSSION

Lens opacity, due to cataract formation, is directly attributed to oxidative processes that occur 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 glutathione, lipid peroxidation, protein oxidation and a decrease in antioxidant enzyme activity [123-125]. Glutathione is an indispensable and primary lenticular antioxidant [126]. There is a wide body of evidence that indicates loss of glutathione occurs because of the oxidation of glutathione to GSSG, because its levels increase drastically once the cataracts develop. In addition, hyperbaric oxygen causes cataracts, as it is characterized by the loss of GSH and protein–SH, as well as protein insolubilization [127]. Therefore, an alternative method for treating or preventing the occurrence of cataracts would be through the use of a potent thiol-exchange compound.

N-acetylcysteine (NAC) is a FDA approved drug which utilizes thiol-exchange properties. One of its current uses is to act as a thinning agent for mucus accumulation [144]. Mucus consists of an aggregation of smaller glycoproteins which are aggregated through disulfide bonds. The more disulfide bonds present the thicker the mucus becomes. By breaking these disulfide bonds, through the thiol-exchange mechanism, between glycoproteins the mucus can be broken apart and made less viscous. NAC has been proven very effective in treating patients with thick mucus and because NAC is not very permeable to cells, this property allows it to maintain high concentration within the areas lined with mucus.

However, to focus NAC's action within cells and tissues is difficult because of this compounds overall net charge inability to cross cellular membranes. It was therefore not applicable to utilize NAC for the treatment of cataracts. N-acetylcysteine amide (NACA),

however, is a derivative of NAC that has the ability to permeate cell membranes and can be further converted into NAC inside cells through an enzymatic process. Results from morphological observations indicate that NACA is able to reverse the formation of cataracts, or at least significantly reduce the severity of cataracts in low grade cataracts: grade I and grade II, within the BSO experimental cataract model. This premise is very evident from this study, in that all lenses which initially contained grade I cataracts prior to treatment with NACA eye drops showed no signs of cataracts after a period of three weeks. Although not all of the rats with grade II cataracts were completely reversed after the period of treatment, all of the lenses showed signs of reduced severity of cataracts. Over 60% of the lenses which originally contained grade II cataracts showed no signs of cataract formation. The remaining lenses in this group all were found to have a change in cataract severity from grade II to grade I (Table 6.1.).

Glutathione is an essential lenticular antioxidant which is present in high concentrations within the lens, providing a first line of defense against oxidative damage [129], as well as playing an important role in antioxidant defense and redox regulation [130]. Studies indicate that loss of GSH will directly affect the activity of the GSH-dependent enzyme glutathione reductase. This enzyme plays an important role in GSH homeostasis: it regenerates GSH from GSSG [131]. Based on the current literature, more than 90% of protein sulfhydryl (protein–SH) groups are lost in the most advanced cataracts [132]. Considering the above two facts, results from this study indicate that treatment with NACA decreases the oxidative damage. GSH levels can be significantly regenerated in lenses already developing and containing cataracts (Figure 6.3). Supporting this data, it was observed that NACA caused an increase in GR activity in the lenses of rats with grade I and grade II cataracts (Figure 6.7). Increases in GSH levels and decreases in GSSG levels (Figure 6.4) may

be attributed to the increased activity of GR by preserving the integrity of cell membranes and by stabilizing the sulfhydryl groups of proteins. Further, NACA itself may act as a sulfhydryl group donor for GSH synthesis and thereby decrease the loss of protein sulfhydryl groups, as well as opacification of the lens.

N-acetylcysteine amide was shown to have a protective effect on other portions of the lens's antioxidant defenses as well. Decreasing the levels of GSH when reactive oxygen species are present can trigger a cascade of further oxidative damage. Lipid peroxidation has been associated with the formation of cataracts in patients [19, 133-135]. The extent of lipid peroxidation was determined in this study by measuring the amount of MDA, a by-product of lipid peroxidation, within the lens. Glutathione depletion is enhanced during the repair of lipid peroxidation due to its use as a substrate for glutathione peroxidase. Once glutathione levels become low, this repair mechanism is compromised and may lead to the formation of lipid damage that is observed in lenses containing cataracts. This unavailability of GSH as a substrate for glutathione peroxidase stalls the process of lipid peroxide decomposition, thus increasing the levels of MDA. It was found that NACA supplied an adequate amount of GSH as a substrate for glutathione peroxidase to effectively decompose lipid peroxides in the rats, effectively reducing MDA levels (Figure 6.5. and Figure 6.6.).

