THE ROLE OF ANTIOXIDANTS IN THE HYDROGEN PEROXIDE-INDUCED OPACIFICATION OF SHEEP LENS

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Jie Lei

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The lens of the eye needs to be transparent with a high refractive index to focus images on the retina. In cataracts the lens becomes opaque, eventually leading to blindness. There are many possible causes of cataract but a lot of evidence implicates oxidative damage as contributing to opacification. This includes epidemiological studies showing that diets rich in antioxidants lowered the prevalence of cataract. This research tested the hypothesis that if cataracts were at least partially caused by oxidative damage then their progression would be slowed by application of antioxidants. The antioxidants used were two plant compounds found in the diet, resveratrol and quercetin. The system used was sheep lenses cultured in Eagles Minimal Essential Medium (EMEM). Lenses remained transparent for up to 7 days in EMEM but became opaque within 24 h when exposed to 1 mM hydrogen peroxide (H_2O_2). The lens is exposed to H_2O_2 *in vivo* as it is found in the aqueous humor. Prior Lenses pre-treated with quercetin reduced but did not prevent opacification. Lens cell death, as determined by measurement of leakage of lactate dehydrogenase, was found to increase with H_2O_2 and the increase was prevented by pre-treatment with antioxidants. The role of the endogenous antioxidant glutathione was also investigated. It was found that H_2O_2 decreased the amount of reduced glutathione in the lens cortex and increased the levels of oxidised glutathione but only at levels of 2 mM and above. Thus the results of this research indicate that H_2O_2 at low concentration (1 mM) is able to damage lens cells and cause opacification without affecting the reduced glutathione levels and that the exogenous antioxidants have some ability to protect the lens.

Key words: Sheep lens, oxidative damage, antioxidant, quercetin, resveratrol, cataract, lactate dehydrogenase, glutathione, Eagles minimal essential medium, hydrogen peroxide.

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Abbreviations

AAH	artificial aqueous humor
ARC	age related cataract
ARN	age related nuclear
BODIPY-FL	4, 4-difuoro-5, 7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-3-propionic acid
BSA	bovine serum albumin
CAT	catalase
DCFH-DA	2', 7'-dichloroflurescin diacetate
DEAE	diethylaminoethyl
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
EMEM	Eagle's minimal essential medium
Em	Emission
Ex	Excitation
FPLC	fast performance liquid chromatography
GCS	glutamylcysteine synthetase
GPX	glutathione peroxidase
GR	glutathione reductase
HLE	human lens epithelial cell
HMW	high molecular weight
LDH	lactate dehydrogenase
LOCH	lens organ culture with hydrogen peroxide
NO·	nitric oxide
$O_2^{\cdot -}$	superoxide
$ONOO^-$	peroxynitrite
PSC	posterior subcapsular cataract

ROS	reactive oxygen species
rpm	revolutions per minute
SJA6017	N-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal
-SH	thiol
SOD	superoxide dismutase
TRP	transient receptor potential
V/V	volume per volume

Chapter 1

Introduction

Cataracts are described as an opacification (cloudiness) of the lens that leads to the scattering of light entering the eye and a loss of vision. Figure 1.1 below indicates the different images received through normal and cataract lenses.

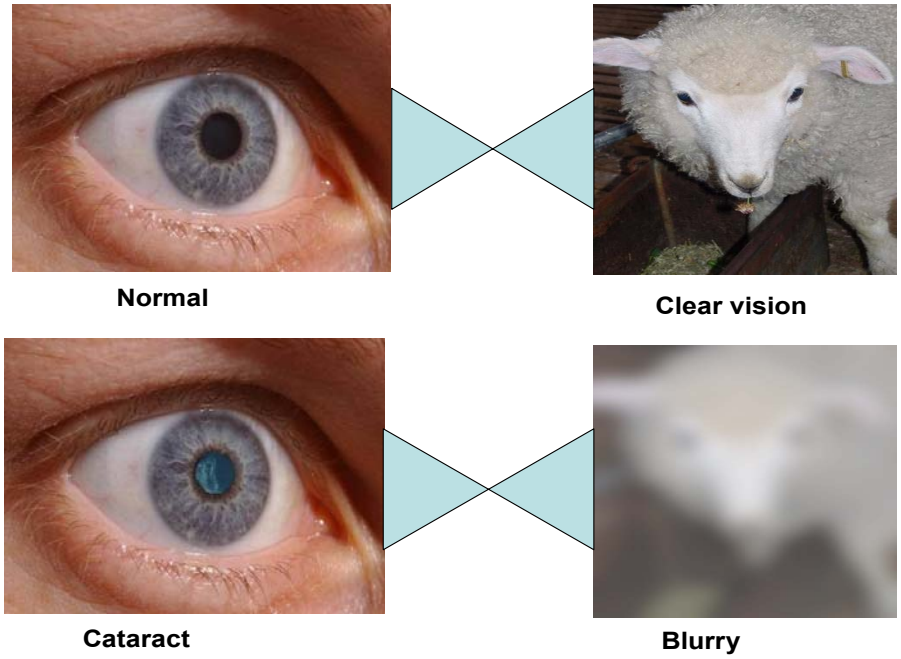


Figure 1.1 Indication of different images through normal and cataract lenses (Courtesy of Dr L. J. Robertson).

Cataracts, which affect more than 50 million people (Taylor & Davies, 1987), are the most common cause of blindness in the world. In first world countries, old age is the single largest cause of cataracts: only about 5% of Caucasian Americans aged 52-64 years have cataracts, whereas18% of those aged 65-75 and 46% of those aged 75-85 are affected by cataracts (Kahn *et al.*, 1977). Figure 1.2 below shows the demographic distribution of cataract in Australian over 40. As the average lifespan increases, the prevalence of cataract also increases.

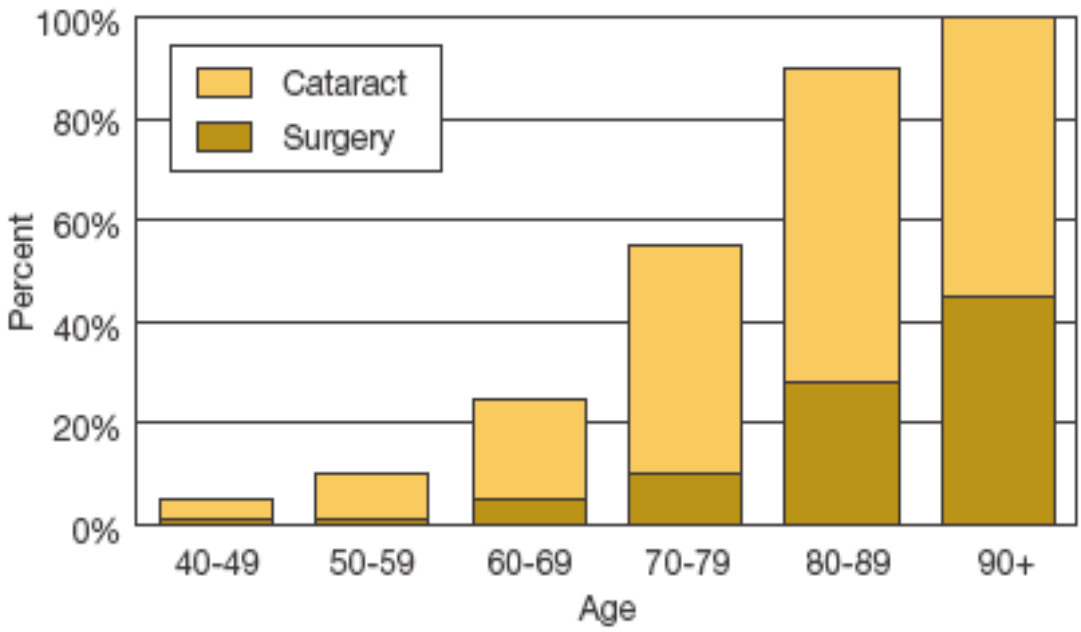


Figure 1.2 Demographic distribution of cataract for Australians (over 40). Data show that around 23% of those aged 60-69, 54% of those aged 70-79 and 90% of those people aged 80-89 have developed cataract. Courtesy of Taylor, H.R. (2004) / Eye Research Australia Clear Insight – Overview 1-17 12. Retrieved from www.cera.org.au on 14 Feb 2006.

Cataract formation cannot be prevented or reversed (Bhat, 1987) and can only be cured by surgical replacement of the lens. There have been significant advances in surgical techniques and refinement of intraocular lens implants which have benefited cataract patients (Zigler *et al.*, 2003). The whole procedure takes only 20 minutes and can be performed under local anaesthetic. In the United States, cataract extraction (involving removal of the affected lens and insertion of a new synthetic lens) is the most frequently performed surgical procedure among Medicare recipients (Congdon, 2001).

However, the more than 1 million cataract operations performed annually in the United States (Steinberg *et al.*, 1993) account for over $3 billion in annual expenditure (Zigler et al., 2003). Thus, although surgical removal of the afflicted lens accompanied by ocular lens implant is highly successful in restoring sight, the procedure is costly, accounting for 12% of the US Medicare budget during the evaluation in 1992 (Steinberg *et al.*, 1993). Estimates indicate that delaying the onset of cataract by ten years could reduce the need for surgery by 45%, thus saving billions of dollars (Bunce, 1993). Therefore, it is desirable to avoid surgery if possible.

In the developing world, where medical services are often unavailable or extremely limited, the situation is even worse. In India alone around 30 million people suffer from

cataract (Bhat, 1987). Thus, the expense and unavailability of surgery mean that non-surgical medical therapy or nutritional treatment to inhibit the formation or slow the progression of cataracts is an important goal in experimental eye research to benefit patients and reduce the huge economic burden.

Developing anti-cataract agents has been difficult because cataract is not a single disease with a single aetiology. There are three major categories of cataract (nuclear, cortical, and posterior sub-capsule), each of which is multifactorial in aetiology and highly variable in severity and rate of progression (Zigler et al., 2003). Further complicating the situation, the factors contributing to age-related cataractogenesis are a combination of pathological and normal aging processes, which have no obvious borders to distinguish them.

Cataracts may be prevented if the mechanisms of formation are known. Based on the available knowledge of the biology of the normal lens and the cataractogenic process, three hypotheses have been proposed for the aetiology of cataract and three approaches have accordingly been adopted in the design of anti-cataract agents (Figure 1.3).

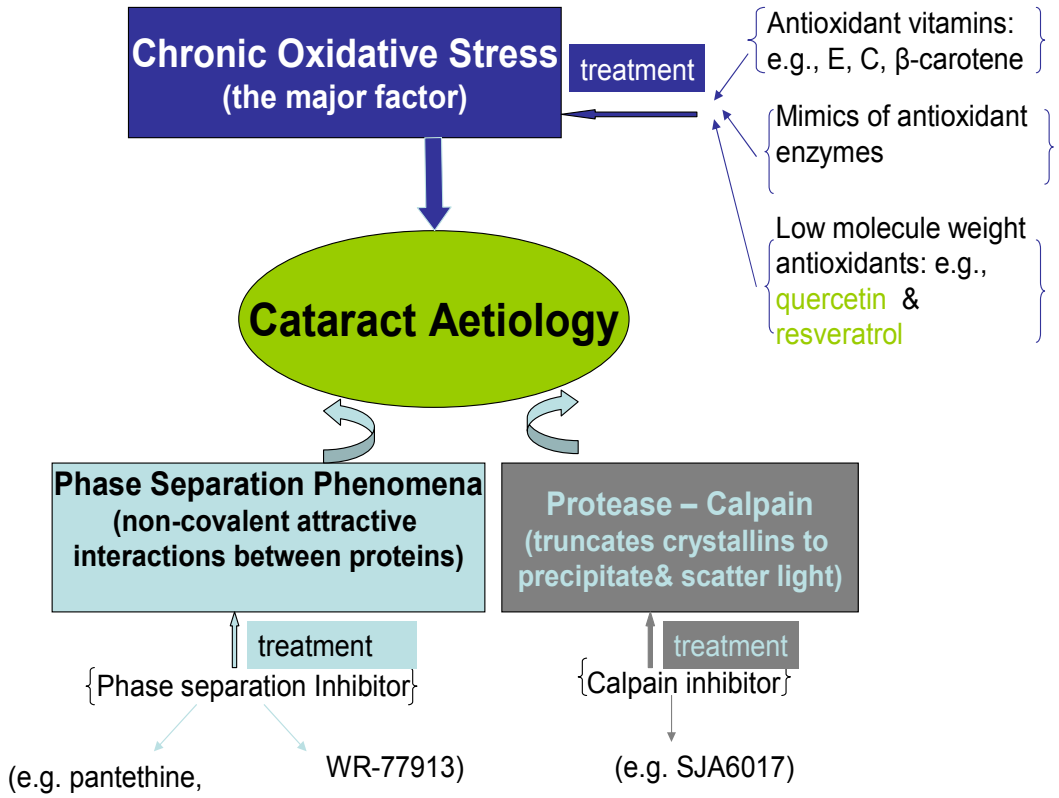


Figure 1.3 Three hypotheses of cataract aetiology and three approaches of anti-cataract agents designed accordingly.

The first hypothesis is that chronic oxidative stress is a major factor in the aetiology of age-related cataract. Experimental evidence suggests that oxidative stress due to the generation of free radicals plays a role in the pathogenesis of cataracts and that the process can be prevented or ameliorated by antioxidants (Robertson *et al.*, 1989). Therefore, agents with anti-oxidative properties have received the most attention. Such compounds comprise of three categories including antioxidant vitamins (e.g., E, C, β-carotene); functional mimics of antioxidant enzymes; and a wide variety of low molecular weight compounds with antioxidant activity (Zigler *et al.*, 2003).

The second hypothesis is that phase separation phenomena is integral to cataract development (Benedek, 1971). Phase separation results from non-covalent attractive interactions between proteins in concentrated solutions, creating protein-rich and protein-poor regions. In the lens, formation of such domains creates light scattering, leading to cataract. Two putative phase separation inhibitors, pantethine and the radioprotective phosphorothioate WR-77913, were tested in several acute animal models of cataract and displayed the delay of the onset of cataract (Zigler et al., 2003).

The third hypothesis is the "protease hypothesis". Calcium activated neutral enzymes, calpains, can induce proteolysis and truncate crystallins to precipitate and scatter light to form cataract (Andersson *et al.*, 1996). Therefore, research on calpain inhibitors is another approach to prevent or inhibit cataract formation. It has been reported that, when lambs with an inherited cataracts were treated with eye drops containing the calpain inhibitor SJA6017 for 4 months, progression of cataracts were slowed down in treated eyes compared with non-treated eyes (Robertson *et al.*, 2005).

In order to study cataracts and possible treatments for cataracts, a number of *in vivo* animal models have been developed. For example, administration of L-buthionine sulfoximine, a specific inhibitor of glutathione biosynthesis, to preweanling mice (aged ≤ 12-days) provides a model system for the induction of cataracts by depletion of lens glutathione (Calvin *et al.*, 1986).The strong sulfhydryl oxidant, selenite, has been used to produce cataract in rats (Shearer *et al.*, 1992). This selenite-induced cataract model has been extensively utilized to demonstrate that calpain-induced proteolysis causes truncated crystallins to precipitate and scatter light. Other *in vivo* experimental animal models such as hyperbaric oxygen, and UVA light, have also been utilized to investigate the mechanism of formation of human senile nuclear cataract (Giblin, 2000).

There are also *in vitro* models of cataract where cataract is induced in cultured lenses, for example, H_2O_2-induced cataract in cultured lenses from rabbit (Giblin *et al.*, 1987) and rat (Lou *et al.*, 1990), diamide (a thiol-specific oxidant)-induced cataract in Sprague-Dawley rat cultured lens model (Azuma & Shearer, 1992), ionomycin cataract in rat cultured lens model (Sanderson *et al.*, 1996), 4-bromo-calcium ionophore A23187 (Br-A23187)-induced cataract in guinea pig and rabbit cultured lens model (Fukiage *et al.*, 1998), and sugar xylose-induced cataract in rhesus monkey lens model (Zigler *et al.*, 2003).

These studies have established the underlying premise that a lens organ culture model system can be used to screen potential anti-cataract agents. The lens, which is avascular and non-innervated *in vivo*, can be maintained in a fully viable state in organ culture. Opacity can be induced in cultured lenses by various chemical or environmental perturbations, and prevention or inhibition of opacification can be observed after addition of appropriate agents to counteract the cataractogenic stresses (Zigler *et al.*, 2003). Sheep lenses are considered to be more appropriate models of the humans lens than the rodent lenses commonly used for lens research. Rats and mice have lenses which are smaller than human and are spherical in shape compared to the flattened disc shape of the human lens. Also in sheep and human, the biconvex lens shape is altered by the ciliary muscle whereas the lens is moved backwards and forwards to focus light on the retina in the rodent eye (Augusteyn & Stevens, 1998).

Oxidative stress, defined as an excess of pro-oxidants relative to antioxidants and a key factor in the gradual loss of lens transparency, is implicated in the initiation of maturity onset cataract which appears late in life and is probably not associated with congenital conditions or other diseases, such as diabetes (Spector, 1995). Evidence from epidemiological studies, model systems and human lenses obtained after cataract surgery, has indicated a role for oxidation in this opacification process. This has fuelled interest in the role of diet and dietary supplements in slowing down the progression of cataract. Halliwell & Gutteridge (1999) concluded that dietary antioxidants have a significant impact on cataract development based on the epidemiological evidence. Experimental studies have shown that, pre-treatment of the plant antioxidant, quercetin, at concentrations of 30 μM for 24 h, inhibited hydrogen peroxide-induced oxidation of the rat lens (Sanderson *et al.*, 1999; Cornish *et al.*, 2002).

The main aims of this research were to investigate the influence that exogenous antioxidants exerted on the lens under oxidative stress induced by H_2O_2 and the resulting

biochemical changes in lens cell. One literature review chapter (2) and three experiment chapters (3, 4 and 5) were included in this thesis:

In chapter 2, the physiology of the lens, the nature of lens proteins and cataracts were introduced briefly. Then, the definition of pro-oxidants and antioxidants were addressed. The role of oxidation in several animal cataract models and the correlation of oxidative stress with cataracts were reviewed. Finally, the enzymatic & non-enzymatic antioxidants and the evidence showing that application of antioxidants was able to slow cataract progression have been discussed.

In chapter 3, the primary aim was to systematically establish the role of exogenous antioxidants on lens opacity in H_2O_2-induced ovine cataract model.

Chapter 4 mainly measured changes in reduced glutathione (GSH), an important endogenous antioxidant. The high concentration of GSH in a normal lens and the decreased concentration in most types of cataracts have led to many hypotheses on its role in cataract formation. GSH may also protect against oxidative damage to the lens. In conjunction with an active glutathione redox cycle located in the lens epithelium and superficial cortex, GSH detoxifies potentially damaging oxidants such as H_2O_2 (normally present in the aqueous humour) and dehydroascorbic acid (Giblin, 2000). Therefore, comparing GSH levels in antioxidant-treated cataract lenses with those non-antioxidant-treated cataract lenses may help in determining the mechanism on how antioxidants slow the progression of cataracts.

The main aim of chapter 5 was to investigate the relationship between the level of oxidative stress and opacity progression in ovine LOCH system. The pro-oxidant capacity of ovine lens fractions (cortex and nucleus) treated with different concentration of hydrogen peroxide (1, 2, and 4 mM) was also evaluated. This may identify the free radicals involved in cataract formation and enable the use of specific antioxidants that target these radicals.

The above objectives were completed using the LOCH (lens organ culture with hydrogen peroxide) model, software to assess the light scatter of the lenses, and a colorimetric glutathione assay to determine the quantification of the reduced glutathione (GSH) and oxidized glutathione (GSSG).

Chapter 2
Literature Review

2.1 Lens Physiology

The lens is an avascular tissue situated in the anterior region of the eye and is involved in focusing light on to the retina. For the light to pass through the lens, it has to be transparent. The orderly arrangement of protein fibres in the lens maintain the lens transparency (Augusteyn & Stevens, 1998).

The lens is surrounded by an elastic capsule which is connected to the muscles of the ciliary body. Contraction and relaxation of ciliary muscles results in the alteration of lens shape required for focussing, a process known as accommodation. Since there are no blood vessels within the lens and it derives nutrients and oxygen by diffusion, the intra-ocular O_2 concentrations are low, which may help to minimize oxidative damage (Augusteyn & Stevens, 1998).

The lens is composed of two types of cells: a single, outer layer of cuboidal epithelial cells on the anterior side of the lens surface differentiates into the elongated (inner) fibre cells which make up the bulk of the tissue (Figure 2.1). These cells have substantially different properties. Epithelial cells, which contain the full complement of sub-cellular organelles, proliferate at the anterior hemisphere and migrate laterally to the equatorial region of the lens where they begin to elongate and differentiate to become fibre cells (Wistow & Piatigorsky, 1988). The newly formed fibre cells are laid down over existing cells. Thus, the old cells cannot be shed and, instead, are pushed towards the centre of the lens to form the lens nucleus, losing water in this process. Hence, the inner region is the oldest part of the lens. Cellular organelles, such as the nucleus and mitochondria, are lost during fibre maturation, along with the ability of the cells to synthesize proteins to replace damaged ones and the ability to degrade proteins (Augusteyn & Stevens, 1998). Fully differentiated fibre cells possess no organelles but are filled with structural proteins known as crystallins, which are organised in a repeating lattice (Bunce, 1994b).

The two types of the lens cells also have substantially different functions. The epithelial cells are in direct contact with the aqueous humour, which is a watery fluid. Therefore, together with immature fibre cells, the epithelial cells control energy-requiring processes such as the ion transport to the lens and protein synthesis. The fibre cells are produced by

terminal differentiation of the epithelial cells. All metabolic activities of the fibre cells decrease following their maturation as enzymes become denatured and are not replaced. Therefore, the function of the fibre cells is to provide a transport medium and the refractive index gradient needed for the focussing of light on the retina. However, this important optical property is not only determined by the regular arrangement of the fibre cells, but also by specific interactions between the intracellular proteins (Augusteyn & Stevens, 1998). The different properties and functions of the epithelial cells and the fibre cells arise as a result of the unique growth pattern described earlier.

Meanwhile, because of the lack of protein turnover in fibre cells, there is a gradient of increased protein concentration and, hence, refractive index and hardness from the outer layers to the central areas. The optical power of the lens would be much lower if the refractive index distribution were uniform, and the resulting refractive index gradient reduces spherical aberration and increases the optical power of the lens (Augusteyn & Stevens, 1998).

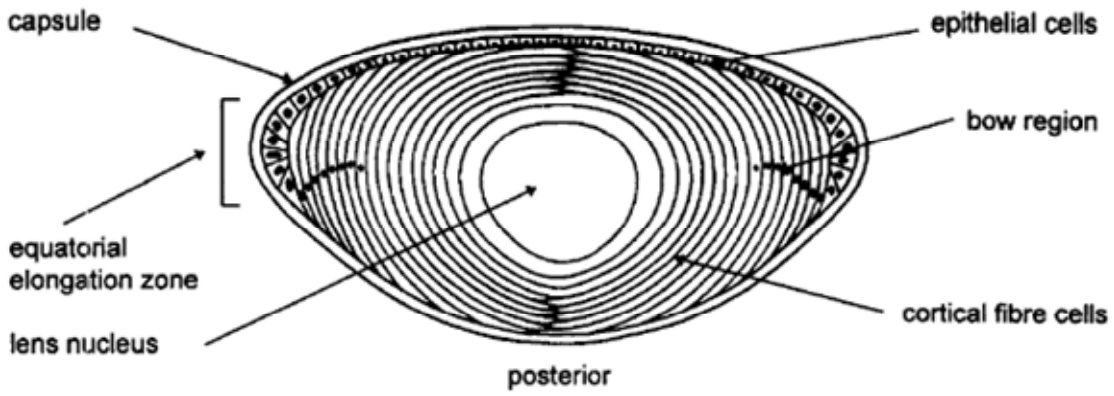


Figure 2.1 Diagrammatic representation of the mammalian lens. Retrieved from Augusteyn & Stevens (1998) / Progress in Polymer Science v23(3) 375-413 377

2.2 Lens Proteins

Apart from water, the major constituents of the eye lens are a family of soluble specialized proteins, the crystallins, which can represent up to 70% of the dry mass of the tissue (Augusteyn & Stevens, 1998). These proteins remain for the lifetime of the organism once they have been laid down (Augusteyn & Stevens, 1998). They are unusually stable and soluble and contribute to the functioning of this important lens organ. For example, lens transparency is achieved via the highly organised way in which the crystallins are stacked (Augusteyn & Stevens, 1998). There are three classes of crystallins: α-, β- and γ-

crystallins, all of which have to last the lifetime of the organism because of low protein turnover in lens fibre cells (Wannemacher & Spector, 1968).

2.2.1 α-Crystallin

α-Crystallin is the most abundant crystallin in the mammalian lens representing 40-50% of the total lens protein content (Augusteyn & Stevens, 1998). It is an aggregate of two types of polypeptide, αA and αB, each of which has a molecular weight of around 20kDa. These polypeptides show extensive post-translational modification including phosphorylation, deamination, truncation, glycation, glycosylation, and oxidation [for a review see Groenen *et al*. (1994)]. They form oligomers with between 15-50 subunits, thus ranging from 300kDa to 1000kDa. The three-dimensional structure of α-crystallin is not available; however, it is known that in α-crystallin oligomers, the ratio of αA to αB is approximately 3:1(Horwitz *et al.*, 1999).

α-Crystallin, is a member of the small heat shock protein family and acts as a molecular chaperone, with the ability to inhibit the thermal precipitation of other proteins, including the β- and γ-crystallins (Horwitz, 1992). Furthermore, α-crystallin inhibits proteases (Ortwerth & Olesen, 1992) and has auto-kinase activity (Kantorow & Piatigorsky, 1994). All three of these roles appear to be associated with the C-terminal domain of the protein (Augusteyn & Stevens, 1998).

2.2.2 The β/γ-family

The other two crystallins β and γ, are structurally related, and belong to the β/γ crystallin superfamily. All proteins in the super family contain the Greek key motif of 4 anti-parallel β-pleated sheets (Augusteyn & Stevens, 1998). The major difference between β and γ crystallins is that β-crystallins contain N- and C-terminal extensions and form oligomers (Augusteyn & Stevens, 1998).

2.2.2.1 β-crystallins

The β-crystallins are a complex group of heteropolymers, with seven different polypeptides found in mammals: βA1 βA2 βA3 βA4 which are acidic and βB1 βB2 βB3 which are basic. The size range of the polypeptides is 23-25kDa for βA and 26-32kDa for βB. The β-crystallins can form different aggregation states ranging from 46kDa to >200kDa (Augusteyn & Stevens, 1998). The two main oligomers of β-crystallins are the octomeric βH (heavy) and tetrameric βL (light). Generally β-crystallins are the second most abundant

crystallins found in the lens but their composition varies during development (Augusteyn & Stevens, 1998).

Although there are seven mammalian β-crystallin genes, not all species express them. For example, mice (Ueda *et al.*, 2002), rat (Lampi *et al.*, 2002), cattle (Shih *et al.*, 1998) and sheep lenses (Robertson, 2003) contain all seven β-crystallins. Whereas, humans lenses don't contain βA2 but contain the other six β-crystallins (Lampi *et al.*, 1997).

2.2.2.2 γ-crystallins

There are six γ-crystallins, γA-F found in mammals (Augusteyn & Stevens, 1998). The γ-crystallins also contain four Greek key motifs arranged in two symmetric domains (Augusteyn & Stevens, 1998). However, while the two α-crystallin monomers and the seven β-crystallin monomers assemble to form a range of differently sized oligomers, γ-crystallins (γA, γB, γC, γD, γE, γF) are monomeric and have a molecular weight of approximately 20kDa (Graw, 1997). One interesting feature of γ-crystallin is that it has more SH groups than an average intracellular protein of the same size. Hence, it can act as a reserve of oxidizing-reducing power, in that it can form internal disulphide bonds without denaturation of the protein, which means if a γ-crystallin molecule is attacked by an oxidizing agent it might be able to cope with this internally without forming a polymer, whereas the other crystallins with fewer SH groups can only do this by polymerizing (Reddy & Giblin, 1984).

Another special but important γ-crystallin is γS-crystallin which possesses properties intermediate of β- and γ-crystallin. This is a monomeric protein which was first described by van Dam who named it βS in (1966) because of its similarities to the β-crystallins in size, amino acid composition, blocked N-terminus and eletrophoretic mobility. The human genome only has coding sequences for five γ-crystallins, γA, γB, γC, γD and γS-crystallins (Srikanthan *et al.*, 2004). The sheep genome for encoding γS-crystallin has been found to locate on the chromosome 1q of Ovis aries (Weiss, 2006), therefore, γS-crystallin is also in sheep lenses.

γ-Crystallins are synthesised only in the fibre cells, principally early in life, and subsequently their levels are higher in the nucleus than in the cortex (Augusteyn & Stevens, 1998). In human lens, γ-crystallin is involved in forming disulphide aggregates with the membrane in human cataract (Garner *et al.*, 1981). There is no evidence of γ-crystallin breakdown in cataract formation, however, missense mutations that alter surface

residues of human γ-crystallins can reduce solubility and cause cataract in children (Srikanthan *et al.*, 2004).

The γ-crystallins are subject to 'attractive' interactions and may therefore play a significant role in the tight packing of proteins in the nucleus. Since high levels of γ-crystallins are observed in the centre of the lens and virtually none in the cortex, comparison of γ-crystallin distribution with the refractive index gradient suggests that they may be directly related (Augusteyn & Stevens, 1998). Meanwhile, the tissues with a high refractive index are also very hard, suggesting that γ-crystallins may also be involved in the regulation of lens hydration (Augusteyn & Stevens, 1998).

2.3 Cataracts

2.3.1 Definition and Symptoms

The function of the lens is to transmit and focus light on the retina, hence it must be transparent. When oxidative damage to the lens and its proteins becomes extensive, the lens becomes sufficiently cloudy to obstruct vision, and the individual is said to have a cataract. Alternatively, events that cause loss of order and induce abrupt fluctuations in refractive index result in increased light scattering and loss in transparency, commonly called cataract (Bunce, 1994b). Defined as a clinically significant opacity of the lens, cataract has been an area of intense research in the last few decades. The first symptom of cataract is blurred vision. A cataract blocks some light from reaching the retina and distorts the light being focused on the retina. Figure 2.2 below shows the mechanism in which vision is affected by cataracts.

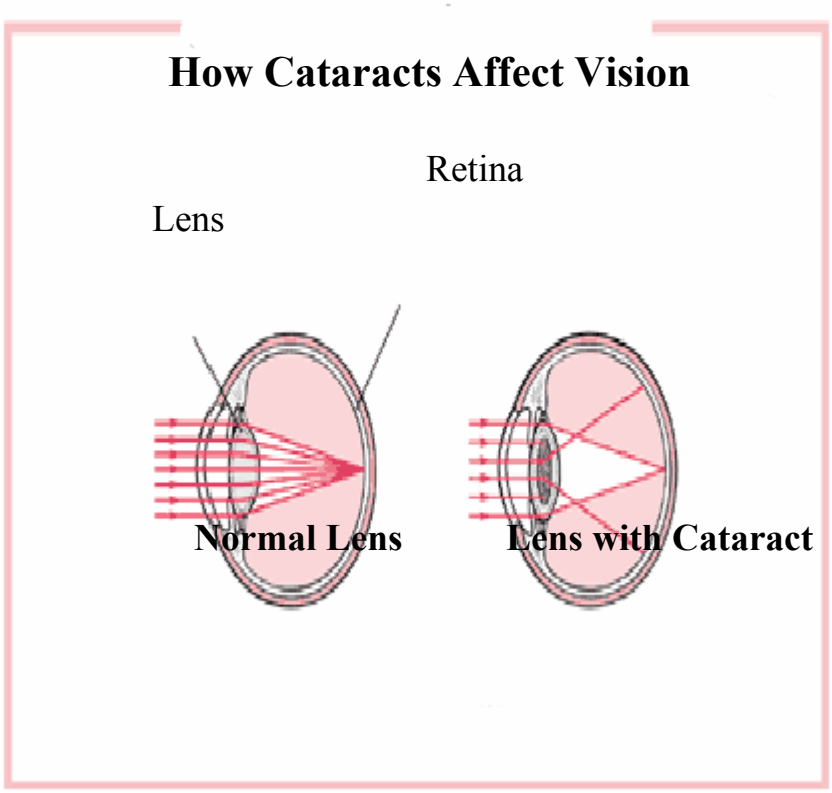


Figure 2.2 Illustration of how cataracts affect vision. Normal lens receive light and focuses it on the retina. In contrast, a cataract blocks some light from reaching the retina and distorts the light being focused on the retina, hence, resulting in blurred vision. Adapted from http://www.merck.com/pubs/mmanual_ha/sec3/ch36/ch36b.html Retrieved on 31 Oct 2005.

2.3.2 Types

There are three major forms of cataracts (nuclear, cortical, and posterior sub-capsule). Cortical cataracts are those opacities that originate in the outer layers of the lens. They typically display over-hydration and eventually liquefaction of the lens fibre cells secondary to electrolyte imbalance (Bunce, 1994b). Nuclear cataract occurs when modification and aggregation of lens structural proteins create light-scattering zones in the central region of the lens (Bunce, 1994b). Posterior subcapsular cataract (PSC) is described as an accumulation of abnormal epithelial cells at the posterior pole of the lens and just inside the capsule (Bunce, 1994b). Instead of differentiating into elongated fibre cells at the equator in the normal manner, the cells assume an irregular form and are displaced toward the posterior pole. This type of cataract probably arises as a result of damage to DNA. Many individuals will develop pure cortical, nuclear, or PSC cataracts, but mixed cataracts are also quite common (Bunce, 1994b). It has been determined that cataract formation in these three forms is a complex and multifactorial process (Spector, 1984).

High molecular weight protein aggregates, accumulation of water insoluble proteins (Harrington *et al.*, 2004) and increase in intracellular calcium content (Marcantonio *et al.*, 1986) have all been implicated in the pathogenesis of cataracts.

2.3.3 Factors Causing Cataract

Truscott (2005) proposed that the lens was analogous to an island because, like an island, the lens was coupled to the rest of the world only via a circulating fluid medium. However, the lens is affected by somatic and developmental factors. For example, although cataract is overwhelmingly a disease of the elderly, developmental impacts at earlier times, such as early childhood may be important. Evans *et al*. (1998) reported that the birth weight at one year of age was a predictor of later cataract development. They speculated that alterations in lens growth or differentiation were key stages leading to abnormal structural or functional changes that predispose to cataract development. Also, external factors may be important in cataract development. Harding (1991) postulated that diarrhoea may be a risk factor for cataract based on the observation that cataract was more prevalent in India.

Age-related cataract is a condition characterized by multiple mechanisms and multiple risk factors including oxidation, osmotic stress, and chemical adduct formation (Bunce *et al.*, 1990). The factors contributing to age-related cataractogenesis are a combination of pathological and normal aging processes. Chronic oxidative stress is widely believed to be a major factor in the aetiology of age-related cataract (Zigler *et al.*, 2003). For example, Anderson *et al*. (1994) reported that free radicals induce lipid peroxidation that leads to cataract formation. This was supported by several clinical, basic and epidemiologic studies (Kurysheva *et al.*, 1997). Other environmental, behavioural, physiological, and biochemical variables are also associated with types of cataract (nuclear, cortical, posterior subcapsular, and mixed) (Mohan *et al.*, 1989).

Lenses age slowly, a process that begins in the second decade of life and progresses more rapidly in middle and older age. The aging process results in the accumulation of light-scattering opacities that interfere with vision; this usually occurs during the sixth or seventh decade of life, but may occur earlier or later depending on the individual (Mares, 2004). Since age is the major risk factor for cataract, understanding changes in the lens with aging may well provide an insight into those processes that are responsible for the onset of cataract in later life.

Many alterations take place in the lens with age. These include changes in elastic properties and total ion content; increased fluorescence and nuclear light scattering; and a possible decrease in the refractive index of the nucleus (Truscott, 2005). Also, there are noticeable changes in the composition of membrane proteins with age. Old fibre cell membranes have very high cholesterol contents, which means that these bilayers are very rigid (Broekhuyse, 1981). The alterations of lipid composition consequently decrease the membrane fluidity – older membranes being more like butter than olive oil (Truscott, 2005).

Roberts (2001) proposed that young and adult eyes avoid damage by intense ambient light because they are protected by a very efficient antioxidant system. However, after middle age, there is a decrease in the production of antioxidants and antioxidant enzymes. At the same time, fluorescent chromophores (lipofuscin) accumulate to concentrations high enough to produce reactive oxygen species (ROS) (Roberts, 2001). Therefore, during the aging process, the (outer) epithelial cells will be most vulnerably damaged through modification of DNA, while the (inner) fibre membranes can be photo-chemically damaged through modification of certain amino acids (such as histidine, tryptophan, and cysteine) (Roberts, 1984) in the main intrinsic membrane proteins and through damage to the lipids (Roberts *et al.*, 1985). Such damage will result in a change in the refractive index of the lens material, leading to aggregation and a predispostion to age-related cataract, as proposed by Benedek in his theory of cataract formation (Benedek, 1971).

Sunlight seems to be an important environmental factor causing cataract, which may involve photo-oxidative processes. An increased risk of cataract was found for job locations in the sunlight, leisure time activities in the sunlight and a history of wearing a hat in summertime (Anonymous, 1991). Mohan *et al*. (1989) found an increased risk of cataract (all types) with decreased cloud cover at place of residence. Leske *et al*. (1991) further reported that occupational exposure to sunlight was a risk factor for nuclear cataract. Tarwadi & Agte (2004) recognised that photo-oxidative stress from x-ray or UV-B radiation of sunlight (wavelength of 290-320 nm) was a cause for cataract.

Lifestyle-related factors, such as cigarette smoking, appear to provide an additional oxidative challenge associated with depletion of antioxidants as well as with enhanced risk for cataract formation (Taylor *et al.*, 1995). Leske *et al*. (1991) also reported that smoking was a risk factor for nuclear cataract. Alcohol consumption appeared to be a minor risk

factor (Truscott, 2005). Another recognised cause for cataract is exposure to environmental pollution (Tarwadi & Agte, 2004).

Diet also plays an important role in cataract formation. Mohan *et al*. (1989) reported that diets low in selected nutrients increased risk for posterior subcapsular, nuclear, and mixed cataracts. They also found an increased risk of cataract (cortical, nuclear, and mixed) with the use of cheaper cooking fuels, and with lower levels of an antioxidant index based on red blood cell levels of glutathione peroxidise and glucose-6-phosphate dehydrogenase and plasma levels of ascorbic acid and vitamin E (posterior subcapsular and mixed). Poor education and lower socioeconomic status are associated with poorer nutritional states and were also significantly related to increased risk for cataract formation (Leske *et al.*, 1991; Taylor *et al.*, 1995). For example, The Italian-American Cataract Study Group (1991) reported an increased risk of all types cataract for persons with less than a high school education.

Excessive caloric intake was also a factor contributing to cataract. Diabetes increases the likelihood of cataract three- to four-fold. Leske *et al*. (1991) reported that diabetes increased the risk of posterior subcapsular, cortical, and mixed cataracts. Obesity, defined as more than 20% overweight, is a major risk factor for non-insulin-dependent, or type II, diabetes and Leske *et al*. (1991) found that body mass index was a risk factor for nuclear cataract. Higher blood pressure was also found to increase the risk of nuclear and mixed cataract (Mohan *et al.*, 1989). Therefore, weight control can be recommended as a prudent, safe, economic, and effective means of lowering risk probability for diabetes and the associated complication of cataract (Bunce *et al.*, 1990).

Certain pharmaceutical substances and possibly acute episodes of dehydration are other recognised causes for cataract (Bunce *et al.*, 1990). For example, aspirin, even when use less than once a month, increased the risk of posterior subcapsular and mixed cataract (Mohan *et al.*, 1989); gout medications increased mixed cataract risk; and oral steroid therapy increased posterior subcapsular cataract risk (Leske *et al.*, 1991). The Italian-American Cataract Study Group (1991) also reported that a history of cortisone use was a risk factor for subcapsular cataract.

Family history, and use of eyeglasses by age 20 years, which is an indicator of myopia, increased risk of mixed cataract (Leske *et al.*, 1991). The Italian-American Cataract Study Group (1991) reported that a positive family history of cataract (which may involve changes in gene expression) was a risk factor for posterior subcapsular, cortical, and mixed

cataracts and that increased serum level of uric acid was a risk factor for PSC. However, a decreased risk of cataract was found for persons with a positive history of arthritis (posterior subcapsular, nuclear, and mixed) and increased handgrip strength (mixed). The results support a role for nutritional, medical, personal, and other factors in cataractogenesis.

2.4 Oxidation and Pro-oxidant

2.4.1 Redox Reaction

Oxidation-reduction reactions are coupled chemical reactions in which one atom or molecule loses one or more electrons (oxidation) while another atom or molecule gains these electrons (reduction). When reduction and oxidation characterize a chemical reduction, it is called a redox reaction (Figure 2.3). Oxidation is always accompanied by an equivalent amount of reduction elsewhere in the system. Figure 2.3 below shows concepts of oxidation and reduction as well as the relationship between them. Redox reactions are at the heart of biological oxidation, the chain of chemical reactions whereby we use oxygen from air to oxidize chemicals in the breakdown of food to provide energy for living (Prior & Cao, 1999).

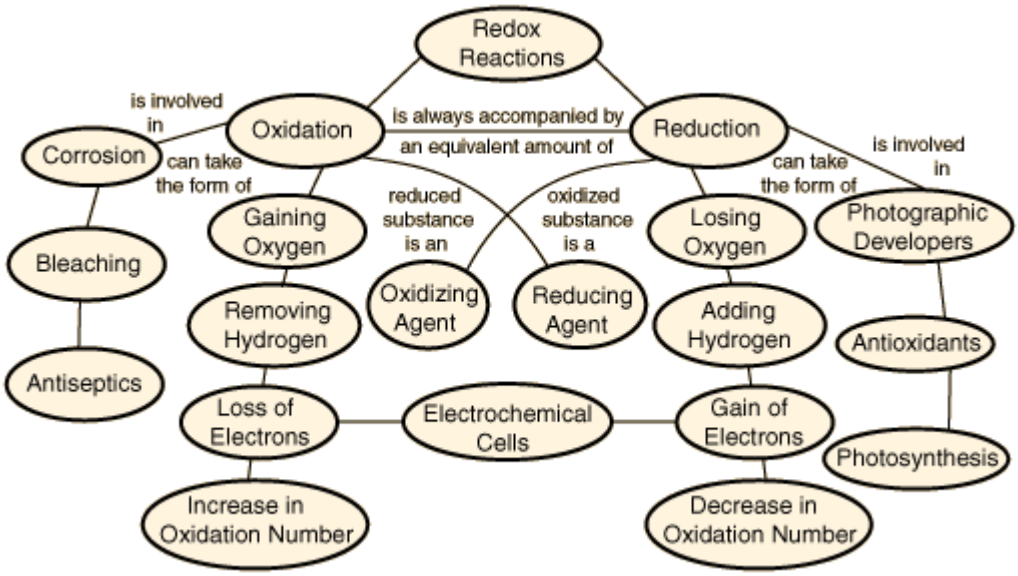


Figure 2.3 Illustration of oxidation/reduction concepts. Retrieved from http://hyperphysics.phy-astr.gsu.edu/hbase/chemical/oxred.html on 19 Sep 2005.

2.4.2 Pro-oxidants

A pro-oxidant is a toxic substance that can cause stochastic oxidative damage to all cellular components including lipids, proteins, nucleic acids (DNA) and carbohydrates (Jarrett & Boulton, 2005), thus resulting in various pathologic events and/or diseases (Halliwell & Gutteridge, 1999). Pro-oxidants are reactive species including not only the free radicals (e.g., superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), and perhydroxyl ($HO_2^{\cdot}$)) but also some reactive non-radical molecules, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3). Reactive species can also be reactive oxygen species (which will be detailed below because of their importance), reactive nitrogen species and other reactive species.

Reactive oxygen species (ROS) (including $O_2^{\cdot -}$, $OH^{\cdot}$ and H_2O_2) are ubiquitous and occur naturally in all aerobic organisms, coming from both exogenous and endogenous sources. ROS are generated during normal cellular metabolism as by-products of numerous oxidases, leakage from mitochondrial electron transfer processes (Slaughter *et al.*, 2002), and during the immune response resulting from the respiratory burst of activated phagocytic cells to eliminate harmful pathogens (Chanock *et al.*, 1994). ROS are not only quite reactive and able to readily damage biological molecules, including DNA, but also arrest cell growth by triggering responses of different cycle checkpoints (Shackelford *et al.*, 2000). Cell-cycle arrest may lead to gene mutations particularly with respect to potential DNA damage (Morel & Barouki, 1999). In this situation, a pro-oxidant is an oxidant of pathological importance.

Although ROS have been considered as harmful molecules that may be associated with cancer and various human degenerative diseases, including Alzheimer, Parkinson, macular degeneration and cataract (Chen *et al.*, 2004), Sen & Packer (1996) mentioned that at moderately high concentrations, certain forms of ROS such as H_2O_2 may act as signal transduction messengers. Moreover, Chen *et al*. (2004) have recently proposed that ROS have the physiological function in the non-phagocytic cells to mediate signal transduction of some growth factors and cytokines to achieve certain cellular function, named as "redox signalling" (Finkel, 1998). They contended that low levels of ROS play an important role in host defence and have the physiological role of redox signalling in the human lens epithelial cells and may play an important role in the development and maintenance of the lens. Several studies (Shibanuma *et al.*, 1990; Sundaresan *et al.*, 1995; Colavitti *et al.*, 2002) showed that the mitogenic stimulus action of some growth factors actually required initial local intracellular ROS generation. Exogenous ROS at low levels could also mimic

growth factors to regulate and activate the mitogenic and stress-associated signalling transduction pathways in the cells, leading to gene expressions for various cellular functions (Rao & Berk, 1992; Duhe *et al.*, 1998; Zhang *et al.*, 1998). Meanwhile, many of these signalling functions can be regulated and controlled by the redox status in the cells (Sen & Packer, 1996). Finkel and Holbrook (2000) further proposed that the levels of ROS are tightly regulated and balanced to provide normal cellular growth and metabolism.

In conclusion, on one hand, excess ROS can inflict random cellular damages and alter specific signalling pathways that contribute to aging, disease and even cell death. On the other hand, lowering ROS levels below homeostasis set point may interrupt the physiological functions and lead to decreased proliferative response and defective host defences.

2.4.2.1 Free Radicals

A free radical is any atom, molecule or compound that presents unpaired electrons and is able to receive or donate them (Ferrari & Torres, 2003). Free radicals are produced during mitochondrial respiration and also released by peroxisomes. Free radicals include oxygen radicals such as superoxide ($O_2^{\cdot-}$); nitrogen radicals such as nitric oxide (NO·) & peroxynitrite ($ONOO^-$); and other free radicals such as tyil (CH_2S^-) (Ferrari & Torres, 2003).

Tarpey & Fridovich (2001) reported that superoxide ($O_2^{\cdot-}$) initiated pro-inflammatory events. The mechanisms involved the transcriptional regulation of gene expression which was sensitive to changes in cellular oxidant production as well as modulation of cell-signalling events. The rate of formation and extent of scavenging of $O_2^{\cdot-}$ have been implicated in various dysfunctions such as atherosclerosis, hypertension, diabetes as well as in postischemic myocardium (Tarpey & Fridovich, 2001). Furthermore, the superoxide radical ($O_2^{\cdot-}$) has been suggested as the cause of the DNA strand breakage observed in cells exposed to cigarette smoke (Spencer *et al.*, 1995).

Tarpey & Fridovich (2001) reviewed reactive nitrogen radicals including nitric oxide (NO·) and peroxynitrite ($ONOO^-$). Nitric oxide is associated with cytotoxic effects because it reacts with cellular thiols, lipids, proteins and DNA. Peroxynitrite is produced by the reaction of $O_2^{\cdot-}$ and NO·. Peroxynitrite is proposed to mediate many of the cytotoxic effects of NO· and therefore, also has the role in pathologic processes.

Thus, potentially lethal free radicals are generated continuously in living systems and they catalyse several redox reactions of various compounds in living tissues and cells. The ocular tissue could be particularly susceptible to free radical injury because of their limited metabolism (Rose *et al.*, 1998).

2.4.2.2 Biologically Important Non-radical – Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is the most stable intermediate of oxygen reduction. The generation of H_2O_2 requires O_2, and H_2O_2 production increases with increasing O_2 tension (Spector *et al.*, 1998). It is generated during the univalent reduction of oxygen mainly by the xanthine/xanthine oxidase system (Lenzi *et al.*, 2000). Also, it can be produced by $O_2^{\cdot-}$ dismutation. Hydrogen peroxide is only a weak oxidizing and reducing agent and is generally poorly reactive (Halliwell & Gutteridge, 1999). It is of biological importance because it can cross cell membranes rapidly and, once inside, can react with iron, and possibly copper, ions to form much more damaging species such as OH·, which is highly reactive and biologically damaging. Hydroxyl radicals account for much of the damage done to DNA in H_2O_2-treated cells since OH· modifies all four DNA bases (Spencer *et al.*, 1995). In addition, H_2O_2 can be converted to OH· by ultraviolet light (UV).

Despite its poor reactivity, H_2O_2 can be cytotoxic because of its ability to oxidize certain keto-acids (Augusteyn & Stevens, 1998). Also, H_2O_2 can degrade haem proteins (including myoglobin, haemoglobin and cytochrome *c*) to release iron ions which can catalyse damaging free-radical reactions and promote DNA degradation as well as stimulate lipid peroxidation (Gutteridge, 1986). Meanwhile, H_2O_2 has been shown to induce apoptotic cell death in whole lenses and to up-regulate expression of some proto-oncogenes (Li *et al.*, 1994). Current evidence suggests that H_2O_2 is the major intracellular ROS to which the lens is exposed (Spector & Garner, 1981; Taylor & Davies, 1987; Spector, 1995) and the major oxidant involved in cataract formation in human.

2.5 Oxidative Stress and Cataracts

Insults such as oxidative-stress, UV exposure, and antioxidant depletion could result in the initiation of cataract formation. “Oxidative stress” has been defined by Helmut Sies as ‘a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage’ (Sies, 1991). Therefore, oxidative stress occurs when redox homeostasis is disturbed in such a way that the ratio of pro-oxidants to antioxidants is increased.

Oxidative stress is believed to be an early event in the development of some cataracts. Spector (1984) first reported that senile cataract, the most common type of cataract in humans, shows progressive and widespread oxidation damage. Subsequently, his review (1995) examined the hypothesis that oxidative stress is an initiating factor for the development of maturity onset cataract and described the events leading to lens opacification. Data were reviewed that indicated that extensive oxidation of lens protein and lipid is associated with human cataract found in older individuals whereas little oxidation (and only in membrane components) is found in control subjects of similar age. In addition, Slaughter *et al*. (2002) showed oxidative stress was caused by excessive production of reactive oxygen species (ROS). Because of the long-term exposure to ROS generating systems in the environment, an aging lens is known to suffer oxidative damage induced by ROS. Numerous studies have shown that oxidation can damage lens proteins (Spector, 1995; Lou, 2003), membrane lipids (Zigler *et al.*, 1983) and DNA (Kleiman *et al.*, 1990), all of which may contribute to lens opacification and cataract formation.

In vivo and *in vitro* animal models of ROS-induced cataract further support the association of oxidative stress and cataractogenesis. For example, Cui & Lou (1993) studied the exposure of rat lenses to H_2O_2 (0.5 mM) for up to 96 h. It was found that the lenses had only patchy opacity at the equator after 24 h, but became hydrated suddenly at 48 h (31% heavier than the control), with an opacity which involved the entire outer cortical region. By 72 h incubation, the nucleus was opacified.

Protection from oxidative damage and hence maintenance of lens transparency is afforded by antioxidant systems that consist of non-enzymatic and enzymatic components. Mates & Sanchez-Jimenez (1999) has reviewed the evidence that the balance between anti- and pro-oxidants played an important role in initiating pathological illnesses such as cataracts. Different types of cataract have been found to be associated with oxidative stress. For example, Altomare *et al.* (1995) found that oxidative stress has been implicated in human diabetic cataract; Spector (2000) reviewed the impact of oxidative stress on cataractogenesis associated with ageing; and Zigman (2000) reviewed the evidence that oxidative stress was implicated in the formation of human cataract associated with UV irradiation.

2.5.1 Oxygen Tension

Light and oxygen are necessary for the function of the eye. However, when present in excess or in uncontrolled circumstances, they appear to be related, probably causally, to the development of cataract (Taylor *et al.*, 1995). The importance of oxygen had been emphasised in Eaton's article with the provocative title 'Is the lens canned?' (Eaton, 1991). He regarded low O_2 in the lens as a vital ingredient in maintaining optical clarity. In this way he proposed that the lens resembles canned food where oxygen is sealed out and ascorbate is used to mop up the residual amounts in order to keep the components in a state close to their original condition (Truscott, 2005). Also, Spector *et al*. (1998) concluded from their experiment that changes in concentration of oxygen level in aqueous humor have profound effects on H_2O_2 concentration and may affect lens viability. Exposure of human patients and experimental animals to hyperbaric O_2 has also been shown to lead to opacification of the lens especially in the nuclear region (Padgaonkar *et al.*, 1989).