Proteins, like lipids, are well known to be oxidized by reactive oxygen species. Oxidative damage to lens proteins is an important factor for cataract formation [136-138]. Protein carbonyls are commonly used as an indicator of protein oxidation in many tissues [139]. Results in this study indicate that protein carbonyls found to be present in cataract containing lenses were significantly reduced upon treatment with NACA eye drops (Figure 6.8.). Depletion of GSH found in cataract containing lenses initiates a myriad of events. There is an initial increase in free radicals that overwhelms the scavenging ability of the

GSH-dependent enzymes (glutathione peroxidase), which leads to oxidation of lipids and proteins. Several studies have indicated a positive correlation between protein carbonyl levels and the genesis of cataract formation [140-142]. The multiple roles of NACA in preventing cataract formation include direct scavenging of free radicals, providing cysteine for GSH synthesis, and nonenzymatic reduction of GSSG into GSH. Thiol-containing compounds have been shown to inhibit cataract formation in previous studies by reducing protein oxidation in the lens [114,115].

These results suggest strongly that N-acetylcysteine amide may have therapeutic advantages in protecting against, delaying, and reversing cataract formation within the lenses by reducing oxidative damage and repairing crystalline proteins. Our results suggest that NACA can prevent the formation of cataracts by maintaining and regenerating glutathione levels in compromised 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 and the ability to maintain antioxidant levels within the lens and, possibly, through disulfide-exchange mechanisms. Treatment with NACA may prove to have a major therapeutic role in cataract patients.

7. CONCLUSIONS

A logical approach to enhancing protection against oxidative damage in eye-related disorders would be the administration of pharmacologic doses of highly-active antioxidants which can be delivered to the sites undergoing stress. Considerable research within this area supports the beneficial effects of antioxidant supplementation provided to decrease these oxidative processes in the retina and retinal pigment epithelium. N-acetylcysteine amide (NACA) provides an excellent source of thiol groups that can be converted by the cells into metabolites capable of stimulating glutathione (GSH) synthesis. NACA can promote intracellular detoxification and act directly as a free radical scavenger. This compound also is very lipophilic and has the ability to pass through cell membranes as well as the blood-retina barrier. Further, NACA acts as a carrier of N-acetylcysteine (NAC) and has the capability to be converted into this FDA-approved drug once it is inside the cell membrane, thereby keeping the compound contained within the cell. The experiments described here support the premise that NACA provides protective effects, both *in vitro* and *in vivo*, for eye-related pathologies.

First, a preliminary study demonstrated that NACA has the ability to protect against oxidative stress induced in the retinal pigmented epithelium upon exposure to METH. Methamphetamine abusers are prone to developing eye related disorders. This research has shown that METH induces toxic effects on ARPE-19 cells by generating reactive oxygen species and intracellular damage. In cells that were pretreated with NACA before exposure to METH, The amounts of reactive oxygen species were mediated and the extent of oxidative damage was greatly reduced. Further studies are needed to determine whether NACA can assist in controlling or preventing macular degeneration.

In the case of cataracts, NACA was found to be capable of both inhibiting the process of cataract formation and reversing the cataract profile once a cataract had already been established in the eye. Not only is NACA a potent antioxidant, it is also an efficient thiol exchanger. Since cysteine is the most readily oxidized amino acid residue in proteins, this typically is the site of protein oxidation. Crystallin proteins found within the lens have cysteine residues available on the lens periphery, which allow for the formation of disulphide bonds. By placing a disulphide exchanger in an environment with many disulphide bonds, the aggregated proteins are allowed to separate ad become less condensed. Utilizing this mechanism is what allows NACA to reverse cataract formation within the lens.

The development of a useful antioxidant agent in the form of eye drops would significantly reduce the oxidative damage in eye-related disorders and reduce the severity of disorders within the retina. NACA has proven effective in cataract treatment as shown by the data presented in the studies described within this paper. This drug appears to have considerable potential for development as a therapeutic agent for successfully treating numerous retinal diseases caused by oxidative stress. One important potential would be the use of NACA eye drops in the aging population since a majority of eye related disorders develop in the elderly.

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