If low O_2 is so important in maintaining lens clarity then what are the O_2 levels inside the lens? The precise answer to this question was not available until new technologies, in particular the optode, made it possible to measure the low levels of O_2 present in the very high protein concentrations of the lens (McNulty *et al.*, 2004). This method has revealed that although oxygen is present in the centre of the lens, there is a steep gradient from the outer parts of the lens into the lens interior. Mitochondria in outer regions appear to be the chief agents responsible for keeping the nuclear O_2 levels low.

2.5.2 Mechanisms of Damage to the Lens by Oxidative Stress

It is important to recognize that age-related cataract in human is a multi-factorial disease that develops over decades. It is believed that oxidation is an initiating or very early event in the overall sequence that leads to age-related cataract. Bunce (1994b) reviewed evidence that oxidative damage may adversely affect membranes, thus driving a disturbance of electrolyte homeostasis; that oxidative stress may also cause modification of proteins leading to protein aggregation and loss of enzymatic function; and that mutations arising from oxidation of purine or pyrimidine bases can generate errors in differentiation of epithelial cells into fibre cells. Sanderson *et al*. (1999) contended that the mechanism also involves disruption of the redox system, proteolysis and a loss of transparency.

As the organ that gathers light, cells of the lens are particularly subject to oxidative stress due to photo-oxidative processes and are consequently prone to cellular oxidative damage by the following mechanisms:

2.5.2.1 Lens Proteins Damage

The oxidative modification of proteins that leads to lens opacity, may occur directly, as in ultraviolet absorption by protein side chains, or indirectly via attack by reactive oxygen species (Slaughter *et al.*, 2003).

Srikanthan *et al.* (2004) found that age-related cataract is associated with increased reactivity of protein thiol. Proteins isolated from cataractous lenses have been found to contain elevated amounts of methionine sulfoxide and end products of oxidative damage (Halliwell & Gutteridge, 1999). In the most advanced cataract lenses, more than 90% of protein sulfhydryl (PSH) groups are lost, and almost half of all the methionine residues in the nuclear proteins become oxidised to methionine sulfoxide (Truscott & Augusteyn, 1977; Garner & Spector, 1980). These oxidised residues in proteins have revealed the extent to which oxidation accompanies age-related cataract. In addition, these oxidative changes to proteins can be detected in the very earliest stages of nuclear cataract and are progressive in the later stages. Truscott (2005) regarded this progression as an important criterion to verify that these measured oxidative changes are not merely one of many processes which take place in the latest phase of cataract once the lens has been severely compromised, but are likely to be linked with the onset of age-related cataract. Given that there is such a close correlation between protein oxidation and age-related cataract, it is highly likely that such oxidative modification of protein is involved in, and may be the underlying mechanism responsible for, the clinical presentation of cataract (Truscott, 2005).

Lens proteins, especially the crystallins, have a very long turnover time and so damage to them is accumulative. Two key biophysical crystallin properties are solubility and long-term stability. Taylor & Davies (1987) proposed that in aged lens, damage, cross-linking, and precipitation of crystallins contribute to a loss of lens clarity. Damage to lens crystallins appears to be largely attributable to the effects of UV radiation and various active oxygen species. Davies & Truscott (2001) reviewed evidence that the oxidative modification of proteins leads to lens opacity, and may occur directly as in UV absorption by protein side chains, or indirectly via attack by ROS. In addition, Spector (1995) suggested that denaturation, oxidation and aggregation of crystallins lead to loss of transparency. Truscott (2005) proposed that insolubilisation of crystallins is the end result of an ongoing process of denaturation and aggregation caused by oxidative damage and resulting in the opacity of lens.

2.5.2.2 Loss of GSH

The mammalian lens contains an unusually high concentration of glutathione, a tripeptide thiol consisting of the amino acids glycine, cysteine, and glutamic acid. Found in virtually all cells, glutathione functions in metabolism, transport, and cellular protection (Meister, 1983). Glutathione in the lens is present largely in the reduced state (GSH) (Anderson, 1985). However, the degree of protein thiolation -- mostly disulfide linkage to glutathione, increases with the progression of age-related cataract. Although it is a minor reaction compared with the overall loss of protein sulfhydryl groups, it is still quantitative and crucial. Reduced glutathione levels can markedly affect the direction of oxidation processes (Halliwell & Gutteridge, 1999). Truscott (2005) stated that GSH above 1mM inhibited hydroxyl radical formation whereas concentrations below 1mM accelerated its production. Also, he proposed that low concentration or loss of reduced glutathione (GSH) in the centre of the lens of the lens may be the common and critical feature that precedes age-related nuclear cataract formation. Decreased levels of GSH is a typical finding associated with nearly all experimental cataract (Truscott, 2005). This loss is due to oxidation of GSH, since the levels of oxidized glutathione (GSSG) rise significantly with cataract development. Therefore, in cataract, the oxidation-antioxidant equilibrium may shift more towards oxidative stress, where the demand for antioxidant micronutrients increases (Tarwadi & Agte, 2004).

2.5.2.3 Damage of Lens Epithelial cells

Epithelial cells are the first line of defence of the lens against stress and play crucial roles in maintenance of the entire organ. In addition, the epithelial cells control transport to the lens. They have direct contact with the aqueous humour and are most vulnerable to phototoxic damage (Roberts, 2001). Oxidation –induced damage to lens epithelial cells includes bulk protein oxidation (Blondin *et al.*, 1986), inactivation of key enzymes (Blondin & Taylor, 1987; Taylor & Davies, 1987; Reddan *et al.*, 1993), DNA breaks (Spector *et al.*, 1989), and lipid peroxidation (Shang *et al.*, 1997). In 1994, Andley *et al*. first successfully established primary and immortalized cell cultures of human lens epithelial cells to better investigate human lens epithelial physiology, aetiology of cataract and eye-related toxicology. They found that damage to epithelial cells readily compromised lens viability (Andley *et al.*, 1994). Furthermore, lens epithelial cells were readily damaged by hydrogen peroxide, showing DNA strand breakage and abnormalities of ion transport, e.g. damage to the Na^+, K^+-ATPase (Spector, 1995). He demonstrated that

the redox set point of the single layer of the lens epithelial cells (but not the remainder of the lens) quickly changes after exposure to oxidative stress, going from a strongly reducing to an oxidizing environment. Almost concurrent with this change is extensive damage to DNA and membrane pump systems, followed by loss of epithelial cell viability and death by necrotic and apoptotic mechanisms. The data suggest that the epithelial cell layer is the initial site of attack by oxidative stress and that involvement of the lens fibers follows, leading to cortical cataract. Also, Kleiman *et al*. (1990) confirmed that significant numbers of DNA single-strand breaks were detected after exposure to as little as 25 μM H_2O_2 for 5 min at 37 °C in primary cultures of bovine lens epithelial cells. The extent of single-strand breakage was concentration dependent and linear from 25 to 200 μM H_2O_2.

2.5.2.4 Oxidation Decreases Lens Ca^{2+}-ATPase Activity

Cortical cataract is associated with increased membrane permeability and intracellular calcium overload (Duncan & Bushell, 1975). The integrity of the calcium pump is essential for maintenance of calcium homeostasis, which is critical for the clarity of the lens. Calcium -ATPase activity is lower (Ahuja *et al.*, 1999) and the degree of lipid hydrocarbon chain order (Bonting *et al.*, 1961) and the level of lipid oxidation (Babizhayev *et al.*, 1988) are higher in cataractous lenses compared with clear lenses. Ahuja *et al*. (1999) observed a 50% decrease in Ca^{2+} -ATPase activity in a human cataractous lens and reported that oxidation of the lens caused an increase in lens calcium (Ahuja *et al.*, 1999). Therefore, they speculated that oxidation of the lens calcium pumps could contribute to cataract formation by inhibiting the pumps, thus causing an increase in lens cytosolic calcium. Elevated calcium concentration enhances calcium binding to cytoplasmic proteins (Jedziniak *et al.*, 1972), lowers Na, K-ATPase activity (Delamere *et al.*, 1993), activates proteases or lipases (Ahuja *et al.*, 1999), and may have a deleterious effect on lens metabolism (Hightower *et al.*, 1985) resulting in increased lens opacity. The mechanism for inactivation of the calcium pump by oxidation has been attributed to oxidation-induced lipid structural changes, oxidation-related protein fragmentation (pump degradation), and direct oxidation of the sulfhydryl groups of the Ca^{2+} -ATPase pump (Ahuja *et al.*, 1999).

2.5.3 Animal Models

A role for oxidation as one of the contributing factors to cataract formation has been reported in several animal models including: selenite (an oxidant)-induced rat cataract model (Tamada *et al.*, 2000); sugar xylose-induced cataract in rhesus monkey lens model (Zigler *et al.*, 2003); diamide (a thiol-specific oxidant)-induced rat cataract model (Obin *et*

al., 1998) and hydrogen peroxide-induced rat cataract model (Lou *et al.*, 1990). Overall, there is evidence that oxidative damage in the lens is part of cataract formation in models of cataractogenesis.

2.5.4 Ovine Model for Cataract Study

In order to study cataracts and possible treatments for cataracts, a number of animal models have been developed including guinea pigs and rabbit (Fukiage *et al.*, 1998), with rat and mouse being the most common models (Tripathi *et al.*, 1991; Shearer *et al.*, 1997). However, sheep lenses are more appropriate models of the human lens than the rodent lenses commonly used for lens research. The spherical rat lens with its very high and steep refractive index gradient is designed for use at close distances (Augusteyn & Stevens, 1998). It cannot accommodate for distance viewing because it is too hard to deform and lacks the necessary accommodative apparatus. By contrast, the biconvex human lens, with its flatter refractive index gradient, is designed to focus further away. The soft tissue is amendable to deformation and this is used during accommodation to change the focal length (Augusteyn & Stevens, 1998). The oval ovine lens resembles the human tissue. From the properties of the lens and the anatomy of its suspensory structure, it would be expected that the ovine lens is capable of accommodation. A genetic cataract of sheep has been characterised and is available for study (Robertson *et al.*, 2005).Therefore, the ovine cataract is an alternative model for cataractogenesis.

2.6 Antioxidants

Oxidative stress elicits an adaptive antioxidant response. As it can be seen from the previous description, reactive species and their derivatives are toxic. Therefore, aerobes only survive in oxygen because they have evolved antioxidant defences. This adaptive antioxidant response is a biological phenomenon which involves cells reacting at a molecular level to acquire greater cellular resistance against a wide range of physiological stresses, including ROS, which may be constantly produced intracellularly as a result of normal metabolic activity, but also produce cellular oxidative damage (Davies, 1995). Halliwell and Gutteridge (1999) defined an antioxidant as “a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly prevents or delays a pro-oxidant initiated oxidation of the substrate”. This is because an antioxidant can effectively reduce a pro-oxidant with the products having no or low toxicity (Prior & Cao, 1999).

As the eye has a lot of potential problems with oxidation, a corresponding degree of protection would be expected. One protective feature is that intra-ocular O_2 tensions are low, as mentioned earlier. In addition, in order to control levels of ROS, nature has evolved antioxidant systems. Intracellular antioxidants include low molecular weight scavengers of oxidizing species, and enzymes which degrade superoxide and hydroperoxides. Such antioxidants systems prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reactions with biological structures. Therefore, protection from oxidative damage and hence maintenance of lens transparency is afforded by these antioxidant systems that are based on a network of non-enzymatic and enzymatic components, including supporting enzymes and metabolic pathways (Poulsen *et al.*, 2000), which will be detailed below. In this way, lens cells use the antioxidant systems and repair mechanisms to ameliorate oxidative insult.

2.6.1 Enzymatic Antioxidants & Non-enzymatic Antioxidants

2.6.1.1 Enzymatic Antioxidants

Antioxidant enzymes are able to catalytically remove free radicals and other reactive species. A wide array of enzymatic antioxidant defences exists, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Halliwell & Gutteridge, 1999). Expression of some of these enzymes is controlled by redox-sensitive transcription factors, allowing the antioxidant system to respond to fluctuations in production of oxidizing species caused by photo-oxidative processes, especially during sustained exposure (Rahman, 2000). For example, Dudek *et al*. (2001) demonstrated that the oxidant-sensitive transcription factor, nuclear factor-*kappa*B (NF-κB), was strongly activated at 1 h and returned to basal levels by 2 h when lens epithelial cells were treated with H_2O_2. However, it is probably the basal expression of the antioxidant system that determines the degree of acute resistance to oxidative stress (Slaughter *et al.*, 2002) and may be key to protection of ocular tissue.

2.6.1.1.1 Superoxide Dismutase

The chemical definition of ‘dismutation’ is a reaction in which the same species is both oxidized and reduced. Superoxide dismutases are present in all eye tissues (Halliwell & Gutteridge, 1999) and are specific for catalytic removal of superoxide. The SOD activities of most eye tissues are inhibited by cyanide and thus most activity can be attributed to copper-zinc superoxide dismutase (CuZnSOD). All CuZnSODs catalyse the same reaction below -- the one-electron dismutation of superoxide into hydrogen peroxide and oxygen.

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \text{ (ground-state)}$$

The copper ions in CuZnSODs appear to function in the dismutation reaction by undergoing alternate oxidation and reduction as below, i.e.

$$\text{Enzyme-}Cu^{2+} + O_2^{\cdot -} \rightarrow \text{Enzyme-}Cu^{+} + O_2$$

$$\text{Enzyme-}Cu^{+} + O_2^{\cdot -} + 2H^+ \rightarrow \text{Enzyme-}Cu^{2+} + H_2O_2$$

Net reaction: $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$ (ground-state)

The Zn^{2+} does not function in the catalytic cycle but helps stabilize the enzyme (Halliwell & Gutteridge, 1999).

There is substantial variation in lens antioxidant systems between different laboratory animal species. Given that differences in the expression of the lens antioxidant system may influence species susceptibility to oxidative stress, Slaughter *et al*. (2003) compared and contrasted a broad spectrum of components of the lens antioxidant system in dogs, rats, marmosets, and rabbits. They found that activities of CuZnSODs were highest in marmoset lens (being 29- and 5.2-fold that in rabbit and rat respectively). In contrast, dog lens had the lowest levels of CuZnSOD (1/63 that in marmoset) (Slaughter *et al.*, 2003).

Moreover, Reddy *et al.* (2004) used transgenic and gene knockouts for CuZnSOD (*Sod1*) to demonstrate that this antioxidant enzyme level correlated with oxidative damage in lens epithelium *in situ* resulting from H_2O_2 exposure of organ cultured lens. The transgenic mice over-expressed this enzyme when the lenses were exposed to H_2O_2, indicating that the potential increase of this antioxidant enzyme was a feedback mechanism for chronic exposure to oxidants.

2.6.1.1.2 Catalase & Glutathione Peroxidase

As it can be seen from above that dismutation of $O_2^{\cdot -}$ generates H_2O_2. Hydrogen peroxide is usually removed in aerobes by two enzymes. The catalases (CAT) catalyse the two-electron dismutation of hydrogen peroxide into ground-state oxygen and water.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Peroxidase enzymes such as GPX remove H_2O_2 by using it to oxidize another substrate, reduced sulphur (written SH_2 below):

$$SH_2 + H_2O_2 \longrightarrow \text{Sulphur} + 2H_2O$$

GPX and CAT are present in all parts of the eye. GPX is the predominant GSH-consuming enzyme and the GPX family uses GSH as a cofactor to destroy hydrogen peroxide and lipoperoxides. Reddy & Giblin (1984) confirmed that there was considerable evidence that GPX was involved in the breakdown of H_2O_2. Furthermore, the major detoxification of H_2O_2 occurred in the epithelium of the lens (Giblin *et al.*, 1982). During their experiment, various concentrations of H_2O_2 were maintained in the culture medium. They found that during a 3 h exposure of lens epithelial cells (from 4-6-day old rabbits) to a 0.03 mM H_2O_2, there was nearly a 10-fold stimulation in hexose monophosphate shunt activity and the cells remained undamaged. The maximum shunt activity of the epithelial cells was twice that of the same number of rabbit skin fibroblasts and, in contrast to the lens epithelial cells, the fibroblasts were severely damaged by 0.03mM-H_2O_2. The results indicate the importance of glutathione metabolism and the epithelium in protecting the whole lens against H_2O_2 –induced oxidative damage.

$$H_2O_2 + 2GSH \xrightarrow{GPX} GSSG + 2H_2O$$

Therefore, the cooperation of glutathione peroxidase and catalase contributes to removal of hydrogen peroxide *in vivo*.

Slaughter *et al*. (2003) reported that rabbit lens contained the highest CAT activity, at up to approximately 3.5-fold that for marmoset and rat and 40% higher than in dog. As for associated H_2O_2-dismutation enzyme GPX, they reported that marmoset lens contained the highest level of GPX, at 1.3-, 5.2-, and 12.8-fold that in dog, rabbit, and rat respectively. Meanwhile, they also found that dog had a very low GR:GPX ratio (reflecting redox turnover of glutathione), which indicates that dog has a relative deficit in the capacity to maintain glutathione in reduced form. In contrast, GR:GPX ratio was highest in rat, which indicates that rat has greater capacity to maintain glutathione in reduced form. The physiological meaning of these results will be explained in section (2.6.1.2.1.3). Sanderson *et al.* (1999) reported in their experiment data that the ratio of GSH/GSSG in freshly dissected rat lenses were 63.

2.6.1.2 Low Molecule Weight Antioxidants

Non-enzymatic scavengers of small molecules such as glutathione (GSH), ascorbate, α-tocopherol (vitamin E), and the carotenoids act as rapid chemical traps or physical quenchers (Chaudiere & Ferrari-Iliou, 1999). Ascorbate and glutathione scavenge oxidizing free radicals in water by means of one-electron or hydrogen atom transfer.

Alpha-tocopherol is a primary scavenger of lipid peroxyl radicals and carotenoids are secondary scavengers of free radicals as well as physical quenchers of singlet oxygen (Chaudiere & Ferrari-Iliou, 1999). Only GSH will be addressed in detail below as one of the low molecule weight antioxidants because GSH plays a role in detoxification and excretion pathways and as a cofactor for enzymatic reduction of peroxides (Hayes & McLellan, 1999). Furthermore, in the lens, GSH is particularly important in preventing accumulation of toxic products of ascorbate oxidation (Sasaki *et al.*, 1995).

2.6.1.2.1 Glutathione (GSH)

Reduced glutathione (GSH), a ubiquitous essential tripeptide thiol (*L*-*γ*-glutamyl-*L*-cysteinyl-glycine; Figure 2.4), is a vital intra- and extra-cellular protective antioxidant against oxidative stress. It contains a side chain sulfhydryl (-SH) residue that enables it to protect cells against oxidants (hydroperoxides) and electrophilic compounds (Rahman & MacNee, 1999). Therefore, maintenance of GSH is known to be vital for lens clarity (Sweeney & Truscott, 1998). Glutathione is not evenly distributed in the lens. Its concentration is higher in the cortex than in nucleus and the highest level is in the epithelium (Reddy & Giblin, 1984). They reported that the concentration of reduced and oxidized glutathione (GSH + GSSG) in rabbit lens epithelium (64 μmol/g) is five times higher than in the cortex. The level of GSH in the nucleus of the lens is relatively low, particularly in the aging lens, and exactly how the compound travels from the epithelium to the central region of the organ is not known. However, Sweeney & Truscott (1998) proposed that the development of a barrier to the migration of GSH from its site of synthesis and regeneration in the cortex, into the nucleus in older normal lenses, may over time allow oxidative modification of protein to take place in the nucleus, resulting ultimately in nuclear cataract. Thus, the relatively low ratio of GSH to protein -SH in the nucleus of the lens, combined with low activity of the glutathione redox cycle in this region, makes the nucleus GSH especially vulnerable to oxidative stress (Giblin, 2000).

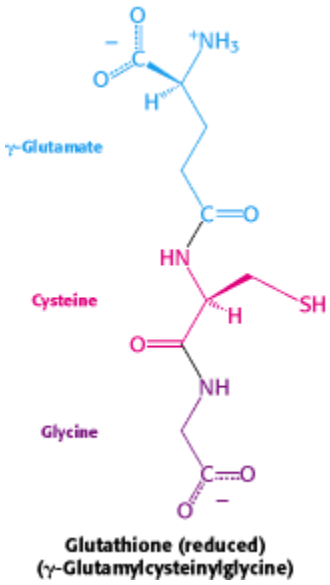


Figure 2.4 Structure of glutathione (GSH) – reduced form.

2.6.1.2.1.1 Correlation between GSH and GSSG

The GSH redox (thiol-disulfide) status is critical for various biological events that include modulation of redox-regulated signal transduction (Brown, 1994), transcriptional activation of specific genes (Arrigo, 1999), storage and transport of cysteine (Meister & Anderson, 1983), protection of cells against oxidative stress (Noctor *et al.*, 1998), detoxification of xenobiotics and heavy metals (May *et al.*, 1998), regulation of cell proliferation (den Boer & Murray, 2000), apoptosis (Hall, 1999), immune modulation (Droge *et al.*, 1994), and inflammation (Rahman, 2000). The majority of intracellular glutathione occurs as reduced GSH (1 to 10mM), depending on the cell type (Rahman & MacNee, 1999). Maintenance of a high intracellular (GSH) / (GSSG) ratio (>90%) minimises the accumulation of disulfides and provides a reducing environment within the cell (Rahman, 2000). Carbohydrate metabolism is also involved in the maintenance of GSH in the reduced state. There is a direct link between the rate of formation of oxidized glutathione (GSSG) and the stimulation of the hexose monophosphate shunt through the generation of NADPH.

$$GSSG + NADPH + H^{+} \xrightarrow[(GR)]{\text{Glutathione reductase}} 2GSH + NADP^{+} \quad *$$

NADPH is supplied by the pentose phosphate pathway at a rate determined by the rate-limiting enzyme, glucose-6-phosphate dehydrogenase (GPDH) (Chaudiere & Ferrari-Iliou, 1999) in the following reactions:

Glucose-6-phosphate dehydrogenase

$$\text{Glucose 6-phosphate} + NADP^{+} \xrightarrow[\text{(GPDH)}]{} \text{6-phosphogluconate} + NADPH + H^{+}$$

$$\text{6-phosphogluconate} + NADP^{+} \xrightarrow{\text{6-phosphogluconate dehydrogenase}} CO_2 + NADPH + H^{+} + \text{ribulose 5-phosphate}$$

NADPH is then used as a cofactor for glutathione reductase (GR) enzymes, which reduces GSSG (glutathione disulfide or oxidized glutathione) to GSH in the reaction (*) above.

2.6.1.2.1.2 GSH Metabolism and Regulation of its Synthesis

Reduced glutathione is the principal lenticular antioxidant of the lens and it is synthesized and regenerated in the lens cortex (Sweeney & Truscott, 1998). In mammals, the GSH metabolism is mediated by the so-called γ-glutamyl cycle (Meister & Anderson, 1983) that includes two ATP-dependent GSH synthesis steps, catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (GS), and a specific GSH degradation pathway, which allows the reutilization of the amino acids for further GSH resynthesis.

Because GSH is considered to be the main storage compound of reduced sulphur (S), GSH degradation is an important process to make S available for an organism in the form of Cys (Lieberman *et al.*, 1996). For its breakdown, gamma-glutamyl transpeptidase (γ-GT) is a membrane-bound enzyme associated with lens epithelium and responsible for catalysing the first step of glutathione (GSH) degradation in the γ-glutamyl cycle in mammals (Storozhenko *et al.*, 2002). This enzyme catalyses the transfer of the γ-glutamyl moiety of GSH to an amino acid to yield a γ-glutamyl amino acid and cysteinylglycine. The γ-glutamyl amino acids are converted to free amino acids and 5-oxoproline by the enzyme γ-glutamylcyclotransferase (lactamase) while cysteinylglycine is split by a peptidase into free glycine and cysteine. The generation of free glutamic acid from oxoproline by the enzyme 5-oxoprolinase thus provides the necessary free amino acids for the resynthesis of GSH (Reddy & Giblin, 1984). The Italian-American Cataract Study Group (Anonymous, 1991) reported that increased serum levels of gamma-glutamyl transpeptidase was a risk factor for mixed cataracts (OR = 1.22).

The avascular lens depends entirely on the surrounding intraocular fluids for the supply of various substances for synthetic reactions. The lens epithelium is a single layer of cells interposed between the aqueous humour and the anterior portion of the lens. It is also the site of much of the aerobic metabolism of the tissue. Although many substances are transported into the lens by the epithelium, there is little GSH in the aqueous humour

(Reddy, 1979). Synthesis is therefore responsible for its abundance in the lens. The synthesis of glutathione requires the presence of two enzymes and the amino acids glycine, cysteine, and glutamate, with cysteine being the rate-limiting substrate. The tripeptide GSH is formed by the consecutive actions of γ-GCS (step 1) and GS (step 2) (Rahman & MacNee, 1999):

$$\text{Glutamic acid} + \text{cysteine} + \text{ATP} \xrightarrow[\text{synthetase}]{\Gamma\text{-Glutamylcysteine}} \gamma\text{-glutamylcysteine} + \text{ADP} + P_i \qquad (1)$$

$$\Gamma\text{-Glutamylcysteine} + \text{glycine} + \text{ATP} \xrightarrow[\text{synthease}]{\text{Glutathione}} \gamma\text{-glutamylcysteinylglycine (GSH)} + \text{ADP} + P_i \qquad (2)$$

In general, the rate-limiting enzyme in *de novo* GSH synthesis is γ-GCS (Hayes & McLellan, 1999). Slaughter *et al.* (2003) confirmed that there was a correlation between GSH levels and GCS activity and Rahman & MacNee (1999) found that the reaction, catalysed by γ-GCS, is feedback-inhibited by GSH.

The mammalian γ-GCS holoenzyme is a heterodimer consisting of a 73 kDa catalytic heavy subunit (γ-GCS-HS) and 30 kDa regulatory light subunit (γ-GCS-LS) (Griffith, 1999). The regulatory properties of γ-GCS-LS are thought to be mediated by a disulfide bridge between the subunits that would allow conformational changes in the active site depending on the oxidative state of the cell (Griffith, 1999).

The reason why GSH/GSSG is very important is because the antioxidant enzymatic and non-enzymatic systems are highly diversified but mostly based on the GSH/GSSG couple. If oxidative stress alters GSH/GSSG ratio, the shift in the GSH/GSSG redox buffer influences a variety of cellular signalling processes. For example, Rahman (2000) proposed that the differences in cellular glutathione levels (or the thiol-disulfide balance) under the influence of oxidative stress might play a direct role in the activation of redox-sensitive transcription factors such as nuclear factor-*kappa*B (NF-κB) and activator protein-1 (AP-1) (Arrigo, 1999) which regulate the protective antioxidant genes in epithelial cells. Since the promoter regions of the human γ-GCS subunits bind AP-1, NF-κB and are regulated by oxidants, γ-GCS heavy subunit transcription in cells subsequently increases following the activation of NF-κB and AP-1, through which glutathione synthesis is thereby regulated by γ-GCS.

2.6.1.2.1.3 Interspecies Variation of GSH Expression and its Enzymes

Expression of the lens antioxidant system varied considerably between species. Slaughter *et al.* (2003) compared and contrasted a broad spectrum of components of the lens

antioxidant system in dogs, rats, marmosets, and rabbits. They reported that marmoset lens contained the highest level of GSH and its respective biosynthetic and recycling enzymes, GCS and GR. Rat and rabbit have intermediate levels of GSH and GCS. The level of GR activity was particularly pronounced in marmoset, which was 33-, 16.1- and 11.3-fold that in dog, rabbit, and rat respectively. However, activities of the NADPH-generating enzyme GPDH was relatively low in marmoset. In contrast, dog lens had the lowest levels of GSH, GCS, GR (1/2, 1/2 and 1/33 that in marmoset) but highest levels of GPDH (the order for GPDH activity was dog>rat>marmoset>rabbit, decreasing by approximately half with each species). Consequently, the GPDH:GR ratio, which indicates NADPH turnover with respect to glutathione reduction, was comparatively high in dog lens and 193-fold greater in marmoset, which had the lowest ratio.

We should be aware of metabolic differences because of the diverse range of species used in ocular investigations. Such considerable interspecies variation in the constitutive expression of lens antioxidant system probably represents a consequence of adaptation to evolutionary pressures, such as the degree of UV exposure dictated by diurnal activity (Reiss *et al.*, 1986). This should be considered when deciding on an appropriate animal species for ocular research, especially when extrapolating outcomes to man. Although marmoset has the highest level of GSH, its nocturnal nature makes it probably not the most appropriate alternative when extrapolating similar GSH level as well as its change regulated by cell redox-state to humans. Rathbun *et al*. (1986) found that glutathione reductase activity was lowest in the dog, the rabbit, and all species of cats. The higher Old World monkeys and hominoids, including the human, exhibited enzyme activities many times greater than any other species except the woodchuck. Since glutathione reductase is the rate-controlling enzyme of the glutathione redox cycle, they concluded that the most suitable model for the human lenticular glutathione redox cycle would be a higher primate.

2.6.1.2.1.4 Functional Role of Glutathione in Lens Cataract Formation

A common feature of most types of cataract is a dramatic decrease in the content of GSH (Reddy & Giblin, 1984). The concentration of this tripeptide also decreases with age in both animal and human lenses (Harding, 1970). Therefore, there has been considerable speculation that GSH may play an important role in maintaining lens transparency. Firstly, GSH might maintain protein thiol (SH) groups in the reduced state and thus prevent the formation of high molecular weight (HMW) protein aggregates, which are believed to be the basis for the scattering of light and lens opacification (Benedek, 1971). Another

function of GSH in the lens may be to protect membrane SH groups that are important in cation transport. It is recognized that the regulation of normal cell volume, involving the transport of electrolytes, is an important factor in the maintenance of lens transparency (Epstein & Kinoshita, 1970). A third role for GSH in the lens may be to protect against oxidative damage. GSH may act either directly as a protective agent by scavenging the damaging free radicals (Giblin (2000) reviewed an important hydroxyl radical-scavenging function for GSH in lens epithelial cells, independent of the cells' ability to detoxify H_2O_2) or by protecting other reactive thiols of crystallins, enzymes or membrane proteins (Reddy & Giblin, 1984). These three aspects of the functional role of GSH should be considered in relevant research of cataract formation.

2.6.2 Anti-cataract Effect of Antioxidants in Human Epidemiology

2.6.2.1 Definition and Types of Epidemiology

A common approach to gathering information about the putative disease-preventing role of antioxidants, and one that had provided much of the evidence for the importance of vitamin E, is epidemiology. Halliwell & Gutteridge (1999) defined "epidemiology" as "the science that deals with the distribution of, and the determinants of, health and illness in populations".

Epidemiological studies may be essentially descriptive (e.g. relating the incidence of a disease to a particular diet) or experimental (e.g. taking a dietary supplement and examining what happens) (Halliwell & Gutteridge, 1999).

The former type measures disease patterns and dietary habits in groups or populations, and correlates the data, searching for association. For instance, Jacques *et al*. (1988) carried out a study to explore relationships between nutrition and senile cataract in 112 subjects aged 40-70 y. Results suggest that risk of cortical cataract was reduced for subjects with the highest plasma level of vitamin D and total carotenoids and that people with cataract may have lower levels of vitamin C and higher levels of vitamin B-6 and selenium. Based on the Jacques *et al*. (1988) finding that cataract patients tended to have lower serum levels of vitamins C, E, or carotenoids than did control subjects, Robertson *et al*. (1989) compared the self-reported consumption of supplementary vitamins by 175 cataract patients with that of 175 individually matched, cataract-free subjects and revealed that the latter group used significantly more supplementary vitamins C and E.

Experimental epidermiology tests directly the reality and importance of the correlations observed and will be detailed below.

2.6.2.2 Experimental Epidemiology of Antioxidant Diet in Human

2.6.2.2.1 Recurring Theme that Antioxidants Reduce Cataract Risk

The putative importance of oxidative stress in the pathogenesis of cataract and in aging in general has focused attention upon antioxidant nutrients because the compromised function of the lens and retina with aging was exacerbated by depleted primary antioxidant reserves, antioxidant enzyme capabilities, and diminished secondary defences such as proteases (Taylor *et al.*, 1995). Some low-molecular mass antioxidants come from the diet, especially ascorbic acid and α-tocopherol (vitamin E). An intimate relationship between nutrition and antioxidant defence has been verified in several ophthalmic epidemiological studies linking high levels of dietary antioxidants with slower progression of cataracts in humans.

Within the past 19 years, eleven papers have reported the results of cross-sectional or retrospective epidemiological studies designed to explore associations between dietary patterns and the incidence of cataract. Nutrient status was evaluated using dietary intake and/or plasma concentrations, and ophthalmological examination was used to detect cataract. Moderate to strong inverse associations were recorded for riboflavin, carotene, and vitamin E and C in some, but not all, of these studies.

Bhat (1987) carried out a comparative study on the biochemical assessment of B vitamin (including thiamine, riboflavin, and pyridoxine) status in cataract patients taking normal subjects without cataract, as controls. Results of this study showed that the riboflavin status was poor in cataract patients while the status of thiamine and pyridoxine appeared to be better than those without cataract. The results suggested an association between human cataract and nutritional deficiency of riboflavin. With regard to vitamin A and vitamin E, it was found that their concentrations in plasma from cataract patients were within normal range (Bhat, 1987).

Leske *et al*. (1991) carried out the Lens Opacities Case-Control Study to evaluate risk factors for age-related nuclear, cortical, posterior subcapsular, and mixed cataracts. The 1380 participants were ophthalmology outpatients, aged 40 to 79 years. They found that dietary intake of riboflavin, vitamins C, E, and carotene, which have antioxidant potential, was protective for cortical, nuclear, and mixed cataract; intake of niacin, thiamine, and iron

also decreased risk. Subsequently, Leske *et al*. (1997) carried out a study to evaluate the association of age-related lens opacities with the use of nutritional supplements (primarily cod liver oil and multivitamins) in 4314 black participants (41-84 years of age) enrolled in the Barbados Eye Study. The findings suggest that regular users of nutritional supplements have a one-fourth lower risk of lens changes and particularly, of cortical opacities; a result seen at ages under 70 years (but not at older ages).

Hankinson *et al*. (1992) examined the association between incidence of cataract extraction and intakes of vitamins C and E, riboflavin, and carotene. The study group was a subset drawn from the Nurse's Health Study, begun in 1976, and included 50,828 American women who were between the ages of 45 and 67 years at the onset of the study in 1980, with eight years follow-up and set in 11 states of the United States. The results indicated dietary carotenoids, a high antioxidant score and long-term vitamin C supplementation were associated with a decreased risk of cataracts requiring removal. Subsequently, researchers from Tufts University studied more than 475 women ages 53 to 73 from the Nurses' Health Study and concluded that nutrient antioxidants played a role in the prevention of age-related nuclear lens opacities (Jacques *et al.*, 1997).

Seddon *et al*. (1994) carried out a population study on 17,744 participants in the Physicians' Health Study. It was a randomized trial of aspirin therapy and β-carotene among US male physicians 40 to 84 years of age in 1982 who did not report cataract at baseline and provided complete information about vitamin supplementation. After 5-year follow-up, it was found that men who took multivitamin supplements tended to experience a decreased risk of cataract.

The Beaver Dam eye research project, which involved adults from 43-84 years of age, also found that the intake of lutein and zeaxanthin, the only carotenoids that are present in the lens (Yeum *et al.*, 1995), reduced the incidence of cataracts (Lyle *et al.*, 1999). Later on, Marse-Perlman *et al*. (2000) found lower risk for cataract among users of vitamin supplements in the Beaver Dam Eye Study cohort and stronger associations with long-term use.

Nadalin *et al*. (1999) examined the association between prior supplementation of vitamin E and early cataract changes in volunteers enrolled in the cross-sectional Vitamin E and Cataract Prevention Study (VECAT) clinical trial. They found that prior vitamin E supplementation may protect VECAT participants from developing at least early cortical

cataracts. However, no apparent protective role in terms of nuclear opacities and nuclear colour was found regardless of the level, regularity or duration of intake.

The cross-sectional Blue Mountains Eye Study found that long-term use of multivitamins, B group (including thiamine, riboflavin & niacin) and vitamin A supplements was associated with reduced prevalence of either nuclear or cortical cataract. A strong protective influence on cortical cataract, from use of folate or vitamin B12 supplements, was a new finding (Kuzniarz *et al.*, 2001).

The Roche European American Cataract Trial (REACT), a three-year trial with 297 adults from the US and England who were outpatients from ophthalmology clinics and had been already diagnosed with early age-related cataract, has shown that a supplement containing β-carotene and vitamins E and C slowed the progression of cataracts. In this randomized, double-masked, placebo-controlled trial, 81 people who consumed a mixture of antioxidant nutrients (containing 18mg/day of β-carotene, 750mg/day of vitamin C and 600 IU of vitamin E) for 3 years had lower rates of progression of lens opacities than the 77 people who did not take these supplements (Chylack *et al.*, 2002).

2.6.2.2.2 No Observed Benefit for High-dose Antioxidant to Reduce Cataract Risk

However, not all trials have shown positive effects. In the Alpha-Tocopherol Beta-Carotene (ATBC) Trial in 28,934 male Finnish smokers, supplementation with α-tocopherol and/or β-carotene or placebo did not lower rates of cataract extraction over 5 to 8 years of follow up (Teikari *et al.*, 1998), nor did it lower prevalence of lens opacities observed in a random sub-sample of participants (Teikari *et al.*, 1997). Cataract extraction was not lowered in U.S. physicians taking β-carotene for 12 years (Christen *et al.*, 2003). A 5-year clinical trial in Linxian, China, in which malnutrition is common, assessed the influence of vitamin supplementation on risk of cataract. Two trials [multivitamin/mineral supplement or matching placebo in trial 1; factorial design to test the effect of four different vitamin/mineral combinations in trial 2 (retinol/zinc, riboflavin/niacin, ascorbic acid/molybdenum, and selenium/α-tocopherol/β-carotene)] were carried out with a duration of 5 to 6 years, followed by end-of –trial eye examinations. In the first trial, it was found that multivitamins were associated with lower prevalence of nuclear cataract. However, in the second trial, no supplement combinations of antioxidants, neither vitamin C with molybdenum nor vitamin E with β-carotene (15 mg) and selenium (50 mg) were associated with lower prevalence of cataracts of any type (Sperduto *et al.*, 1993). However,

a significant decrease in nuclear cataract and a small increase in posterior sub-capsular cataract were associated with riboflavin/niacin supplementation (Bunce, 1994a).

Furthermore, McNeil *et al.* (2004) found that in a randomized controlled trial vitamin E given for 4 years at a dose of 500 IU daily did not reduce the incidence of or progression of nuclear, cortical, or posterior subcapsular cataracts. These findings do not support the use of vitamin E to prevent the development or to slow the progression of age-related cataracts.

Mares (2004) concluded that the evidence from clinical trials alone suggested that supplementation with high dose antioxidants containing β-carotene, vitamin E and/or vitamin C for 3 to 12 years did not influence the rate of cataract development. Although these clinical trials of antioxidants generally have not supported the benefits of a few select antioxidants given in high doses, collectively they have provided consistent evidence that β-carotene is not likely to protect against cataract (Sperduto *et al.*, 1993; Teikari *et al.*, 1997; Teikari *et al.*, 1998; Christen *et al.*, 2003). Mares did not think of these results as surprising because β-carotene, the major carotenoid in human serum and other tissues, is not found in human lenses (Yeum *et al.*, 1995).

Mares (2004) also noted observational studies indicating lower rates of some types of cataract or cataract extraction among people who use multivitamin supplements, and that the protection against cataract was strongest among people who used multivitamin supplements for more than 10 years (Hankinson *et al.*, 1992; Seddon *et al.*, 1994; Jacques *et al.*, 1997; Mares-Perlman *et al.*, 2000; Kuzniarz *et al.*, 2001). Based on the finding of lower occurrence of cataract among people who used multivitamins but no further reduction among people who took higher dose of antioxidants, Mares hypothesised that once adequate antioxidant blood levels were attained, there might have been diminished benefit to higher doses. In another word, there was no dose-dependent relationship between nutrient intake and cataracts.

Moreover, Mares (2004) pointed out that high-doses of antioxidants may pose health risks. For example, high-dose antioxidants given to people who were also taking high doses of niacin and statin drugs caused an unexpected reduction in high-density lipoprotein cholesterol (the protective increase in HDL2 with simvastatin plus niacin was attenuated by concurrent therapy with antioxidants) and an increase in the risk of heart attacks and strokes (Brown *et al.*, 2001). Similarly, vitamin E supplementation (50 mg/day of all rac-α-tocopherol acetate) increased risk for hemorrhagic stroke by 50% in the ATBC study (Anonymous, 1994). High-dose β-carotene (20 mg or greater) increased the risk of lung

cancer (Anonymous, 1994). Supplementation with high-dose β-carotene decreases absorption of lutein and zeaxanthin (Micozzi *et al.*, 1992), the only carotenoids that are present in the lens (Yeum *et al.*, 1995), and which may protect the lens against cataract development (Mares-Perlman *et al.*, 2002).

Therefore, a large body of current data does not suggest health benefits of high-dose antioxidants, particularly β-carotene. On that basis, Mares (2004) contended that there is little evidence that high-dose antioxidants provide benefit beyond that which may be provided by multivitamins or nutrient-rich diets. Consistently, Taylor *et al*. (1995) also contended that optimizing nutrition, including diets rich in fruit and vegetables, [for example, lutein increases in the broccoli diet (Micozzi *et al.*, 1992)] may provide the least costly and most practicable means to delay cataract.

2.6.3 Anti-cataract Effect of Antioxidants in Laboratory Animal Model

A number of *in vivo* animal models have been treated with antioxidants to observe the effect of antioxidants. For example, Ross *et al*. (1982) reported the protective *in vivo* effects of vitamin E on cataractogenesis in diabetic rats. Devamanoharan *et al*. (1991) reported that vitamin C had a significant preventive *in vivo* effect on rat cataracts induced by selenite. This finding confirmed their view that ascorbate functioned as an anticataractogenic substance.

A lens organ culture model system can also be used to screen potential anti-cataract agents. Opacification of lenses maintained in culture is induced by specific insults such as H_2O_2 or the cataractogenic sugar xylose. Potential anti-oxidant agents are then added to the culture medium and their ability to inhibit opacification and certain biochemical changes associated with the opacification are assessed.

Cui & Lou (1993) showed the rat lens clarity and biochemical changes partially recovered if the oxidant was removed within 24 hr, indicating a potential therapeutic role for antioxidants. Furthermore, several reports have confirmed that application of antioxidants slows cataract progression in laboratory animal model. For example, Zigler *et al.* (2003) published that Tempol-H, the hydroxylamine of the nitroxide antioxidant Tempol, significantly inhibited opacification of rat lenses in an H_2O_2-induced cataract system as well as opacification of rhesus monkey lenses induced by xylose. Therefore, the antioxidant activity of Tempol-H makes it an attractive candidate as a therapeutic agent for

the prevention of age-related cataract. Also, Shang *et al.* (2003) showed that Vitamin C and Vitamin E compensate for GSH depletion to protect against H_2O_2 –induced epithelial cell death of rabbit lens, which supports the preventive or therapeutic use of vitamin C and E to combat age- and pathology-associated decline in GSH. In addition, Sanderson *et al.* (1999) confirmed that low micromolar concentrations of the naturally-occurring flavonoid, quercetin, inhibit cataract formation in a rat lens organ cultured model exposed to the endogenous oxidant hydrogen peroxide. Quercetin was active both when incubated in culture medium together with hydrogen peroxide, and was also active when the lenses were pre-treated with quercetin prior to oxidative insult. Based on the laboratory experiment, they proposed a mechanism where quercetin protected the lens from calcium and sodium influx, which are early events leading to lens opacity.

Chapter 3
Effectiveness of Quercetin & Resveratrol in Preventing H_2O_2-induced Opacity in Cultured Sheep Lenses

3.1 Introduction

The purpose of this research is to use the organ cultured lenses from sheep as a model system to examine the role of oxidants in cataract formation. To achieve this, two potential antioxidants were tested with Lens Organ Culture with Hydrogen Peroxide (LOCH), aiming to clarify whether H_2O_2 is relevant to sheep cataract and assess antioxidants' ability to inhibit opacification. Furthermore, calpain was assayed in the presence of the antioxidants to assess whether the antioxidants also act as calpain inhibitors *in vitro*.

Laboratory studies have established the underlying premise that a lens organ culture model system can be used to screen potential anti-cataract agents (Zigler *et al.*, 1985; Zigler *et al.*, 1989; Kamiya & Zigler, 1996). Opacity can be induced in cultured lenses by various chemical or environmental perturbations, and then prevention or inhibition of opacification can be observed after addition of appropriate agents (Zigler *et al.*, 2003).

Cultured sheep lenses were exposed to H_2O_2 to induce opacification in this research. This model was referred to as the LOCH. Because H_2O_2, at concentrations found in pathological cataractogenesis, can cause lens opacification in organ culture (Giblin *et al.*, 1987; Lou *et al.*, 1990) and produces a pattern of oxidation similar to that found in human cataract, it was concluded that H_2O_2 is the major oxidant involved in cataract formation in man. This model of oxidative cataract has been well characterized by Spector (1995). Exposure of lens epithelial cells, as well as whole lenses, to H_2O_2 is known to cause protein oxidation and aggregation (Smith *et al.*, 1997), lipid oxidation (Ahuja *et al.*, 1999), decreased ion transport (Giblin *et al.*, 1987), ubiquitin-mediated intracellular proteolysis (Shang *et al.*, 1997), and apoptotic cell death in whole lenses (Li *et al.*, 1994), each of which is thought to contribute to cataract formation (Taylor, 1999).

Most of the previous studies of hydrogen peroxide induced oxidation have used rodent lens, e.g. (Lou *et al.*, 1990; Sanderson *et al.*, 1999; Cornish *et al.*, 2002). However, the

sheep lens is a more appropriate model of the humans lens than rodent lenses. Therefore, these experiments aimed to clarify whether H_2O_2 was relevant to sheep cataract.

The two antioxidants chosen in the current work were flavonoids: quercetin and resveratrol. Flavonoids are polyphenolic antioxidants naturally present in vegetables such as onions; fruits such as apples; and beverages such as tea and wine. Robertson *et al*. (1991) reported that a decreased risk of cataract was associated with a daily tea consumption of greater than 500 mL. Tea is a major source of quercetin (Figure 3.1), and flavonoid intake was found to be highly correlated with consumption of tea (Hertog *et al.*, 1993). Therefore, quercetin in regularly consumed foods may reduce the risk of cataract. Sanderson *et al*. (1999) reported that low micro-molar concentrations of quercetin inhibited cataractogenesis in a rat lens organ cultured model exposed to endogenous hydrogen peroxide. They found that quercetin, was active both when incubated in culture medium together with hydrogen peroxide, and when the lenses were pre-treated with quercetin prior to oxidative insult. The present experiment tested this phenolic antioxidant in the sheep LOCH model.

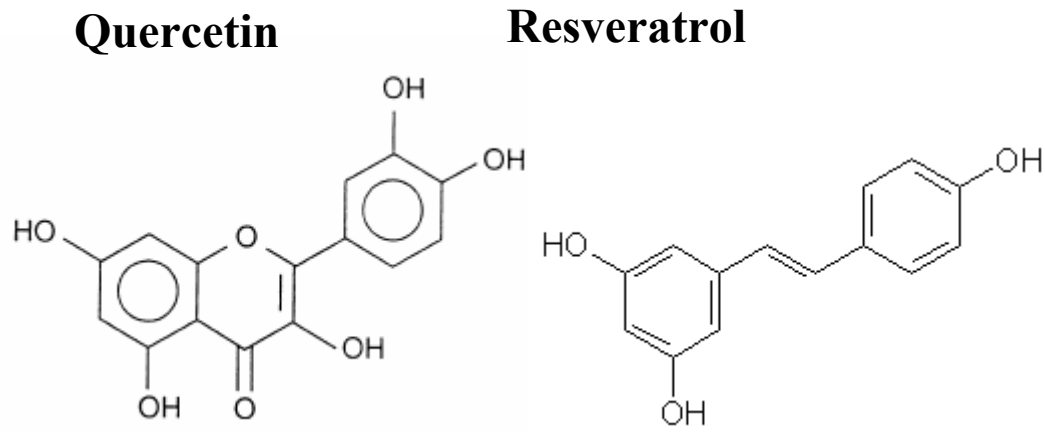


Figure 3.1 Structures of Quercetin & Resveratrol.

The other novel antioxidant used —resveratrol (3, 5, 4'-trihydroxystilbene) (Figure 3.1), exists in cis and trans configurations in a narrow range of spermatophytes of which vines, peanuts and pines are prime representatives. It is classified as a phytoalexin anti-fungicide conferring disease resistance in the plant kingdom (Soleas *et al.*, 1997). *In vitro* and animal experiments have shown that it possesses many biological attributes that favour protection against atherosclerosis, including antioxidant activity, modulation of hepatic

apolipoprotein and lipid synthesis, inhibition of platelet aggregation as well as the production of pro-atherogenic eicosanoids by human platelets and neutrophils (Soleas *et al.*, 1997). Red wine represents its main source in the human diet (Soleas *et al.*, 1997), and it is a major constituent of the polyphenol fraction to which the health benefits of red wine consumption have been attributed. In addition to its cardioprotective and cancer chemopreventive activities (Jang *et al.*, 1997; Hsieh *et al.*, 1999), Savaskan *et al*. (2003) reported that resveratrol may be neuroprotective through maintaining cell viability and exerting an anti-oxidative action by enhancing the intracellular free-radical scavenger glutathione. Therefore, resveratrol, the naturally grape-derived polyphenol was examined as a possible candidate to slow or prevent cataract formation in sheep LOCH assay.

In the current experiment, ovine lens organ culture was set up and opacity induced in cultured ovine lens by endogenous H_2O_2. After ovine lenses were pre-treated with candidate antioxidants, the ability of antioxidants to inhibit the opacification was assessed and compared with lenses exposed to H_2O_2 alone.

The aim of this research was not only to assess the effect of antioxidants on lens transparency but also to clarify mechanisms of protection. Sanderson *et al*. (1999) proposed a mechanism where quercetin protected the lens from calcium and sodium influx, which are early events leading to lens opacity. Although quercetin and resveratrol are antioxidants, they may also be involved in other reactions, for example, such as protease inhibition. As discussed earlier in the "protease hypothesis" for cataractogenesis (Chapter 1, *p* 4), calpain may be important in the formation of cataract. Therefore, the antioxidants were assayed with BODIPY casein to assess whether were able to inhibit calpain *in vitro*. This substrate, a fluorescent-derivative of casein, is highly quenched. If a small quantity of protease is introduced, the casein is cleaved, and fluorescence increases. The change in fluorescence value is a measurement of proteolytic activity. Relative calpain activity is therefore measured as the change in fluorescence caused by calpain.

3.2 Materials and Methods

3.2.1 Lens Culture

3.2.1.1 Preparation of Culture Medium

Eagle's Minimal Essential Medium (EMEM) (pH 7.4) is a bright-light red solution (due to the presence of the Phenol Red·Na pH indicator as a component), prepared from Minimum essential medium powder (purchased from Sigma-Aldrich, product number 034K8308)

with gentamycin (0.02 mg/mL), Amphotericin B (2.5 μg/mL) and 26 mM $NaHCO_3$ in dH_2O.

Artificial aqueous humour (AAH) is a colourless solution that was adapted from the formulation described by David *et al*. (1984): NaCl 130 mM; KCl 5 mM; $CaCl_2$ 1 mM; $MgCl_2$ 0.5 mM; D-glucose 2.5 mM; $NaHCO_3$ 5.0 mM; HEPES 20.0 mM; pH 7.3.

All the culture media mix (including EMEM and AAH) were sterilised immediately using 0.2 μM pore size filters (VacuCap® 60 Filter Unit: 0.2 μM pore size Super® Membrane (hydrophilic polyethersulfone)) into autoclaved bottle.

3.2.1.2 Preparation of Lens

Sixteen pairs of lamb (9-12 months old) whole-eye globes were removed immediately after slaughter, and the lenses were dissected from the globes of the animal within 2 h of death using a posterior approach. After dissection, the lenses were transferred to the EMEM culture medium. The entire lens was submerged with its anterior epithelium upward in the medium. To ensure that freshly dissected lenses do not grow mould and fungus during a culture experiment, the lenses were pre-incubated in the above EMEM containing the antibiotic/antimycotic solution -- APS [Amphotericin (0.25 μg/mL) + Penicillin (100 unit/mL) + Streptomycin (100 μg/mL)] in the Petri dish for half an hour in the laminar flow hood. Then the APS pre-incubated lenses were transferred into cell culture dishes and each lens was incubated at 37 °C in 5% CO_2 with EMEM 10 mL (pH 7.4) for an overnight settling period. Following the overnight incubation, lenses were inspected for damage and 24 undamaged lenses (no opacities or fungal growth) were selected for the experiment.

3.2.1.3 Antioxidants Tested in LOCH Model

Twenty four lenses were divided into four groups according to different treatment: (1) Control -- 6 lenses were pre-incubated with EMEM (10 mL/lens) only for 24 h; (2) H_2O_2 -- 6 lenses were pre-incubated with EMEM (10 mL/lens) only for 24 h too, but would be treated with H_2O_2 later; (3) Q -- 6 lenses were pre-incubated in EMEM (10 mL/lens) containing 30 μM quercetin for 24 h prior to H_2O_2 treatment; and (4) R-- 6 lenses were pre-incubated in EMEM (10 mL/lens) containing 30 μM resveratrol for 24 h prior to H_2O_2 treatment.

Quercetin and resveratrol were prepared as 30 mM stock solutions dissolved in 1 mL dimethyl sulfoxide [DMSO, $(CH_3)_2SO$]. Then 10 μL of the appropriate stock was added to 10 mL EMEM for each lens. Therefore, the final concentration for quercetin (or

resveratrol) was 30 µM, and the final concentration of DMSO in the quercetin or resveratrol solution was 10 µL/10 mL = 0.1% v/v.

It is possible that residual quercetin or resveratrol on the lens surface would react with H_2O_2 in the culture medium, and so prevent radicals reaching the lens. To prevent this, lenses were pre-incubated with quercetin or resveratrol (30 µM) in EMEM in the incubator at 37 °C, 5% CO_2 for 24 h, as outlined in Table 3-1. Then lenses were transferred to artificial aqueous humour (AAH) for approximately 1 h for washing prior to exposure to 1 mM H_2O_2 in AAH for 6 h to remove quercetin or resveratrol from lens surface. Control lenses remained in AAH for the duration of the experiment. The lenses in culture were photographed at 0, 2, 4 and 6 h with a Sony Cybershot DSC-F505V digital still camera.

Table 3-1 Experimental scheme for ovine lens organ culture with hydrogen peroxide (LOCH) experiment. Each group has 6 lenses.

Treatment group	**Pre-incubation → (24 h)**	**Inducing oxidative stress (6 h)**
1. Control (**C**)	EMEM	AAH
2. H_2O_2 (**H**)		AAH + 1 mM H_2O_2
3. Quercetin + H_2O_2 (**Q**)	EMEM + 30µM quercetin	
4. Resveratrol + H_2O_2 (**R**)	EMEM + 30µM resveratrol	

3.2.2 Visual Monitoring of Culturing Lenses

Lenses were photographed using digital camera (Sony Cybershot DSC-F505V) fitted to a stand (Haiser R 3XA). A transparent flat bottom culture dish with a lens and medium was placed on black grid lines (1x1 mm) with white background light (Radiographic Supplies, Christchurch, New Zealand). Images of the anterior epithelium of the lens were taken with an image resolution of 1856 x 1392 pixels as a RGB true colour JPEG image.

3.2.3 Measure of Opacity

The software used to grade the opacification captured was Image Pro-Plus v4.1. A macro script was created to automatically analyse the digital images of lenses (pixel size 1856 x 1392) placed on a 1mm x 1mm black grid. The grading system was developed on the basis of selecting predefined pixel RGB values, after area defining and sharpening of the image. 1. The programmed system predicted total blobs (which were clear squares formed by the 1mm x1mm black grid) from the total lens area. Due to the consistent measurements of the

camera position and settings, the measurement of pixel/cm was a constant. This allowed the macro to analyse multiple user specified images automatically.

2. The system sharpened the image using a sharpening filter built into the software. The macro identified the actual blobs visible through the opaque parts of the lens by selecting predefined pixel RGB values.

3. From the constant pixel/cm ratio, the macro was able to compare the calculated actual blobs against the total predicted blobs calculated from the lenses size. This value was shown as a percentage and used as the score of the lens. The macro value scaled from 1 to 10. Fully opaque lenses were graded 10, while clear/transparent lenses were graded 1.

3.2.4 BODIPY Assay (Inhibitory Effect of Antioxidants on m-Calpains)

Calpain *II* (m-calpain) was assayed using BODIPY-FL casein as the substrate. The assay was run in a 96-well black microtiter plate (to minimise background fluorescence) and the total volume for each well was 200 µL. BODIPY-FL (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid) was purchased from Molecular Probes (Eugene, OR), and the BODIPY-FL casein was prepared as described by Thompson *et al*. (2000). The information on how to purify the specific m-calpain was detailed in Appendix H.

As shown in Table 3-2, 10 µL pooled sample (quercetin & resveratrol) made up at a series of concentrations in triplicate were diluted to 100 µL with dH_2O (40 µL) and m-calpain (50 µL). Assays of EDTA, calcium, DMSO blanks and m-calpain controls were also carried out respectively at a volume of 100 µL in triplicate, with the corresponding contents detailed in Table 3-2. All the equal amount (100 µL) of substrate solution (5 µg BODIPY-casein/mL, 10 mM $CaCl_2$, 0.1 mM NaN_3, 0.1% mercaptoethanol, 10 mM Tris-HCl, pH 7.5) was automatically injected to each well in the assay at last by the Fluostar machine before starting to measure the fluorescence reading. Sample fluorescence was read immediately every min for 10 min in a BMG ELISA plate reader (Fluostar, BMG Labtechnologies GmbH. Offenburg, Germany) with an excitation wavelength of 485 nm, emission 538 nm and a gain of 60. The software used was version 3.02-0.

Thus, calcium-independent changes in fluorescence were determined by assaying the sample in the presence of 12.5 mM EDTA. Relative calpain activity was measured as the change in fluorescence caused by calpain per minute and was recorded individually for

calpain control, DMSO blank and antioxidants respectively. The calpain inhibitory effect for samples containing antioxidants was calculated by the following equations:

Equation 1: The fluorescence change caused by sample activity = [(fluorescence of the sample at 10 min) - (fluorescence of the sample at zero min)] - [average (calcium + EDTA blanks)]

Equation 2: The fluorescence change caused by calpain activity = [(fluorescence of m-calpain control at 10 min) - (fluorescence of m-calpain control at zero min)] - [average (calcium + EDTA blanks)]

Equation 3: Inhibitory Rate = {1 – [Fluorescence (eqn 1) / Fluorescence (eqn 2)]} x 100%

The real inhibitory effects for varying concentrations of antioxidants were calculated by dividing them by the calpain control and then removing the DMSO inhibitory effect on calpain to get the final inhibitory rate for antioxidants to specific m-calpain *in vitro*.

Table 3-2 Protocol for calpain inhibition assay.

(µL)	Blanks				Assay
	EDTA	**Calcium**	**m-calpain**	**DMSO**	
100 mM EDTA	25				
dH_2O	25		50	40	40
m-calpain	50		50	50	50
Calcium chloride		100			
BODIPY substrate	100	100	100	100	100
Inhibitor					10
DMSO				10	

3.3 Results

3.3.1 Ovine Lens Culture

The diameter of lenses from a 9-12 months old sheep was between 10-13 mm (Figure 3.2), with a wet weights between 750 and 1000 mg. Black suspensory ligaments remained attached around the equatorial region of dissected lens and indicated the outer boundary of a lens fully suspended in the culture media (Figure 3.2).The grid could be seen as clear prominent black lines through a transparent lens, or as blurry lines as the lens became opaque and scattered light. 75% lenses (24 lenses out of 16 pairs) survived through the dissection and culture for the experiment.

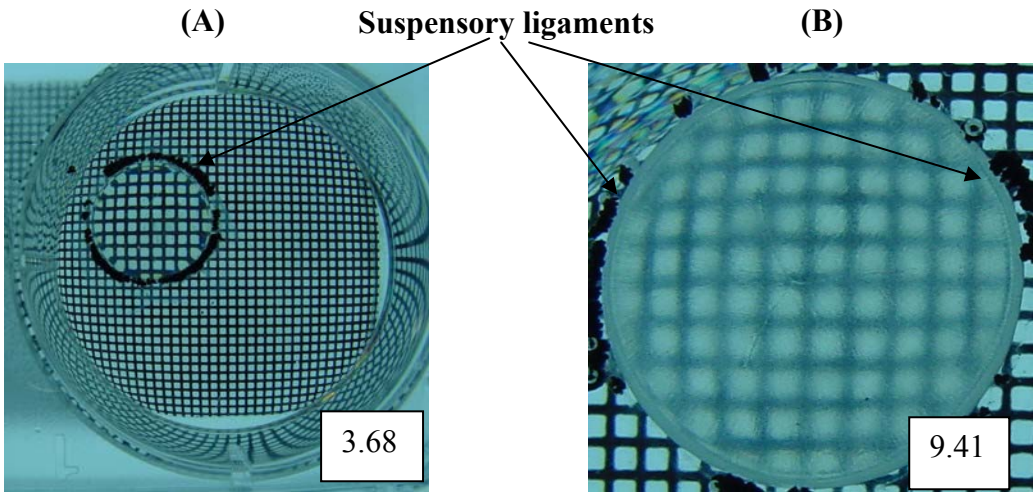


Figure 3.2 Digital images of two ovine lenses in the culture media (AAH) on a grid including their opacification scores (or relative transparency score system) based on digital visual analysis. Two lenses with two end scale of degree of opacity were compared. (A) clear/transparent control lens with the score of 3.68, while (B) fully opaque lens with the score of 9.41 induced by treatment with 1 mM hydrogen peroxide over 6 h. The scale of the black grid was 1 mm x 1 mm.

With the exposure of sheep lenses to 1 mM H_2O_2, the opacification grade increased over the time course of the experiment (Figure 3.3).

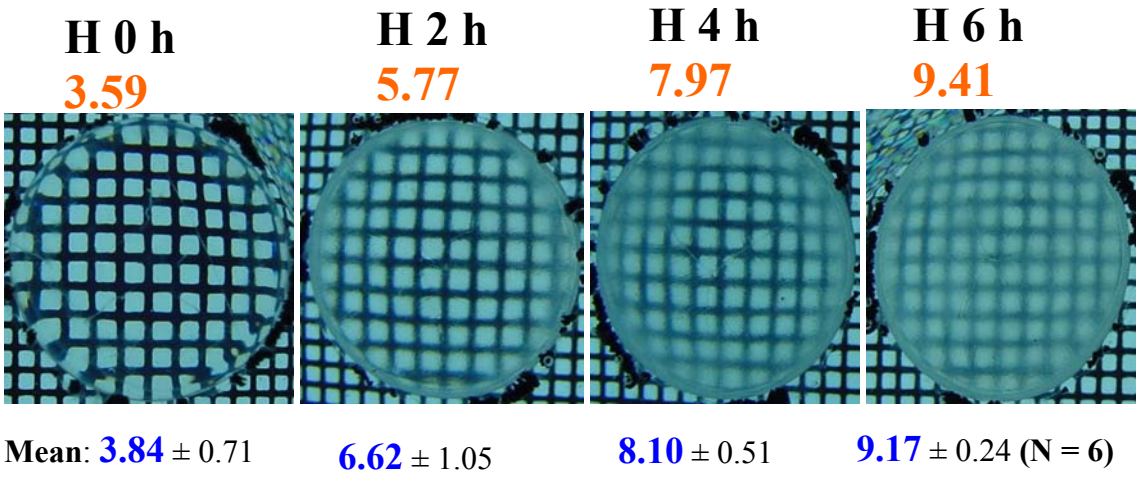


Figure 3.3 H_2O_2 –induced ovine lens opacification over the time course. Numbers at the bottom of the image represented mean opacity ± SD of 6 lenses at different time interval (0, 2, 4, and 6 h). The number on the top of the image represented the actual opacity grading score of the specific lens, whose opacity was closest to the mean value. With exposure of sheep lenses to 1 mM H_2O_2, the opacity grade increased over the time course of the experiment.

Incubation with quercetin (30 µM) significantly reduced lens opacity starting at 4 h ($p < 0.05$, n = 6; Figure 3.5). At 6 h, the opacity of lenses pre-treated with quercetin was 78% of that of H_2O_2-controlled lenses ($p < 0.05$, n = 6; Figure 3.4). Quercetin prevented the progression of H_2O_2-induced lens opacity over 6 h by 30%. However, there was no

significant difference between lenses treated with 30 µM resveratrol and those with hydrogen peroxide alone in Student's *t*-test ($p = 0.172$, n = 6).

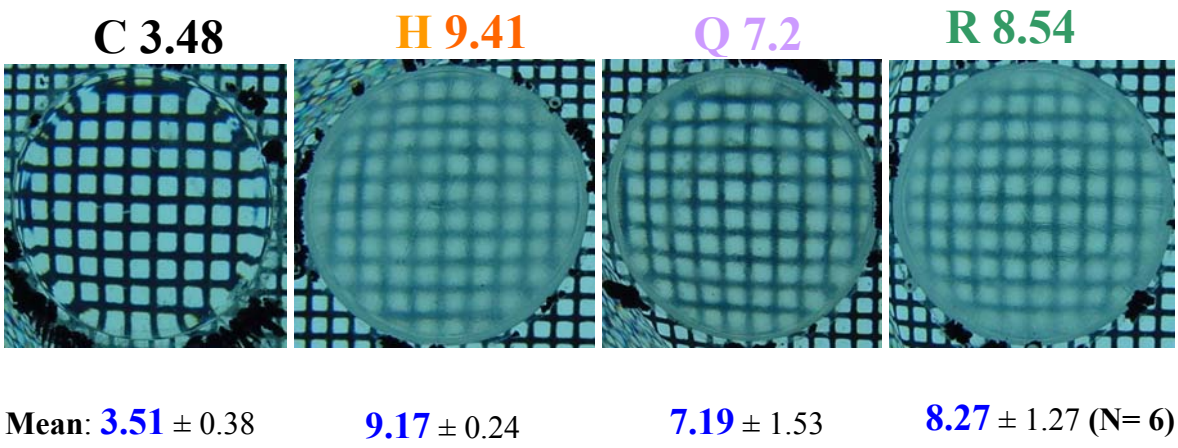


Figure 3.4 Digital image of ovine lenses with antioxidant pre-treatment in the LOCH experiment and their opacification grading scores (mean ± SD) at 6 h. Sheep lenses were cultured in control AAH (C), 1 mM H_2O_2 (H), 30 µM quercetin prior to 1mM H_2O_2 exposure (Q) and 30 µM resveratrol prior to 1mM H_2O_2 exposure (R). Photographs were taken after 6 h. Specific image of the lens (with the opacity score on the top) closest to the mean value (number at the bottom -- mean opacity ± SD) was chosen to represent the opacity after 6 h in each different treatment group. The extent of lens opacity at 6 h was significantly different between H and Q group ($P < 0.05$, n=6).

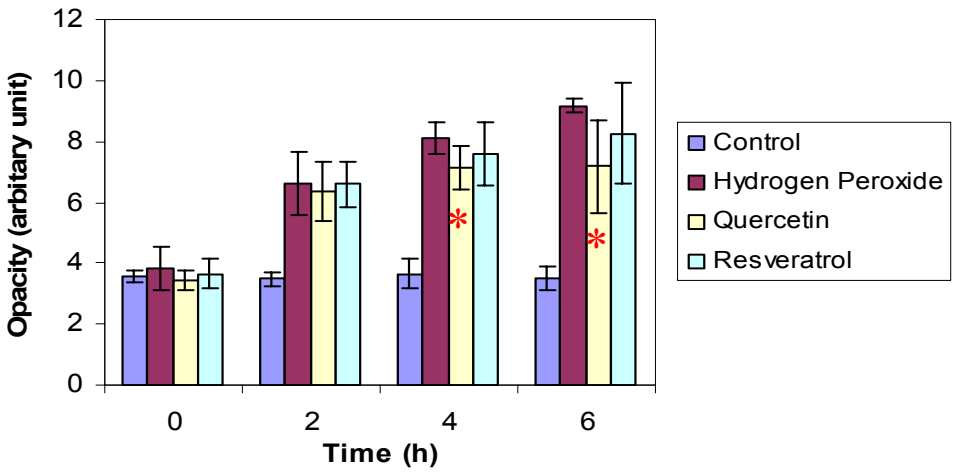


Figure 3.5 Opacity progression during 6 h incubation period with different treatments as explained in Figure 3.4. * indicates that the opacities of quercetin at 4 and 6 h significantly different from those of H_2O_2-treated lenses at the same time using the two-tailed Student's *t*-test ($P < 0.05$). The actual opacification score for H_2O_2, Quercetin and Resveratrol at 0, 2, 4, 6 h were shown in Appendix A.

3.3.2 Effect of Antioxidants on m-Calpain Activity

Quercetin and resveratrol were not soluble in water and required DMSO to dissolve. Therefore, DMSO blanks were necessary to determine the inhibitory effect of quercetin or resveratrol. Different volumes of DMSO were tested to determine the calpain-inhibitory effect. There was a linear relationship between DMSO control and calpain inhibition (Figure 3.6). For example, there was 95% inhibition when DMSO (% total volume) increased to 25% in the assay. The concentration of DMSO used in the quercetin and resveratrol assays, 5% or 10 µL in 200 µL total volume (Table 3-2), caused 16% inhibition.

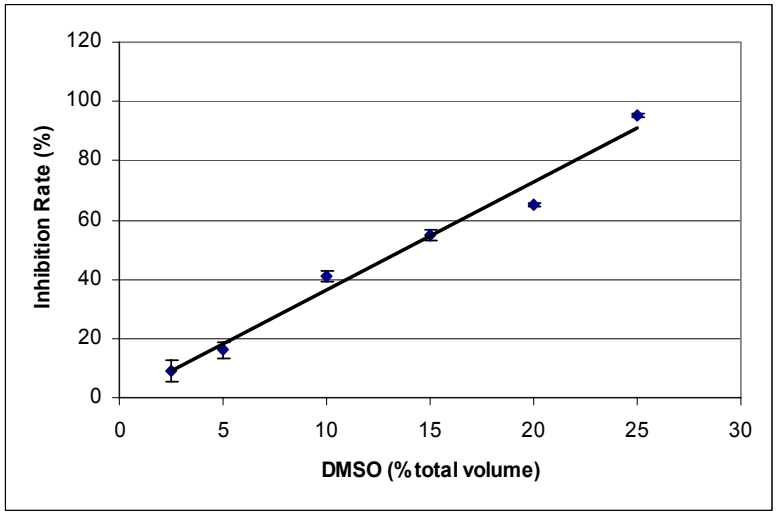


Figure 3.6 The linear relationship between different concentrations of DMSO in BODIPY assay and the inhibition rate for calpain (R^2 = 0.98).

The calpain inhibition rate of varying concentration for quercetin and resveratrol were presented in Table 3-3 and Table 3-4 respectively.

Table 3-3 The effect of quercetin concentrations (0 - 125 µM) on inhibition rate for calpain. In this assay, 10 µL DMSO blank showed 13% inhibition rate for calpain. The data in this table have been corrected for DMSO inhibition rate removed, and thus, are the real inhibition rates for quercetin. The assays of series of concentrations of quercetin were undertaken in triplicates (n=3).

Concentration of Q (µM)	Calpain Activity Inhibition Rate (%)
15	13 ± 2.0
31	**19** ± 1.7
62	28 ± 1.2
125	55 ± 8.7
10 µL DMSO Blank	*13* ± 1.7

It was concluded that IC_{50} (concentration of antioxidant required to inhibit calpain *II* by 50%) of quercetin is about 115.7 µM and that quercetin at concentrations of 30 µM inhibited about 15% of calpain activity according to the equation in Figure 3.7.

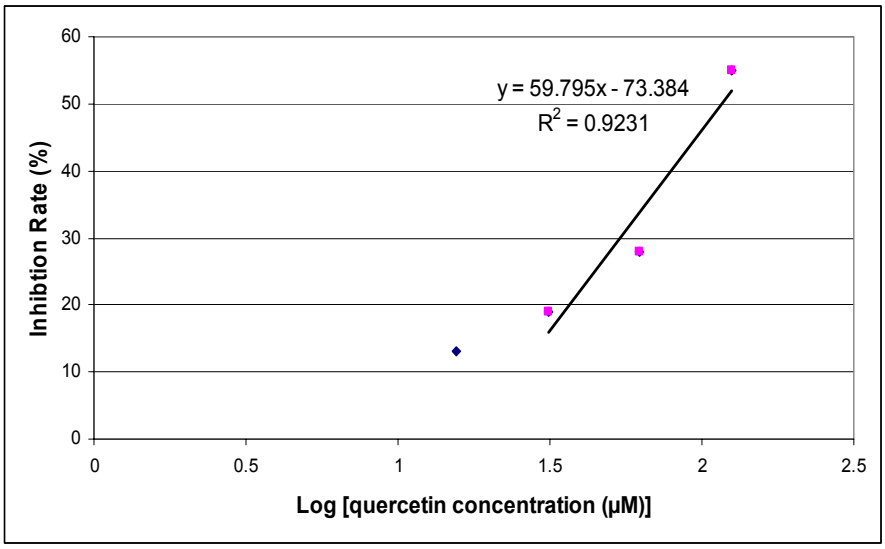


Figure 3.7 Calculation of IC_{50} of quercetin according to the equation of linear trend line: y = 59.795x – 73.384. X-axis represents log [quercetin concentration (µM)]. Y-axis represents the inhibition rate for calpain (%).

Table 3-4 suggested that resveratrol at concentrations of 30 µM inhibited about 8% of calpain activity. It was calculated that IC_{50} of resveratrol is about 1.37 mM according the equation in Figure 3.8.

Table 3-4 The effect of resveratrol concentrations (0 - 2 mM) on inhibition rate for calpain. In this assay, 10 µL DMSO blank showed no inhibition for calpain. Therefore, the data from resveratrol BODIPY assay were the actual inhibition rates for resveratrol. The assays of series of concentrations of resveratrol were undertaken in triplicates (n=3).

Concentration of R (µM)	Calpain Activity Inhibition Rate (%)
2000	59 ± 2.7
1800	55 ± 0.6
1500	53 ± 1.0
1000	41 ± 0.6
500	29 ± 1.0
250	18 ± 1.2
125	13 ± 3.2
61	10 ± 1.2
31	**9** ± 1.5

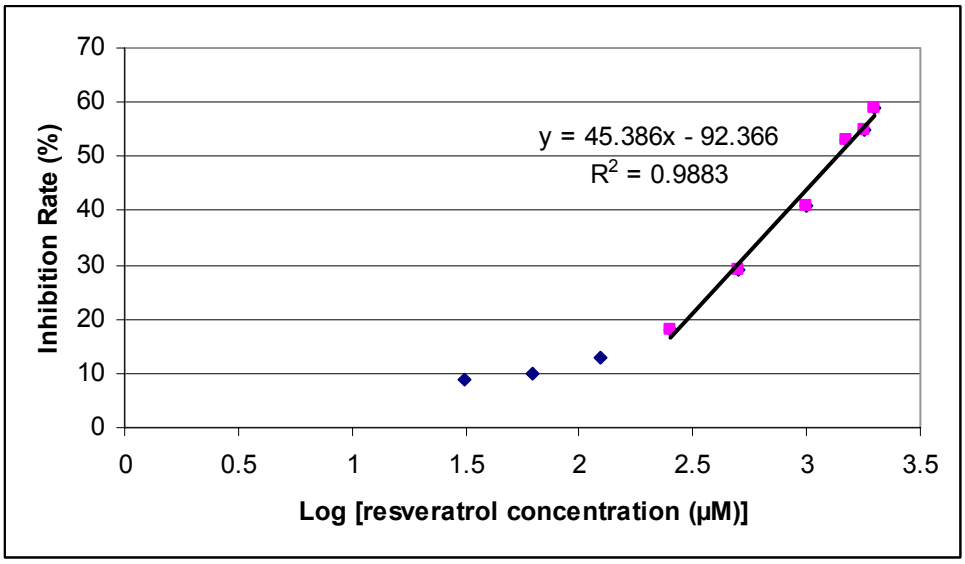


Figure 3.8 Calculation of IC_{50} of resveratrol according to the equation of linear trend line: y = 45.386x - 92.366. X-axis represents log [resveratrol concentration (µM)]. Y-axis represents the inhibition rate for calpain (%).

3.4 Discussion

Ramachandran (1991) reported the presence of 0.03 mM H_2O_2 in human aqueous humor and so the lens is in contact with this oxidant *in vivo*. Significantly higher levels (0.08-0.19 mM) have been found in cataract patients (Giblin *et al.*, 1984; Spector, 1984; Ramachandran *et al.*, 1991). Spector & Garner (1981) reported even higher concentrations of H_2O_2 (0.66 mM) in the aqueous humour of cataract patients. The variation in published H_2O_2 levels may in part be the result of the conditions under which the aqueous humor was obtained, stored, and used for assay (Spector *et al.*, 1998). In *in vitro* rat lens culture experiments, 0.5 mM H_2O_2 caused lens opacification within 24-72 h and produced a pattern of oxidative damage similar to that found in human cataract (Cui & Lou, 1993). Cornish *et al*. (2002) showed in their *in vitro* rat lens experiment that 0.5 mM H_2O_2 was sufficient to induce light scatter in the equatorial cortical regions of the lens over a 4 h period. Also, Sanderson *et al*. (1999) showed that rat lenses exposed to 1 mM H_2O_2 for 8 h resulted in loss of transparency. Spector (1995) concluded that the oxidative damage resulting from exposure to H_2O_2 paralleled changes occurring in the human lens as a result of cataractogenesis, including loss of GSH, increases in intracellular calcium and lens opacification. In addition, Zigler *et al*. (1985) found that H_2O_2 generated in the fluids surrounding the lens posed a much greater oxidative stress than superoxide or hydroxyl free radicals. This was the reason why 1 mM H_2O_2 was used to induce the ovine lens

opacification as an oxidant in the current experiments. These have shown that exposing sheep lenses to 1 mM H_2O_2 for 6 h resulted in the loss of lens transparency.

In this first ovine LOCH assay, quercetin reduced H_2O_2-induced lens opacification and improved transparency at concentration of 30 µM, whereas resveratrol of 30 µM was not effective at reducing opacity progression ($P > 0.05$, n=6). This study also showed that the mechanism of action was not dependent on directly removing or inactivating H_2O_2 in the culture medium. The effect of pre-incubation with quercetin suggests that quercetin is either taken up by the lens, or elicits some change(s) in the lens that protects it from opacification in the LOCH model.

The biological variability of the lenses was reflected in the high standard deviations (±1.59 for quercetin and ±1.52 for resveratrol) of opacity progression during 6 h in this experiment. This LOCH experiment was carried out by choosing random lenses. The difficulty in using pair lenses was that both needed to be maintained through the culturing procedure. If one lens died, the other could not be used any more. However, it was found that the biological variation might have masked the effectiveness of the antioxidants in reducing the opacity progression.

Quercetin at concentrations of 30 µM (the same concentration as used in the culture system) inhibited 15% of calpain activity and 30 µM resveratrol inhibited only 8% of calpain activity *in vitro* assay. However, considering that BODIPY assay was an *in vitro* assay, the calpain inhibition by quercetin would be much lower at the concentrations of quercetin with the lens. Hence, this suggested that the mechanism for quercetin to reduce the H_2O_2-induced opacification was not as a calpain inhibitor.

DMSO [$(CH_3)_2SO$], a by-product of the wood industry, has been in use as a commercial solvent since 1953 (Muir, 2005). It is an amphipathic molecule with a highly polar domain and two apolar methyl groups, making it soluble in both aqueous and organic media. Due to its relatively small (molecular weight of 78.13) and compact structure as well as its physicochemical properties, DMSO is a very efficient solvent for water-insoluble compounds (Santos *et al.*, 2003) and capable of penetrating living tissues without causing significant damage (Szmant, 1975). Therefore, since quercetin and resveratrol are water-insoluble, DMSO was used as solubilizing agent in their sample preparation for the lens culture and for BODIPY assay as well. Although Santos *et al*. (2003) reported that DMSO is a hydroxyl radical scavenger, the prevention on lens opacification through this radical-

scavenging mechanism should be neglected since its concentration used in the lens culture was very low; 10 µL/10 mL = 0.1 % v/v.

From the BODIPY assay, it was found that DMSO had the inhibitory effect on calpain activity *in vitro*. For example, 2.5% v/v caused 9% calpain inhibition (Figure 3.6). However, as only 0.1% v/v of DMSO was used in LOCH experiment, the role of DMSO to prevent lens opacification through calpain inhibition should be excluded.

3.5 Conclusions

In conclusion, quercetin, not resveratrol, significantly protected cultured ovine lenses against H_2O_2-induced opacification. On the other hand, the BODIPY assay clarified that the mechanism for quercetin to reduce H_2O_2-induced opacification or to improve lens transparency was not through a calpain inhibition. High standard deviations on opacity progression were found in the present experiment. A reduction of the biological variation could possibly improve the system sensitivity towards detecting any effects for antioxidants, which thus led to the planning and execution of the second experiment (in the next chapter) using paired lenses for LOCH model.

Chapter 4
Biochemical Changes in Cultured Sheep Lenses in the Presence of H_2O_2 and Antioxidants

4.1 Introduction

In the first experiment, a large standard deviation probably masked antioxidant's effects in the LOCH system. The reason was probably due to the biological variation because the first LOCH experiment had not treated lenses in pairs to get the comparison effect. Therefore, a second LOCH experiment was carried out using paired lenses.

Several studies have shown that glutathione is the most abundant non-protein thiol in the cell (Dickinson *et al.*, 2002). Reduced glutathione is the major intracellular low-molecular-weight thiol and plays a critical role in the cellular defence against oxidative stress in mammalian cells (Meister, 1983). It participates in the reduction of disulfides and other molecules, and conjugates with compounds of exogenous and endogenous origin. It protects cells against the destructive effects of reactive oxygen intermediates and free radicals (Meister, 1983).

After potential antioxidant agents (quercetin & resveratrol) were put into culture media, the following laboratory work was carried out: 1) the ability of antioxidants to inhibit the opacification in a paired-model to minimize biological variation; 2) LDH assay to determine the viability of the lens cells; 3) biochemical changes associated with the opacification, e.g., GSH & GSSG level in lenses which have been treated with antioxidants in LOCH model, were assessed and compared with their paired H_2O_2 controlled ones; 4) HPLC assay to detect the uptake and presence of antioxidants (and their metabolites) both inside the lens and in the culture media outside the lens.

4.1.1 Biochemical Assays of LDH

It was important in the experiment to determine the viability of the lens cells following H_2O_2 treatment and whether this was improved in antioxidant-treated lens cells. Cell viability is assessed by monitoring signs of cells death, which are normally evaluated by the quantification of plasma membrane damage. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. Consequently, the amount of LDH

activity detected in the culture medium correlated to the proportion of lysed cells (Szekeres *et al.*, 1981; Decker & Lohmann-Matthes, 1988; Martin & Clynes, 1991). Therefore, a precise, fast and simple colorimetric method was used to quantify cytotoxicity/cytolysis based on the measurement of LDH activity released by damaged cells as described by (Decker & Lohmann-Matthes, 1988). Comparisons of LDH activity within the lens culture media between antioxidant treatment and the paired hydrogen peroxide treatment would indicate whether the antioxidants protected the cells.

4.1.2 Assay of Glutathione – Endogenous Antioxidant

One possible important function of GSH in the lens is to protect the thiol (-SH) group of crystallins, preventing them from aggregating to form opaque clusters, which are high molecular weight (HMW) protein aggregates through disulphide bond formation (Reddy & Giblin, 1984). Reduced glutathione in the lens may also protect critical SH groups involved in regulating cation transport and permeability. Studies with mammalian lenses indicate that lowering lens GSH concentration leads to increased membrane permeability to cations and inactivation of Na^+-K^+-ATPase (Reddy & Giblin, 1984). One consequence of the changes in ion distribution is the inhibition of protein synthesis, which may explain the cessation of growth in cataractous lenses (Reddy & Giblin, 1984).

The majority of tissue "total glutathione" [i.e., GSH (reduced glutathione) + GSSG (oxidised glutathione), in GSH equivalent] is in the reduced form, GSH (Anderson, 1985). In the human lens, the GSH concentration is 6 -10 mM with the GSH/GSSG ratio >10 (Halliwell & Gutteridge, 1999).

There are a number of procedures (e.g., chemical, enzymatic, chromatographic) for the determination of GSH and/or glutathione disulfide (GSSG) in biological samples (Meister, 1983; Meister & Anderson, 1983). An example of a chemical method is where GSH forms a thioether with 4-chlorol-1-methyl-7-trifluoromethyl-quinolinium methylsulphate (Slaughter *et al.*, 2002, 2003). This is subsequently converted by sodium hydroxide to a coloured thione, which is measured at 400 nm to determine GSH level.

Enzymatic methods, which include fluorometric (i.e., monochlorobimane) and colorimetric (i.e., DTNB) detection means, are more efficient and accurate than chemical method to determine GSH levels. Two are commonly used:

i. A dye, monochlorobimane, appears to form an adduct exclusively with GSH in a reaction catalysed by glutathione S-transferase. As unbound monochlorobimane is

almost non-fluorescent, whereas the dye fluoresces blue (Ex. = 380 nm; Em. = 461 nm) when bound to glutathione. Thus, the changes in glutathione level can be easily detected using a fluorometer or a 96-well fluorometric plate reader (Nauen & Stumpf, 2002).

ii. The colorimetric method uses DTNB and GSSG reductase recycling to determine GSH and GSSG levels not only in cells but also in tissue, plasma and erythrocytes. The recycling system dramatically improves the sensitivity of total glutathione detection, allowing quantification of 1-100 ng glutathione in a 200 µL reaction.

A number of sensitive and specific chromatographic methods are also available for the determination of GSH, but they are somewhat lengthy (Moore & Stein, 1954; Purdie & Hanafi, 1971; Tabor & Tabor, 1977; Hsiung *et al.*, 1978). The advances in high-performance liquid chromatography (HPLC) and the variety of HPLC columns now available have led to development of several rapid chromatographic methods. For example, both derivatization of GSH with 2-vinylpyridine followed by ion-exchange chromatography & ninhydrin detection and the derivatization of GSH with Kosower's monobromobimane reagent followed by reverse-phase HPLC and fluorescent detection were described by Anderson (1985).

After the advantages and disadvantages were compared among the above various methods, the convenient, colorimetric enzymatic method [5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) –GSSG reductase recycling assay] based on the glutathione recycling method of Tietze (1969) was used in the current research for quantitative determination of nanogram amounts of either the reduced (GSH) or the oxidised form of glutathione (GSSG) using a microtiter plate reader. The reduced form of glutathione (GSH) can be specifically detected by omitting the glutathione reductase from the reaction mixture. The determination of GSSG in biological samples was often difficult because GSSG was normally present at very low levels as compared to GSH (Anderson, 1985). Therefore, oxidation of GSH must be minimized and rapid sample preparation was important. The oxidized glutathione content was determined by modification of the glutathione recycling method, which removed GSH from the assay by derivatization with 2-vinylpyridine first, based on GSH and other thiols reacting rapidly at pH ~7 with 2-vinylpyridine to form a conjugate.

4.1.3 HPLC Assay

It was also essential to investigate the uptake of quercetin (or resveratrol) in lens and stability of quercetin (or resveratrol) and their metabolites in aqueous media. Therefore, HPLC method described by Price and Rhodes (1997) was used to detect quercetin (or resveratrol) and their metabolites not only inside the lens but also in the aqueous medium outside the lens and thus to clarify the place where the reaction occurred for antioxidants.

4.1.4 SDS-PAGE Assay

It was possible that H_2O_2 could cause changes in lens protein synthesis or turnover and antioxidants should have some protection on it. Therefore, protein assays were done to determine the protein concentration for each lens sample. Proteins were separated on Sodium Dodecyl Sulphate –Polyacrylamide Gel Electrophoresis (SDS-PAGE) using mini-gels according to Laemmli (1970) to show whether there were differences at the protein level between antioxidant treated lenses and the paired hydrogen peroxide control ones.

4.2 Materials and Methods

4.2.1 Assessment of Opacification in Paired Model

25 pairs of lamb (9-12 months old) whole-eye globes were collected from a local abattoir immediately following slaughter and delivered to the laboratory for lens dissection. Pairs of lenses from the eyes of each animal were kept together. Lenses were dissected and cultured as the same way in the first LOCH experiment (detailed in previous chapter). Following the 48 h incubation, lenses were inspected for damage and 30 undamaged lenses (no opacities or fungal growth) were selected for experiment. For the two lenses from the same sheep, one lens would be pre-incubated with quercetin (or resveratrol) overnight, then quercetin (or resveratrol) would be washed off and the lens would be treated with hydrogen peroxide; while the other lens would be treated with hydrogen peroxide only. Therefore, the design structure for those 30 lenses was as demonstrated in Table 4-1, among which there were 12 paired lenses.

Table 4-1 Experimental scheme for 2nd ovine LOCH experiment in pair model. Lenses in group 2 and 4 were paired with group 3 and 5 respectively. There were 6 individual lenses in each group (n=6).

Treatment Group	Pre-incubation (24 h) ⟶	Inducing oxidative
1. control **(C)**	EMEM	AAH only
2. H_2O_2 control paired with Q **(Hq)**	EMEM	AAH + 1mM H_2O_2
3. Quercetin + H_2O_2 **(Q)**	EMEM + 30 µM quercetin	
4. H_2O_2 control paired with R **(Hr)**	EMEM	
5. Resveratrol + H_2O_2 **(R)**	EMEM + 30 µM resveratrol	

As in the previous chapter, all lenses were photographed and their opacities were graded by the image analysis system. The significance of opacity increase over 6 h between antioxidant groups Q & R and the hydrogen peroxide controls Hq & Hr was determined by using Student's paired *t*-test.

4.2.2 Lactate Dehydrogenase (LDH) Leakage Assay to Test Lens Cell Viability

4.2.2.1 Test Principle

An increase in the amount of dead or plasma membrane-damaged cells results in an increase of the LDH enzyme activity in the culture supernatant. Lactate dehydrogenase activity was determined colorimetrically. In the first step NAD^+ is reduced to NADH as LDH catalyzes the oxidation of lactate to pyruvate. In the second step diaphorase transfers electrons from NADH to the tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl] -5-phenyltetrazolium chloride) reducing it to formazan (Figure 4.1) which absorbs at 500 nm.

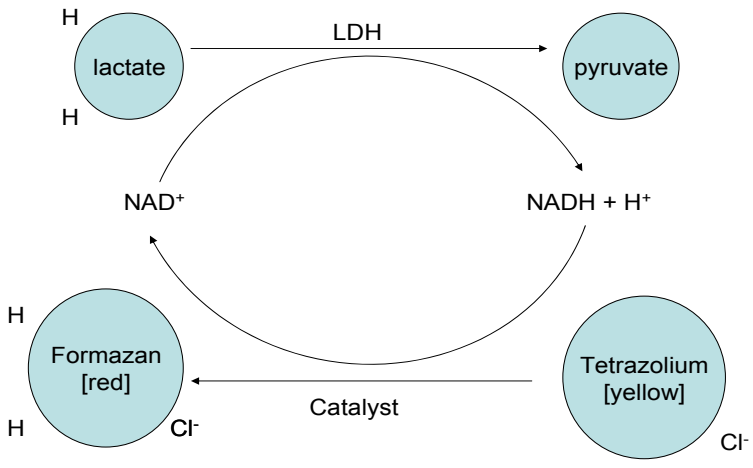


Figure 4.1 Principles of LDH assay. In the first step, released lactate dehydrogenase (LDH) reduces NAD^+ to NADH + H^+ by oxidation of lactate to pyruvate. In the second enzymatic reaction 2 H are transferred from NADH + H^+ to the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl] -5-phenyltetrazolium chloride) by a catalyst.

4.2.2.2 Collection of Culture medium

1 mL aliquots of each culture medium were collected at the end of the 6h experimental period and stored at 4 °C for LDH measurement (the other 9 mL aliquots of each culture medium were left for HPLC assay).

4.2.2.3 LDH Measurement

For quantitative measurement of LDH levels in the samples, 1000 U/mL of LDH (Lactate Dehydrogenase from bovine heart, Sigma, Catalog L3916) was diluted with assay medium (AAH containing 1mM H_2O_2) from 0 (background control) to 10 mU/100µL to generate a standard curve. In a clear flat bottom 96 well microplate, 100 µL aliquots of culture media were mixed with 100 µL LDH reaction mixture using a cytotoxicity detection assay kit (Roche Applied Science, Cat. No.1 644 793). All samples were assayed in triplicate, incubated at room temperature for 30 min in the dark, and the absorbance was measured at 492 nm. The background at 660 nm was subtracted. Therefore, the LDH activity was determined by comparison with the plotted standard curve of the known amount of LDH (0-10 mU/100µL).

4.2.3 Glutathione Assay (DTNB-GSSG Reductase Recycling Assay for GSH and GSSG)

4.2.3.1 Principle of Glutathione Assay

This assay was based on the glutathione recycling method of Tietze (Tietze, 1969) by using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and glutathione reductase (Figure 4.2). DTNB and GSH reacted to generate 2-nitro-5-thiobenzoic acid and GSSG. Since 2-nitro-5-thiobenzoic acid was a yellow coloured product, GSH concentration could be determined by measuring absorbance at 412 nm. GSH could be generated from GSSG by glutathione reductase & NADPH, and reacted with DTNB again to produce more 2-nitro-5-thiobenzoic acid.

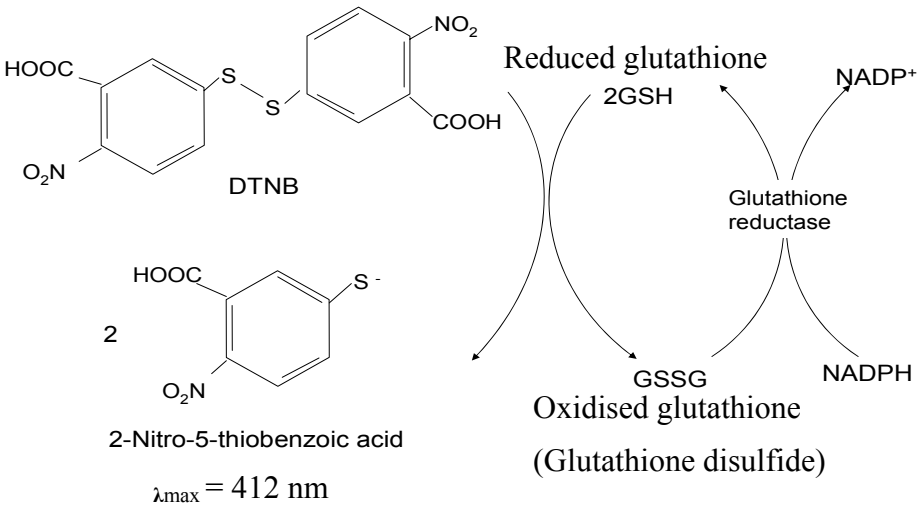


Figure 4.2 Principle of Glutathione Assay.

4.2.3.2 Preparation of Sheep Lens Extract for Glutathione Assays

The assays were carried out as described by Anderson (1985). After being photographed, the lenses were immediately transferred to 4 mL of chilled assay stock buffer [125 mM sodium phosphate, pH 7.5, containing 6.3 mM EDTA (ethylenediaminetetraacetic acid, tetra sodium salt)] and then homogenised on ice for 20 s at 13,000 rpm using a PT-MR 3100 polytron (Littau, Switzerland). A 2 mL aliquot for GSH measurement was mixed with 0.5 mL of 5% 5-sulfosalicylic acid (SSA) to precipitate protein from samples and prevent oxidation of GSH. Therefore, the total volume of each acidified homogenate was 2.5 mL and it was centrifuged at 10,000 × g for 10 min at 4°C. After the centrifugation, the supernatant was transferred to a fresh tube and split 4:1 for the oxidised and reduced glutathione assays respectively and stored on ice for up to an hour before glutathione assay. A further 1ml aliquot was used for subsequent HPLC assay and the remainder was for measurement of protein concentration and analysis by SDS-PAGE.

4.2.3.3 Quantification of Reduced Glutathione (GSH)

Five-fold dilutions of the sample were made using 1% SSA to make sure the readings were within the range of standard calibration curve. On a 96-well plate, 140 µL of 0.28 mM NADPH [prepared daily as described by Sanderson *et al*. (1999)] and 20 µL of assay stock buffer were added to each well and mixed well. After incubation at room temperature for 10 min, the diluted lens sample (20 µL) was added and incubated at room temperature for another 5-10 min. Each lens sample was assayed in duplicates. Then 6 mM DTNB (20 µL)

from frozen stock was added using multi-channel pipettes and the assay incubated for 10 min on a FLUOstar spectrophotometer (BMG ALPHATECH System, Auckland, NZ) at 25 °C and the absorbance at 412 nm was determined. The reduced glutathione of lens extracts was calculated by comparison to a 0 -50 ng/μL GSH standard curve.

4.2.3.4 Quantification of Oxidized Glutathione (GSSG)

In the fume hood, reduced glutathione was removed from the assay by derivatization with 2-vinylpuridine; 2 μL was added to sheep lens extract (100 uL). Auto-oxidation was prevented by placing 5 μL triethanolamine on the inside of each vial above the levels of the liquid which contained the sheep lens extract supernatant. Vials were then vigorously mixed for 30 s (final pH would be 7 - 7.5) (Griffith, 1980). The samples were left to derivatize at ambient temperature for 60 min and then diluted to 5-fold with 1% SSA. The assay was as described for GSH assay except that 0.2 unit glutathione reductase (20 μL) replaced the 20 μL assay stock buffer in GSH assay. Each lens sample was assayed in duplicates. The oxidised glutathione content of lens extracts was calculated from a standard curve by comparing its rate of absorbance change at 412 nm over 6 min with the rate of known concentration of GSSG (0 – 1 ng/μL).

4.2.4 HPLC Analysis

After 1ml media samples (AAH) was removed for LDH assay, 9 mL of each media was snap frozen and stored at -70 °C for subsequent HPLC analysis. Media samples (AAH) were freeze dried overnight, and 200 μL of 80% methanol/water/1.6 mM ascorbic acid (to stabilize the quercetin or the resveratrol) added, followed by centrifugation at 11,000 × *g* for 5 min at 4 °C. To test the recovery rate for quercetin and resveratrol in EMEM culture medium, EMEM recovery test was also undertaken with the same extraction approach as above. The EMEM culture medium recovery rate was calculated by comparison with a standard curve of known concentration of antioxidants (0 -60 μM). The recovery of quercetin was about 55% for the culture media.

Similarly, freeze dried lenses homogenates (original 1ml aliquot from the lens homogenate) were prepared for analysis of metabolites. 0.5 mL of 80% methanol/water/1.6 mM ascorbic acid was added and sonicated for 4 min, then centrifuged for 10 min at 11,000 × *g* at 4 °C.

The supernatant (including samples of lens homogenates and media) was filtered through 0.22 μM PTFE filter units (LabServ Filtration, BIOLAB Company, Australia & New

Zealand) into HPLC vials. Quercetin & resveratrol were analyzed on a Waters HPLC system consisting of a model 717 Plus autosampler, a model Waters 600-MS pump, and a model 996 photodiode array detector with Millennium software system. A Luna 5 µ C18, 4.60×250 mm column (phenomenex) was used with a modified version of the analytical HPLC Method described by Price and Rhodes (1997). Solvents A (water:tetrahydrofuran:trifluoroacetic acid, 98:2:0.1) and B (acetonitrile), were run at a flow rate of 1ml/min, using a gradient of 17% B (2 min), increasing to 25% B (5 min), 35% B (8 min), 50% B (5 min) and then to 100% B (5 min), which was modified by Day *et al* (1998). A column clean-up stage maintained B at 100% (5 min) followed by a re-equilibration at 17% B (10 min). The column was packed with Luna 5 µM C 18(2) reverse-phase silica, 250 mm by 4.6 mm id (Phenomenex, Macclesfield, UK). Diode array detection monitored the effluent at 250 and 450 nm (the detector was set at 372 nm for quercetin and its metabolites; at 306 nm for detection of *trans*-resveratrol and at 273 nm for *cis*-resveratrol). The concentration of antioxidants (quercetin or resveratrol) in culture medium extracts was calculated from a standard curve generated by plotting the rate of area (uV * sec) against concentration of antioxidants from the range of 0 - 30 µM.

Also, the recovery of quercetin and resveratrol from lens was tested. A 2 µL of 30 mM quercetin or resveratrol was added to 2 mL of the control lens homogenate supernatant. After the lens homogenate was freeze dried, 1 mL of 80% methanol/water/1.6 mM ascorbic acid was added to extract flavonoids for the HPLC with the method as above (the final concentration of antioxidants was thus 2 µL*30 mM/1 mL=60 µM). The lens recovery rate was calculated by comparison with a standard curve of known concentration of antioxidants (0 -60 µM). The recovery of quercetin was about 65% for the lens and about 77% for resveratrol in the lens.

4.2.5 Protein Assay and SDS- PAGE

Protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, Illinois, USA) according to manufacturer's instructions. Samples were diluted 500 times to fit into the standard calibration curve plotted using dilutions of 2 mg/mL Albumin Standard (Pierce, Rockford, Illinois, USA) with concentrations ranging from 0 - 0.5 mg/mL. The samples were analysed for their protein concentration in triplicate and the average mg/mL protein concentration calculated.

Twenty-five µg total proteins from each lens (soluble and insoluble) was applied to the gel. 12% SDS-PAGE of the lens proteins of all groups were performed by running samples on

gels with the size of 9 x 5 cm (NuPAGE; Invitrogen Life Technologies, Carsbad, CA) for 50 min at 200 V using the MOPS buffer system and then stained with Coomassie Brilliant Blue (Sigma Chemical Company, St. Louis, MO).

4.3 Results

4.3.1 Effects of Quercetin or Resveratrol on H_2O_2 –induced Opacification in Paired Ovine Lens Model

Prior incubation with quercetin reduced but did not prevent opacification. The opacity of lenses pre-incubated with quercetin (7.8 ± 0.6) was significantly (P <0.05, n=5) less than the opacity of paired H_2O_2 control lenses (8.8 ± 0.4) at 6 h in the ovine LOCH experiment (Figure 4.3). The opacity of control lenses (C group) remained unchanged (0.03 ± 0.59; n=4) over 6 h of incubation period. The opacity of Hq group lenses progressed 5.1 ± 0.44 (n=5) over 6 h of incubation period. The opacity of Q group lenses progressed 3.6 ± 0.91 (n=5) over the same incubation period. Difference in opacification between Hq group and Q group was highly significant using Student's paired *t*-test (P < 0.01).

In contrast, prior incubation with resveratrol did not reduce opacification. There was no difference (P > 0.05) of opacity scale at 6 h between R group (8.3 ± 0.5) and Hr group (8.7 ± 0.4) (Figure 4.3). However, the opacity scale of R group at 0 h (4.7 ± 0.4) was higher (P < 0.05) than lens opacity scale of Hr group at 0 h (3.6 ± 0.4) (Figure 4.4).

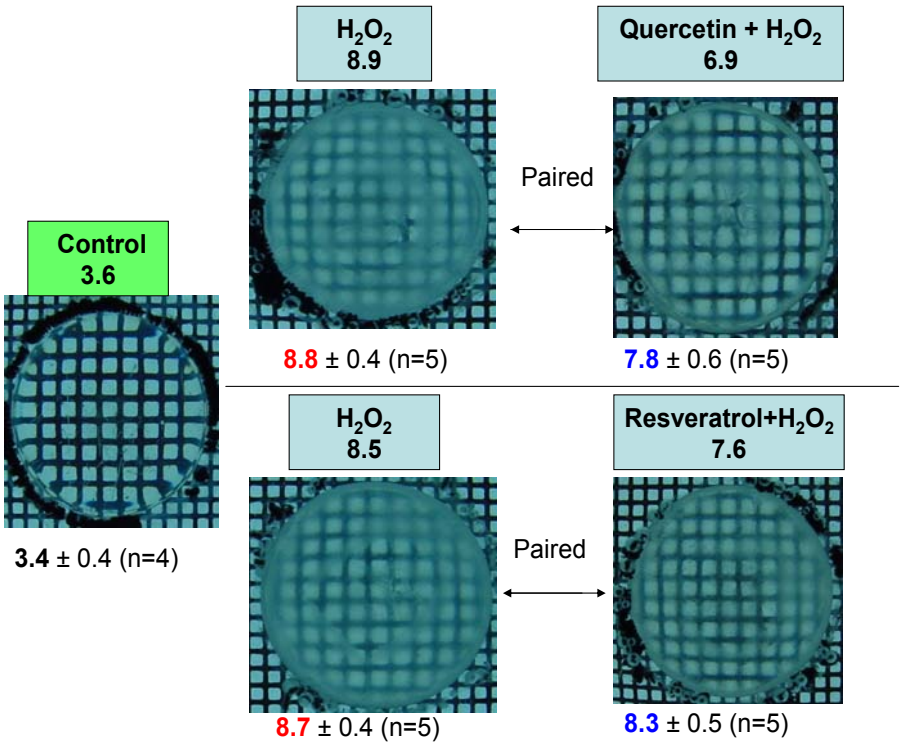


Figure 4.3 Typical digital images of ovine lenses and their opacification grading scores (mean ± SD) on paired lenses after incubation with H_2O_2 with or without pre-treatment with antioxidants (resveratrol or quercetin) at 6 h incubation time. The extent of lens opacity was significantly different between H_2O_2 (1 mM) and Quercetin (30 µM) + H_2O_2 (1 mM) group * ($P < 0.05$). The actual opacification score for each lens from each group at 6 h was shown in Appendix B.

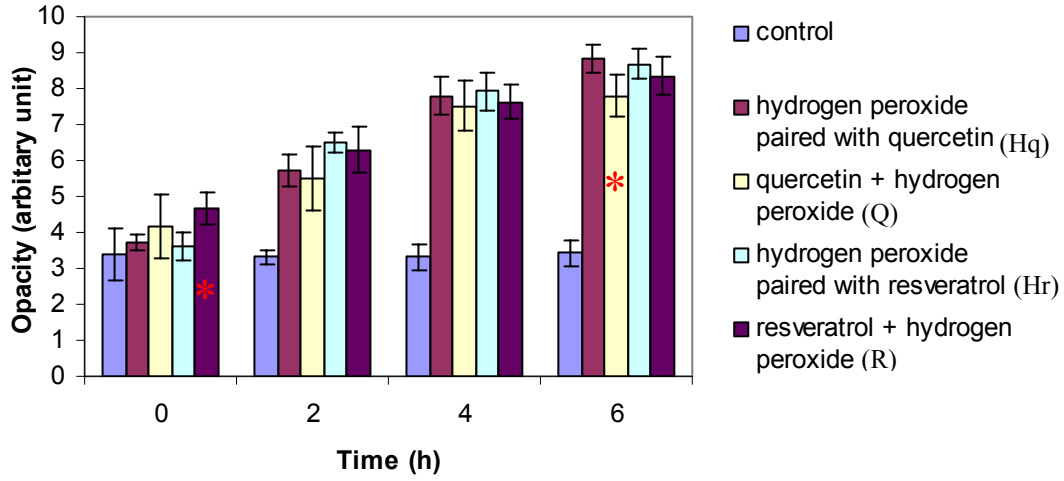


Figure 4.4 Opacity progression during 6 h incubation period with different treatments as outlined in the previous Figure. * indicates that the opacities of resveratrol + H_2O_2 treatment at 0 h significantly higher than their paired lenses treated with H_2O_2 at the same time using the Student's paired *t*-test ($P < 0.05$) and quercetin + H_2O_2 treatment at 6 h significantly lower than their paired lenses treated with H_2O_2 at the same time using the Student's paired *t*-test ($P < 0.05$). Error bars shows standard deviation.

4.3.2 Effects of Antioxidants on LDH Leakage in Ovine LOCH Model

Lens cell death as determined by measurement of lactate dehydrogenase was found to increase with hydrogen peroxide and the increase was prevented by pre-treatment with antioxidants (Figure 4.5).

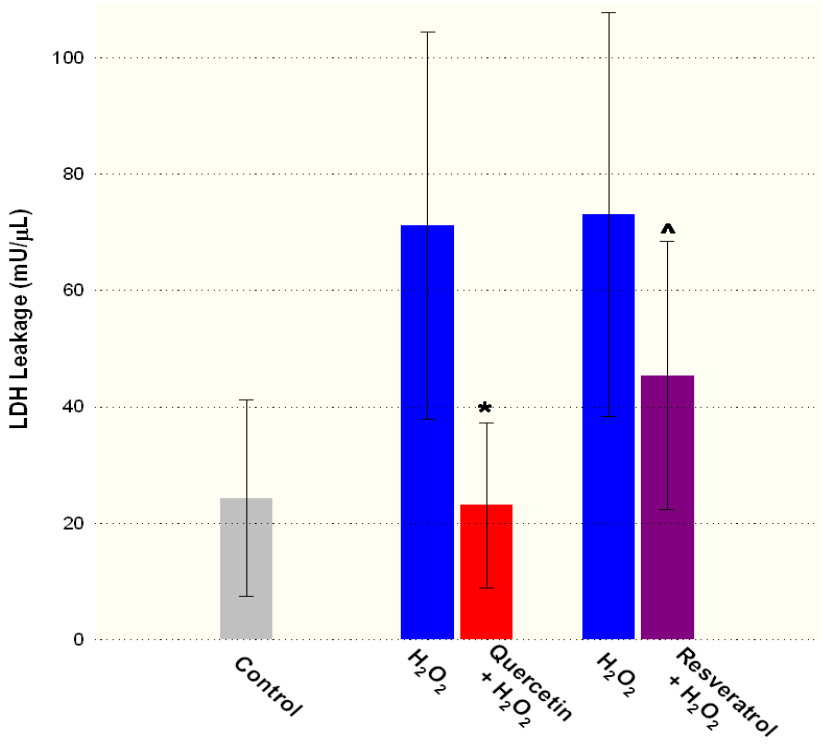


Figure 4.5 A plot of mean LDH leakage levels detected in 5 treatment groups at the end of 6 h experimental period. Paired *t*-test: * and ^ = $P < 0.05$ relative to its corresponding H_2O_2 treated pair. Significant difference between Control group and H_2O_2 groups (P values < 0.05) was found using the Student's 2-sample *t*-test. No significant difference either between Control and (Quercetin + H_2O_2) group or between Control and (Resveratrol + H_2O_2) group (both of the P values > 0.05) using the Student's 2-sample *t*-test. Error bars shows standard deviation.

Actual individual data of LDH leakage (mU/100 µL) is in Appendix D.

After 48 h of lens settling period, the LDH leakage for control lens was 24.3 ± 16.9 mU/100 µL AAH culture media (n=5). After 6 h of exposure to H_2O_2, the levels of leaked LDH to culture media was about 3 times greater than in the controls. Pre-incubation with antioxidants significantly ($P < 0.05$, n=6) reduced LDH leakage by 70% and 40% for quercetin and resveratrol, respectively, compared with paired H_2O_2 treated lenses.

4.3.3 Effects of Antioxidants on GSH/GSSG Ratios in Ovine LOCH Model

After 48 h pre-settling period, the control sheep lenses contained 16.3 ± 2.8 μmol GSH /gram lens (50.5 ± 14 nmol GSH/mg protein) and 0.42 ± 0.11 μmol GSSG /g lens (1.26 ± 0.22 nmol GSSG/mg protein) (n=6) with a GSH/GSSG ratio of 40. The levels of GSH, GSSG and GSH/GSSG ratio in ovine lenses treated with 1 mM H_2O_2 were not significantly different from the control or paired antioxidant-treated lenses (Table 4-2).

Table 4-2 Concentrations of GSH, GSSG (μmol/g lens) and GSH/GSSG ratio (mean ± SD) in 5 treatment groups at the end of 6 h experimental period. Lenses of Hq group were paired with lenses of Q group. Lenses of Hr group were paired with lenses of R group (n =6).

Description	Abbreviation	GSH	GSSG	GSH/GSSG Ratio
Control	**C**	16.3 ±2.8	0.42 ±0.11	40.4 ±9.4
H_2O_2 (1 mM)	**Hq**	16.6 ±2.9	0.34 ±0.07	50.1 ±13.5
Quercetin + H_2O_2	**Q**	15.0 ±1.2	0.41 ±0.11	38.0 ±8.2
H_2O_2 (1 mM)	**Hr**	16.6 ±1.5	0.43 ±0.13	41.3 ±12.5
Resveratrol + H_2O_2	**R**	17.0 ±2.2	0.39 ±0.06	45.3 ±12.9

Actual individual data of GSH & GSSG concentrations (μmol/g lens) and actual individual data of GSH/GSSG ratio are in Appendix C respectively.

After 6 h of exposure to 1 mM H_2O_2, the levels of GSH & GSSG had no significant difference with control lenses. Lenses in Hq group contained 16.6 ± 2.9 μmol GSH/g lens and 0.34 ± 0.07 μmol GSSG/g lens with a GSH/GSSG ratio of 50. Lenses in Hr group contained 16.6 ± 1.5 μmol GSH/gram lens and 0.43 ± 0.13 μmol GSSG/g lens with a GSH/GSSG ratio of 41. GSH/GSSG level in lenses pre-treated with quercetin or resveratrol were not significantly different either from controls (2-sample *t*-test) or from hydrogen peroxide treated lenses (paired *t*-test).

4.3.4 HPLC Analysis

In the lens extract sample, HPLC result showed a chromatogram peak (first peak, retention time - 20 min) with the same retention time and spectrum as the quercetin standard (shown in Figure 4.6) and a second peak (retention time 23 min) which was assumed to be the metabolite of quercetin -- 3'-O-methyl quercetin (Figure 4.7; as explained in 4.4 Discussion). The 2nd peak area (3'-O-methyl quercetin) was much larger than the first peak (quercetin). But it could not be quantified because no 3'-O-methyl quercetin standards were available for plotting a calibration curve. As for the AAH culture media, there was a very small quercetin peak (first peak, retention time - 20 min) and a second small peak

separated at 23 min (Figure 4.8). The 2nd peak area (3'-O-methyl quercetin) was larger than the first peak (quercetin).

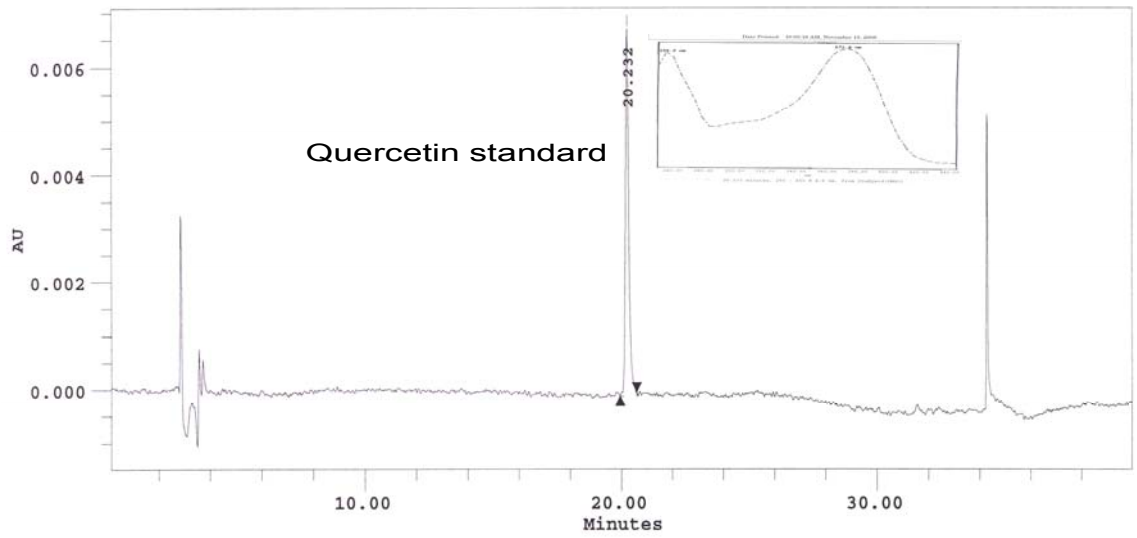


Figure 4.6 Typical chromatogram of quercetin standard (dissolved in DMSO) analysis using HPLC. The retention time for the peak is 20 min. The small window shows the spectrum of the peak with the maximum absorbance at 372 nm.

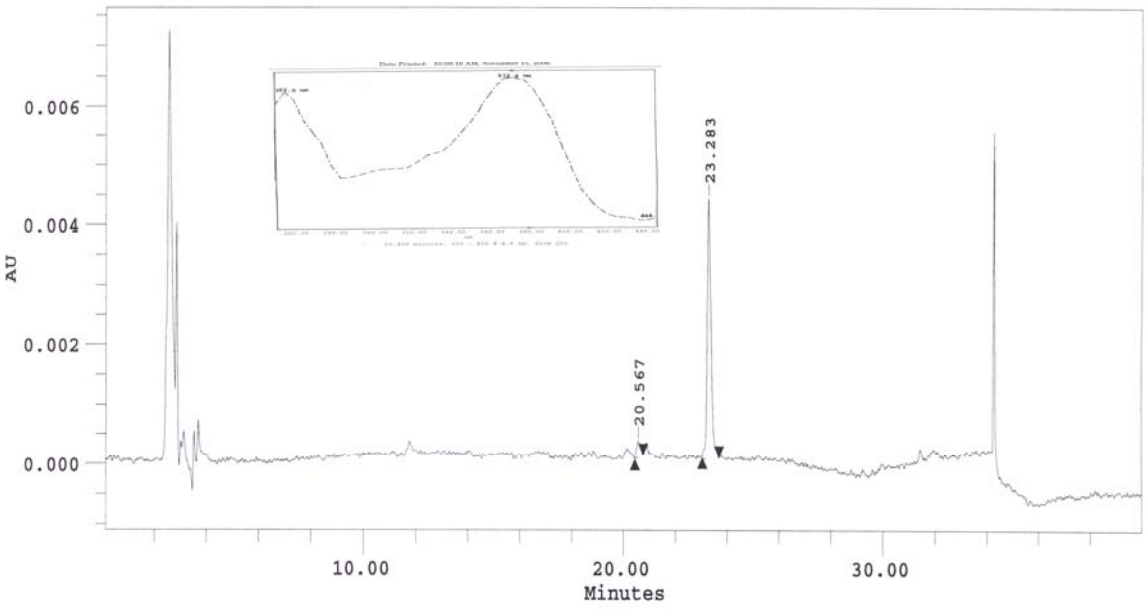


Figure 4.7 Typical chromatogram of quercetin and its metabolites in the whole ovine lens extract. Flavonoid content was analysed by HPLC. Chromatograms show peak for quercetin (first peak, retention time 20 min, the spectrum could not be seen due to too small peak) and the second peak (retention time 23 min) which was assumed to be the metabolite of quercetin --3'-O-methyl quercetin after 6 h exposure to H_2O_2. The small window shows the spectrum of the second peak with the maximum absorbance at 372 nm.

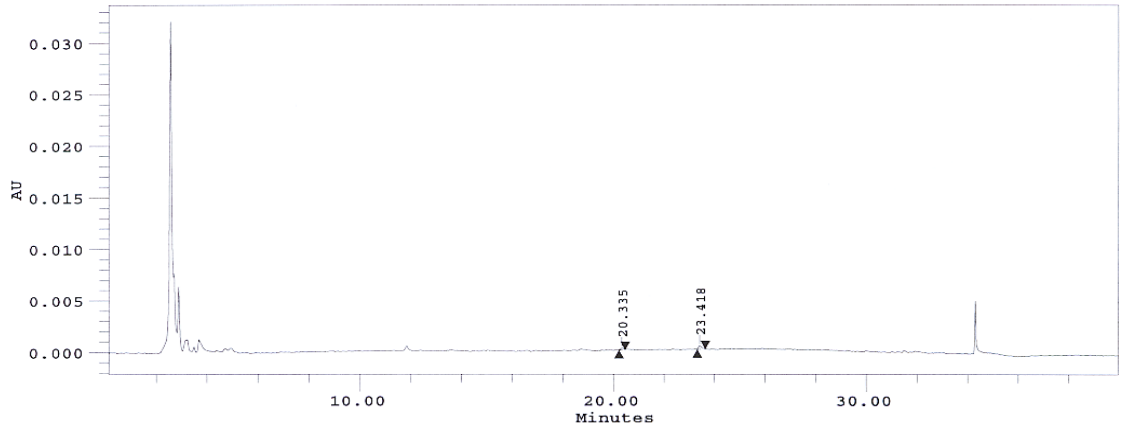


Figure 4.8 Typical chromatogram of quercetin and its metabolites in the experimental AAH culture media. Flavonoid content was analysed by HPLC. Chromatograms show peak for quercetin (first peak, retention time 20 min) and the second peak (retention time 23 min) which was assumed to be the metabolite of quercetin --3'-O-methyl quercetin after 6 h exposure to H_2O_2. Both spectrums of the two peaks could not be seen due to their too small area.

The retention time of the *trans*-resveratrol standard was about 18 min with the maximum absorbance at 307 nm in its spectrum (Figure 4.9). In the lens sample extract for HPLC, there was very tiny peak of *trans*-resveratrol separated at retention time of 18 min with maximum absorbance of 302 nm (Figure 4.10). The AAH culture media had a peak for *trans*-resveratrol (retention time 18 min with the maximum absorbance at 302 nm Figure 4.11). There was another peak separated at 16 min with a maximum absorbance of 274 nm, which was assumed to be *cis*-resveratrol (reasons are discussed in section 4.4; Figure 4.12). The area of *trans*- and *cis*-resveratrol peak was similar in the AAH culture media. The concentration of *trans*- resveratrol in culture media was 0.95 ± 1.36 µM (converting the area to concentration of *trans*-resveratrol according to the plotted standard calibration curve, n = 4). There were traces of *trans*-resveratrol found inside the lens. Therefore, the result showed that there was much more *trans*-resveratrol (almost twice) detected outside the lens than inside the lens. There was no peak for *cis*-resveratrol detected in lens sample extract.

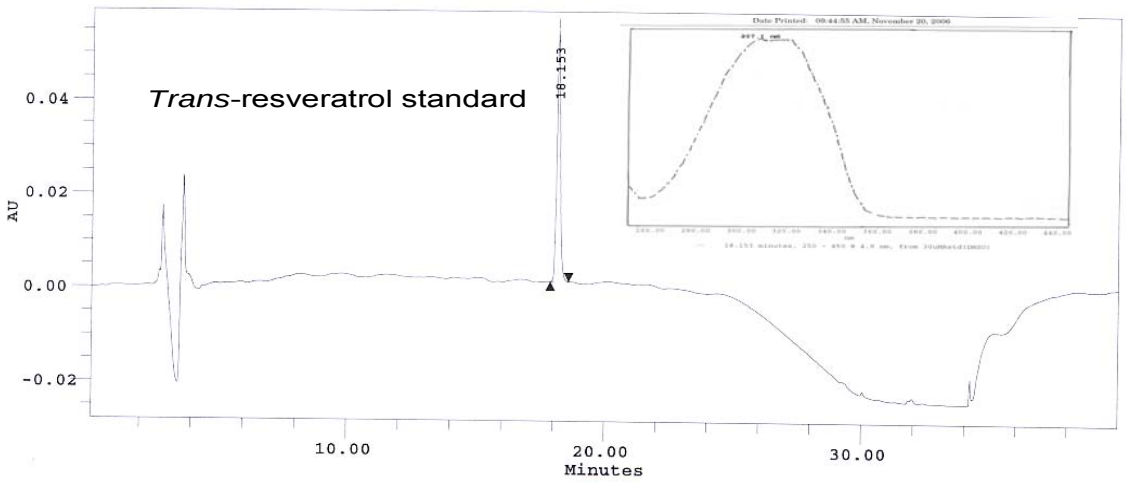


Figure 4.9 Typical chromatogram of *trans*-resveratrol standard (dissolved in DMSO) analysis using HPLC. The retention time for the peak is 18 min. The small window shows the spectrum of *trans*-resveratrol standard with the maximum absorbance at 307 nm.

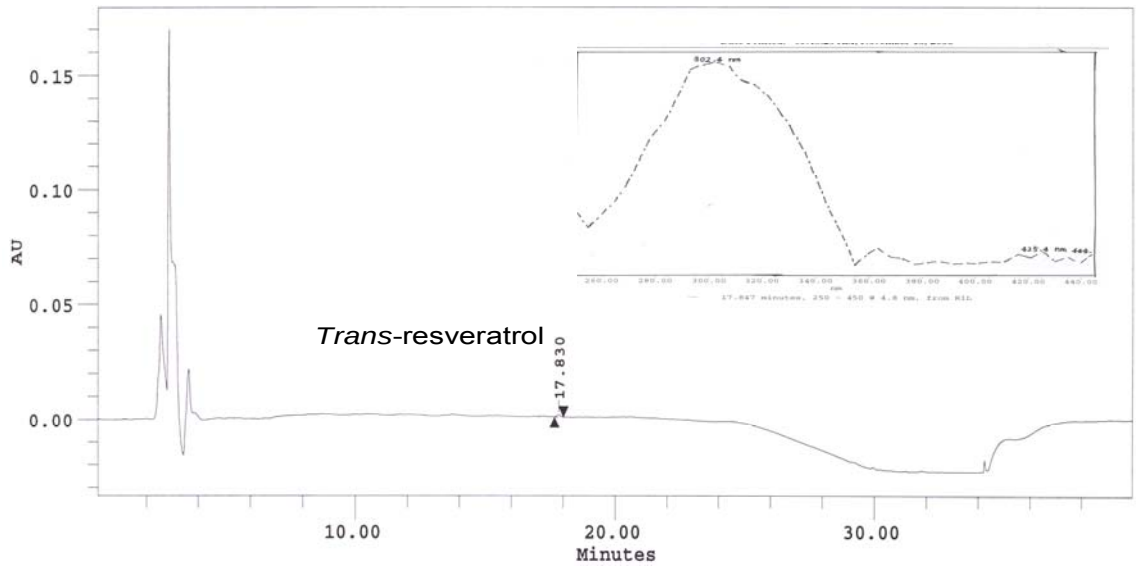


Figure 4.10 Typical chromatogram of *trans*-resveratrol in the whole ovine lens extract. Flavonoid content was analysed by HPLC. Chromatograms show *trans*-resveratrol peak (retention time is 18 min) after 6 h exposure to H_2O_2. The small window shows the spectrum of the peak with the maximum absorbance of 302 nm.

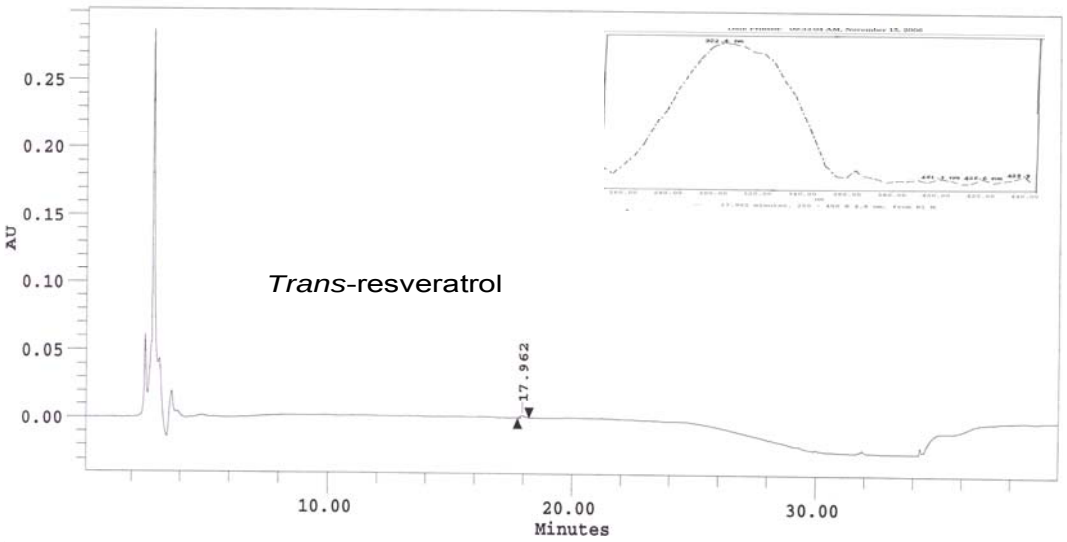


Figure 4.11 Typical chromatogram of *trans*-resveratrol in the experimental AAH culture media. Flavonoid content was analysed by HPLC. Chromatograms show *trans*-resveratrol peak (retention time is 18 min) after 6 h exposure to H_2O_2. The small window shows the spectrum of the peak with the maximum absorbance of 302 nm.

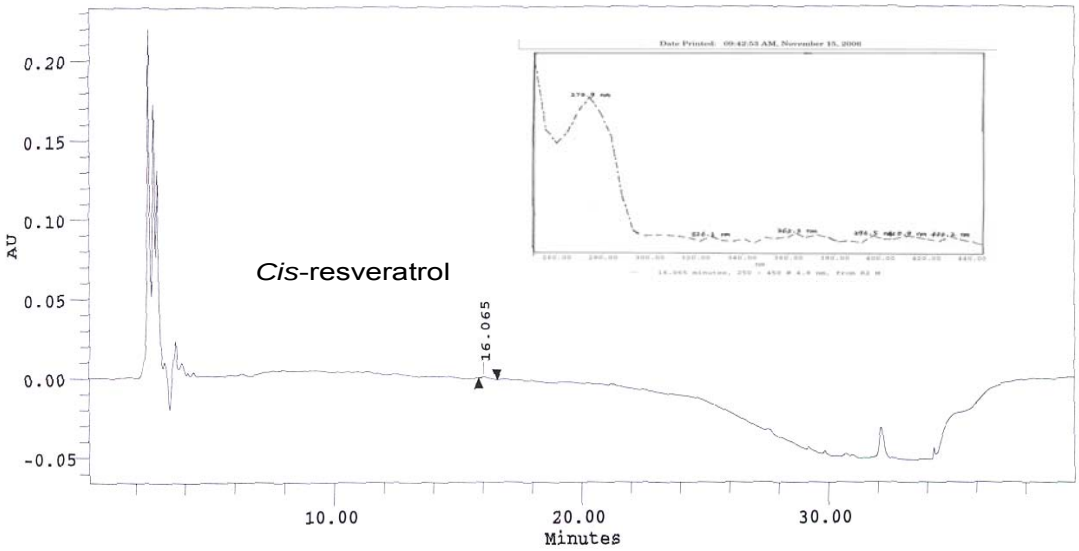


Figure 4.12 Typical chromatogram of *cis*-resveratrol in the experimental AAH culture media. Flavonoid content was analysed by HPLC. Chromatograms show *cis*-resveratrol peak (retention time is 16 min) after 6 h exposure to H_2O_2. The small window shows the spectrum of the peak with the maximum absorbance of 274 nm.

4.3.5 SDS-PAGE

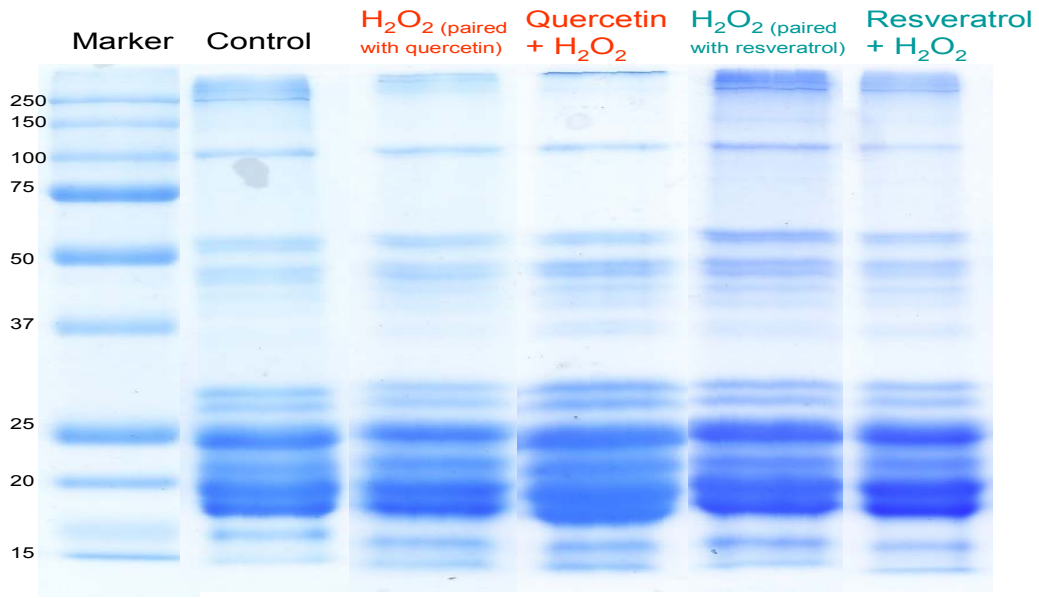


Figure 4.13 12 % SDS-PAGE of 25 µg total proteins extracted from ovine lens homogenates in the paired ovine LOCH model.

SDS-PAGE was carried out for total protein (soluble and insoluble) from each lens sample. The major proteins in the samples from sheep lenses were identified using SDS-PAGE gels stained with Coomassie blue. Crystallins appeared as low-molecular-weight proteins < 40 kDa and non-crystallin proteins appeared at high molecular weights > 40 kDa. The SDS-PAGE in Figure 4.13 did not reveal any apparent differences or changes in the proteins following H_2O_2 or antioxidant treatments.

4.4 Discussion

The current paired LOCH experiment showed that prior incubation with quercetin reduced but did not prevent opacification. As expected, the standard deviation in the paired 2nd LOCH model was much less than the first non-paired LOCH experiment.

At 6 h, there was no significant difference of the opacity between lenses pre-incubated with resveratrol and their paired H_2O_2 controlled lenses using Student's paired *t*-test. Hence, it was not possible to confirm a protective role for resveratrol in lens opacification. It was observed that pre-incubation of resveratrol for 24 h had resulted in increased lens opacity before the lens was exposed to H_2O_2, which might be caused by experimental error or the fact that resveratrol itself was capable of irritating the lens or increasing the lens opacity. Therefore, the opacity-reducing role of resveratrol was uncertain.

The LDH assay showed that both antioxidants (quercetin and resveratrol) protected cell viability of ovine lenses.

Oxidative stress caused by accumulation of free radicals is involved in the pathogenesis of senile cataracts (Robertson *et al.*, 1991). If so, appropriate amounts of antioxidants might be expected to prevent or retard the process. The majority of previous laboratory experiments on cataract have focused on the antioxidant vitamins C and E. Both have been shown to have protective effects in some *in vivo* and *in vitro* models of cataract (Creighton & Trevithick, 1979; Ross *et al.*, 1982; Devamanoharan *et al.*, 1991). However, it is noteworthy that *in vitro* experiments with vitamin C (Shang *et al.*, 2003) tend to report a protection against oxidative damage (e.g., it restored the resistance of GSH-depleted lens cells to H_2O_2) rather than protection of transparency. Similarly, resveratrol was not shown to reduce lens opacification in the current experiment, but protected the cell viability.

Sanderson *et al.* (1999) found that the rat lenses after 48 h pre-settling period contained 24.0 nmol GSH/mg protein and 0.38 nmol GSSG/mg protein with a GSH/GSSG ratio of 63. These remained unchanged after incubation with quercetin. The current experiment also quantified GSH or GSSG concentration expressed in forms of nmol/mg protein after the protein amount was determined for each lens sample. The ovine lenses after 48 h culture contained 50.5 nmol GSH/mg protein and 1.26 nmol GSSG/mg protein with a GSH/GSSG ratio of 40. Further, Sanderson *et al*. (1999) reported that the levels of GSH decreased by 45% and GSSG increased by 12.5-fold after 4 h of exposure of rat (10-15 wk old) lenses to 1 mM H_2O_2. Pre-treatment with quercetin did not affect these changes.

In the present experiment [6 h of exposure of sheep lenses to the same concentration (1 mM) of H_2O_2], there were no significant changes in GSH or GSSG after 6h exposure to 1 mM H_2O_2. These results may be due to species differences. One difference could be the different size of the lens. Since rat lens is much smaller than sheep lens, rat lens may be more susceptible to oxidative stress. Other differences might involve the antioxidant regulation in rat and sheep lens. Zigler *et al*. (1989) compared opacification in cultured monkey lenses and rat lenses exposed to the same oxidizing systems. Rat lenses were damaged by lower concentrations of oxidants than were monkey lenses. They concluded that oxidative stress affected both rat and monkey lenses by similar mechanisms but that lenses from monkeys, and probably other primates, were more resistant to these effects because they have better endogenous antioxidant defences. This may be why sheep lenses

did not show significant decrease in GSH level when exposed to the same concentration of 1 mM H_2O_2.

Because 1 mM H_2O_2 exposure of 6 h did not affect GSH/GSSG level in the sheep lens, it was not possible to determine whether quercetin or resveratrol protected GSH/GSSH ratio. The mechanism by which they inhibited opacification was unknown as the opacity of lens was not correlated with GSH depletion or GSSG increase. However, Sanderson *et al*. (1999) proposed a mechanism where quercetin protected the lens from calcium and sodium influx, which are early events leading to lens opacity.

The second peak presented in the chromatography for lenses pre-incubated with quercetin was probably quercetin's metabolite – 3'-O-methyl-quercetin because the retention time (23 min) and maximum absorbance (372 nm) in the spectrum of that peak was consistent with 3'-O-methyl-quercetin, which had been identified by Sanderson *et al.* (1999) using mass spectrometry. In the current experiment, the lenses were washed in AAH for 1 h to eliminate the residual quercetin (or resveratrol) adhering on the lens surface before they were transferred to new culture media. Therefore, there shouldn't be any quercetin or resveratrol detected in the culture media unless they were coming out from the inside of the lens to scavenge H_2O_2 or free radicals. HPLC assay demonstrated that the main reaction for quercetin with H_2O_2 occurred both inside and outside the lens but with the inside as the main location, since there was much more assumed 3'-O-methyl-quercetin detected by HPLC inside the lens than in the culture medium.

As for resveratrol, the peak with a retention time of 16 min with the spectra of maximum absorbance at 274 nm was identified to be *cis*-resveratrol from the literature (Lamuela-Raventos *et al.*, 1995). The *trans* isomers are transformed to the *cis*- forms under UV light or direct oxidation. The spectra and elution time for *trans*- resveratrol in lens and culture media were slightly different from the *trans*- resveratrol standard, e.g., double shoulder sort peak (Figure 4.9) is shifted to the left side (Figure 4.10; Figure 4.11). This can be explained by binding of *trans*- resveratrol to the lens proteins as demonstrated by (N' Soukpoe-Kossi *et al.*, 2006). There was more *trans*-resveratrol peak area present in the culture medium (0.95 μM) than inside the lens (almost twice) and no *cis*-resveratrol inside the lens. These supported the following possibilities. Firstly, resveratrol equilibrated from the inside to the outside of the lens since original culture media did not contain any *trans*-resveratrol. However, because the result showed much less *trans*- resveratrol detected inside the lens, resveratrol was more possible to have been leaking or diffusing from the

inside to the outside of the lens. Savaskan *et al*. (2003) reported that resveratrol exerted an anti-oxidative action by enhancing the intracellular free-radical scavenger glutathione. The leakage of resveratrol to the culture media led to smaller concentration of resveratrol remaining in the lens, which resulted in the lack of significant effect of resveratrol in the present study.

SDS- PAGE did not show any apparent changes in the crystallin proteins between H_2O_2-treated lenses and their paired lenses pre-incubated with antioxidant. Since the oxidative stress and antioxidant treatment did not show effect on the protein profile and the alteration of lens transparency was not caused by proteolysis due to the calpain protease activity in the current experiment, phase separation phenomena may explain this result. In the mammalian lens, a reorganisation of the cellular structure is necessary for the transition from the normal transparent state to the opaque, cataractous state (Clark & Carper, 1987). Lens cells are transparent because of short-range order in the organisation of cytoplasmic proteins, which are largely crystallins. The transition in early stages of cataract development is associated with a phase separation in the lens cytoplasm (Clark *et al.*, 1999). A phase separation is a low-energy mechanism of cytoplasmic restructuring that is reversible. It was assumed that weak non-covalent interactions are responsible for transparent organisation of cytoplasmic proteins. In the transparent state, short-range order in the organisation of cytoplasmic protein allows the cytoplasm exists as a single homogeneous phase. In the opaque state, the short-range order is disrupted, and the cytoplasm exists as two separate phases with the difference in index of refraction to cause scattering of light (Clark & Carper, 1987). Interactions between cytostructural proteins and crystallins may be required for the initial organisation of lens proteins into the transparent homogeneous structure necessary for maintenance of transparency. Clark *et al*. (1999) mentioned that in the absence of a cytoskeletal scaffold, attractive interactions between lens proteins may dominate and accelerate the organisation of proteins into large light-scattering aggregates. In the current experiment, SDS-PAGE gel separated proteins according to their molecular weight. During the preparation to run SDS-PAGE gel, proteins had been denatured. Since the phase separation is a low-energy and reversible mechanism, the weak non-covalent bond leading to phase separation could have been lost during this procedure. That was perhaps why, despite the transparency of the lens being significantly different, there was no apparent difference in the protein profile. However, it was worthwhile to notice that cytoskeletal proteins were soluble in the urea. Therefore,

future work could use urea to extract the cytoskeletal proteins and run SDS-PAGE to see if there are differences in the cytoskeletal protein profile.

4.5 Conclusions

Pre-incubation of ovine lenses with quercetin reduced H_2O_2-induced opacification and cell damage in the paired LOCH model, while resveratrol showed a protective effect against oxidative damage on lens cell viability rather than protection of transparency. Exposure of ovine lenses to 1 mM H_2O_2 did not affect GSH & GSSG levels. Since hydrogen peroxide was able to damage lens cells and cause opacification without affecting the reduced glutathione levels, GSH decline or GSSG increase was not the leading cause of opacification for H_2O_2-induced ovine cataract. Antioxidants may maintain cell viability.

Published reports showed that H_2O_2 decreased GSH and increased GSSG and it may be that 1 mM was insufficient for our lens model. Further, possibly only some region of the lens was affected by H_2O_2 and thus the overall GSH/GSSH in lens homogenate would still remain in the normal range. Therefore, a third LOCH experiment was designed to treat lenses in different concentrations of H_2O_2 (1, 2, and 4 mM) and measure the GSH/GSSG level in three fractions of ovine lenses (epithelium, cortex and nucleus).

Chapter 5
Response of Lens Fractions to Hydrogen Peroxide

5.1 Introduction

In this experiment, the GSH/GSSG content was quantified in three different fractions of sheep lens (epithelium, cortex and nucleus) following treatment with different concentration of hydrogen peroxide (1, 2, and 4 mM). This was to determine whether hydrogen peroxide only affected GSH/GSSG levels in some fractions of the lens rather than in the whole lens. Moreover, a pro-oxidative detection assay was used to evaluate the pro-oxidant capacity of ovine lens fractions (cortex and nucleus) treated with different concentration of hydrogen peroxide (1, 2, and 4 mM).

In chapter 4, 1 mM H_2O_2 treatment did not cause significant changes in GSH or GSSG content in the whole lens compared with controls. Since there is no information regarding the effect of H_2O_2 on GSH/GSSG equilibrium in ovine lens, 1 mM H_2O_2 might be able to trigger changes at certain regions of the lens that would be lost in whole homogenized lens and/or higher concentrations of H_2O_2 are required to induce GSH/GSSG changes in ovine lens. To examine that hypothesis, a third experiment was planned where higher concentrations of H_2O_2 were used and the lenses were dissected into three fractions (epithelium, cortex and nucleus) to investigate reduced and oxidized glutathione content in those three ovine lens fractions.

The measurement of free radicals has increased awareness of the functional implications of radical-induced impairment of the oxidative/antioxidative balance (Vendemiale *et al.*, 1999). Molecular probes and pro-oxidative detection assays can help identify free radicals that might be involved in cataract formation and enable us to use suitable antioxidants that target these radicals. The molecular probe 2', 7'-dichloroflurescin diacetate (DCFH-DA) is readily diffusible through the cell membrane and is trapped in the cell by being incorporated into cellular lipids and enzymatically deacetylated by intracellular esterases to 2'-7'-dichlorofluorescin (DCFH), which is oxidant-sensitive and an oxidizable substrate for the various radicals (Wrona & Wardman, 2006). The direct oxidation of DCFH to the fluorescent product 2'-7'-dichlorofluorescein (DCF) with H_2O_2 can occur in the presence of a catalyst, such as heme, peroxidase or cytochrome *c* (Wrona & Wardman, 2006). Therefore, the DCFH-DA pro-oxidant assay was carried out in the third LOCH experiment

to elucidate the role of pro-oxidation in cataract formation. The method used was a modification of the described by Driver *et al*. (2000). This method was chosen because it is simple and can be used on same sample used for other biochemical analyses.

Furthermore, SDS-PAGE was carried out to see whether there was significant difference or change at protein level among control, 1, 2, and 4 mM H_2O_2 treatment in epithelium, cortex and nucleus fractions of ovine lenses.

5.2 Materials and Methods

5.2.1 Opacification of Lenses Tested with Different Concentration of H_2O_2 in lens culture

Ten pairs of lamb (9-12 months old) whole-eye globes were collected from a local abattoir immediately after slaughter and transferred to the laboratory for lens dissection. Lens culture (including EMEM and AAH) was set up as described in the previous experiments. Following the 48 h incubation period in EMEM, lenses were inspected for damage and 12 undamaged lenses (free from opacities or fungal growth) were selected for the experiment. The treatment groups were 3 lenses treated with 1 mM H_2O_2 in AAH; 3 lenses treated with 2 mM H_2O_2 in AAH; 3 lenses treated with 4 mM H_2O_2 in AAH and 3 lenses remained in AAH without H_2O_2 as controls. Lenses were scored for opacity using image analysis system (Image Pro-Plus v4.1) at 0, 2, 4 and 6 h of incubation with H_2O_2. The differences in opacity score at different incubation times over 6 h between treated groups and the controls were determined using Student's 2-sample *t*-test.

5.2.2 GSH/GSSG Quantification in Three Sections of Lens

Sheep lenses were dissected into three fractions: epithelial fraction included the epithelial cells and capsule peeled off from the lens and had a mean weight of 30 mg (3.8% of the total weight of the lens); cortex fraction was the jelly part peeled off from the inner hard core with a mean weight of 500 mg (64% of the total weight of the lens) and the remaining part was the nuclear fraction with a mean weight of 250 mg (32% of the total weight of the lens).

Each dissected epithelium fraction (including capsule) was transferred to 120 µL of chilled phosphate stock buffer (125 mM, pH 7.5, containing 6.3 mM EDTA). The tissue suspension was sonicated (Model W-225, Heat Systems-Ultrasonics, INC, supplied by Waston Victor LTD, New Zealand) for 20 s to lyse all epithelial cells. A 20 µL aliquot was removed for protein measurement after sonication. For GSH measurement, 20 µL of 5%

SSA (5-sulfosalicylic acid) was added to the remaining lysates (80 µL) then mixed well and centrifuged at 10,000 × g for 10 min at 4°C.

Each dissected cortex fraction was transferred to 2 mL of chilled phosphate stock buffer and sonicated as above for 20 s to lyse all cortical cells. A 100 µL and 200 µL aliquots were removed for protein measurement and pro-oxidant assay, respectively. For GSH measurement, a 375 µL of 5% SSA was added to 1.5 mL aliquots of lysates then mixed well and centrifuged as described with the epithelium fraction.

Each dissected nuclear fraction was transferred to 1 mL of phosphate stock buffer and sonicated for 20 s to lyse all nuclear cells. Similarly, a 100 µL and 200 µL aliquots were removed for protein measurement and pro-oxidant assay, respectively. For GSH measurement, 150 µL 5% SSA was added to 600 µL aliquots of lysates then mixed well and centrifuged as described for epithelium and cortex factions. All the supernatants were stored on ice for up to 1 h prior to assay GSH and GSSG. GSH/GSSG was assayed and quantified as in chapter 4.

5.2.3 Pro-oxidant Assay (DCFH-DA)

The pro-oxidant activity of lens was measured using fluorescent dye - DCFH-DA, based on the method described by Driver *et al.* (2000) with modifications. The activity was determined on either lens cortex or nucleus lysates [0.5 g lens cortex sonicated in 2 mL phosphate sodium buffer (125 mM, pH 7.5, containing 6.3 mM EDTA) or 0.25 g lens nucleus was sonicated in 1 mL of the above buffer]. A 60 µL of either cortex or nucleus diluted sample (200X dilution) was transferred to 96-well plate (200 µL/well) and 100 µL of PBS buffer (without EDTA) was added. To each well, DCFH-DA was added to a final concentration of 1.25 mM. Samples were incubated at 30 °C for 1 h and the fluorescence was measured using FLUOstar fluorescence plate reader (BMG ALPHATECH System Ltd & Co. Auckland, NZ) with excitation at 485 nm and emission at 520 nm. Samples were measured in triplicate and the pro-oxidant activity was expressed as the rate of change of fluorescence units during 60 min measurement per second per gram lens tissue (ΔDCF/s. g).

5.2.4 Protein Assay and SDS PAGE

Aliquots of epithelium, cortex and nucleus (20, 100, 100 µL respectively) were used to determine the protein concentrations using the BCA Protein Assay Reagent (Pierce, Rockford, IL). For the protein assay, epithelium, cortex and nucleus samples were

appropriately diluted (100, 200 and 300-fold, respectively) to fit into the standard calibration curve plotted at protein concentrations of 0 - 0.5 mg/mL bovine serum albumin (BSA).

Fifteen µg soluble proteins (after centrifugation) from each lens epithelial, cortical and nuclear fraction were applied to 12% SDS PAGE as described in chapter 4.

5.3 Results

5.3.1 Lens Opacification Progression

All control lenses were transparent over 6 h incubation periods. In contrast, hydrogen peroxide at concentrations of 1, 2 and 4 mM induced lens opacification ($P < 0.05$) after exposure for 6 h (Figure 5.1) The opacity of treated samples progressed 4.28 ± 0.65; 5.81 ± 0.44; and 4.38 ± 1.30 (arbitrary units) for treatment of 1, 2, and 4 mM H_2O_2 respectively. The opacity progression over 6 h at 2 mM was higher (P <0.05) than 1 mM H_2O_2 treatment, whereas there was no significant difference between 1 mM and 4 mM, neither was there significant difference between 2 and 4 mM H_2O_2 treatment.

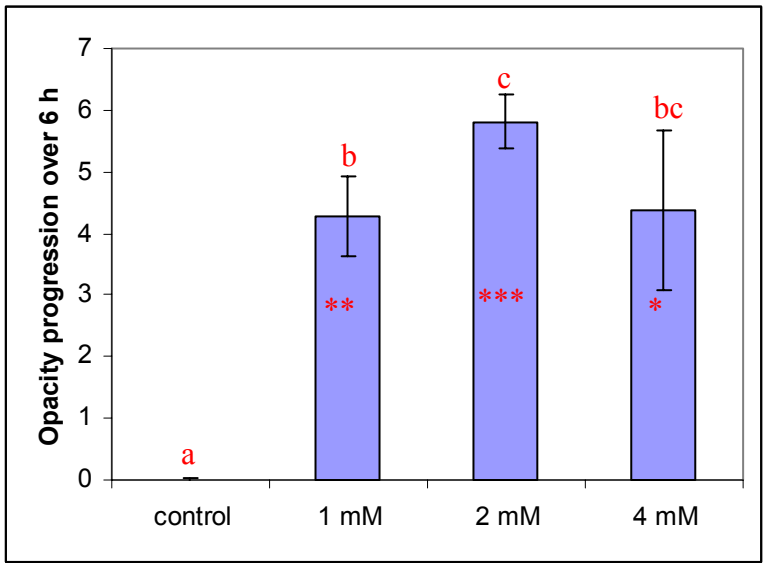


Figure 5.1 Effect of treatment with different concentrations of H_2O_2 (1, 2, and 4 mM) on lens opacity in the LOCH assay. Results were expressed as mean ± SD. Lenses were cultured in EMEM for 48 h and then incubated in AAH medium containing 1, 2 or 4 mM H_2O_2 for 6 h. The opacification of lenses were measured over 6 h periods and scored using image analysis system. Comparisons of opacity progression between H_2O_2 treatments and controls were made using Student's 2-sample *t*-test. The actual opacification score for 1, 2, and 4 mM H_2O_2 at 0, 2, 4, 6 h were shown in Appendix E. * = ($p < 0.05$), ** = ($p < 0.01$) and * = ($p < 0.001$), bars lacking a common letter differ, $P < 0.05$.**

5.3.2 The effect of H_2O_2 concentration and lens fractions on changes in GSH and GSSG

5.3.2.1 Epithelium

Control epithelial fractions of the lenses after 48 h settling period contained 3.7 ± 2.0 µmol GSH/g (epithelium fraction included the lens capsule) and 0.23 ± 0.04 µmol GSSG/g. The GSH/GSSG ratio for the control epithelial fractions was about 15 (Table 5-1). The levels of GSH in treated lenses after 6 h of exposure to H_2O_2 of different concentrations were not different ($P > 0.05$) from the controls. However, GSSG content was increased ($P < 0.05$) by around 6.6-folds in the 2 mM H_2O_2 treated lenses (1.74 ± 0.29 µmol GSSG/g) and 6.1-folds in the 4 mM H_2O_2 (1.64 ± 0.53 µmol GSSG/g) treated lenses (Table 5-1). The ratios of GSH/GSSG were 5.7, 1.5 and 3.3 at 1, 2 and 4 mM H_2O_2, respectively and no differences ($P > 0.05$) were found relative to controls (GSH/GSSG ratio of 15) as shown in Table 5-1.

Table 5-1 The effect of incubation with different concentrations of H_2O_2 (1, 2, and 4 mM) for 6 h on GSH and GSSG concentrations and GSH/GSSG ratio in the epithelial region of the lenses.

Group	GSH(µmol/g)	GSSG(µmol/g)	GSH/GSSG Ratio
Control	3.7 ± 2.0	0.23 ± 0.04	15.4 ± 6.5
1 mM	2.9 ± 3.2	0.42 ± 0.17	5.7 ± 4.4
2 mM	2.8 ± 1.8	1.74 ± 0.29*	1.5 ± 0.8
4 mM	6.0 ± 4.3	1.64 ± 0.53*	3.3 ± 2.2

* = (P < 0.05)

Actual individual data of GSH and GSSG concentrations (µmol/g epithelium) are in Appendix F (1).

Actual individual data of GSH/GSSG ratio in epithelium are in Appendix F (4).

5.3.2.2 Cortex

The cortical part of the control lenses, after 48 h settling period, contained 21.85 ± 1.31 µmol GSH/g cortex tissue and 0.53 ± 0.03 µmol GSSG/g cortex tissue with a GSH/GSSG ratio of about 41. After 6 h of exposure to H_2O_2, the level of GSH at 1 mM H_2O_2 was not different (18.47 ± 1.67 µmol GSH/g, $P > 0.05$) from controls, whereas lower GSH concentrations were found in 2 mM (16.74 ± 1.40 µmol GSH/g cortex, $P < 0.05$) and 4 mM (14.63 ± 1.49 µmol GSH/g cortex, $P < 0.01$) H_2O_2 treated lenses (Figure 5.2). Hydrogen peroxide caused a concentration-dependent reduction in GSH level (23% and 33% for 2 and 4 mM H_2O_2, respectively).

Oxidised glutathione level for 1 mM H_2O_2 (0.46 ± 0.05 µmol GSSG/g cortex) was not different ($P > 0.05$) from control. However, GSSG level was increased in 2 mM (1.06 ±

0.18 µmol GSSG/g cortex, $P < 0.05$) and 4 mM (1.43 ± 0.21 µmol GSSG/g cortex, $P < 0.05$) H_2O_2 treated lenses (2 and 2.7-fold for 2 and 4 mM H_2O_2 treatments, respectively) compared with the controls (Figure 5.2).

The ratios of GSH/GSSG were lower [16 ($P < 0.01$) and 10.3 ($P < 0.001$) for 2 and 4 mM H_2O_2 treatments, respectively] compared to the controls (GSH/GSSG ratio of 41) (Figure 5.2).

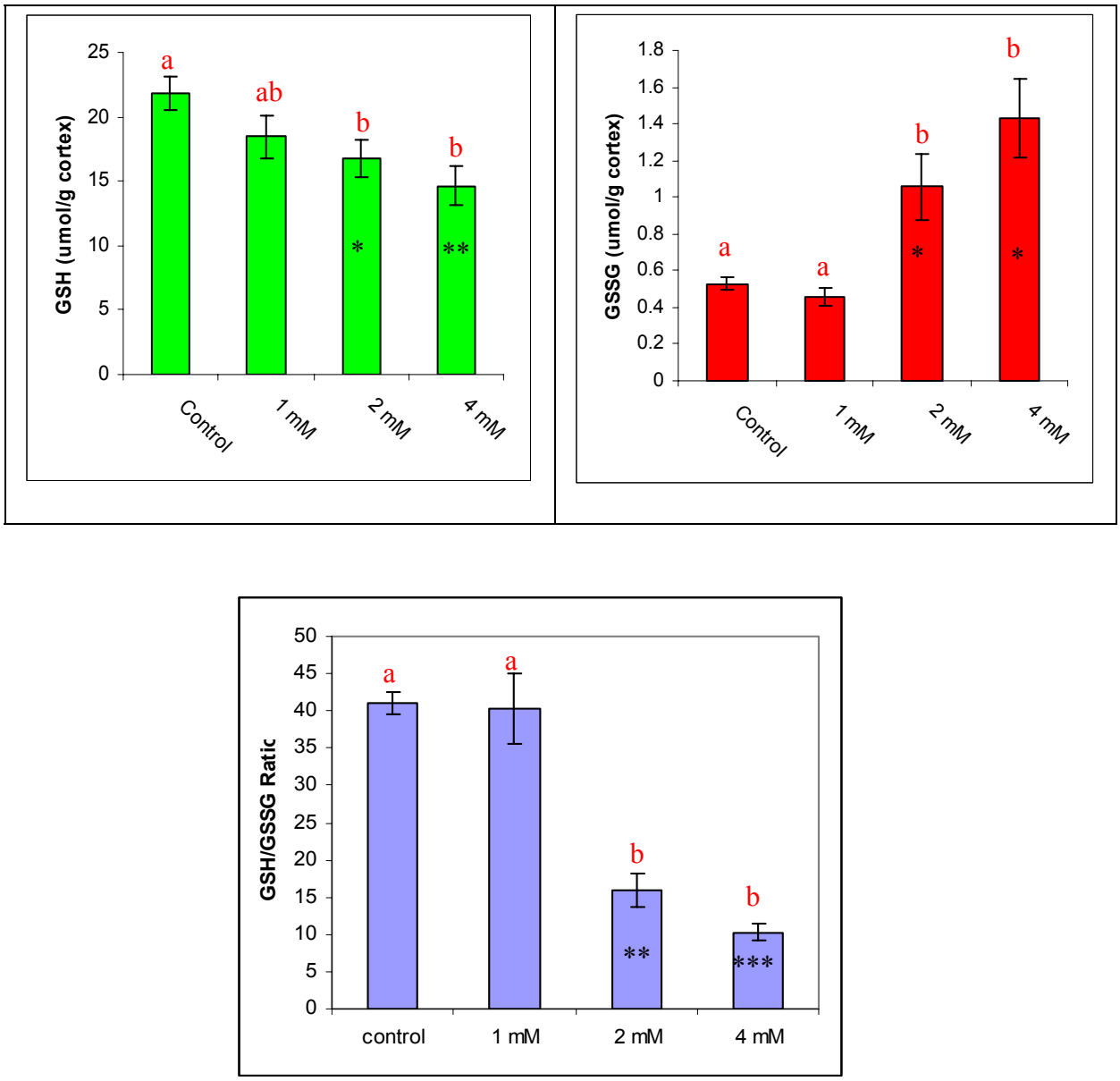


Figure 5.2 The effect of incubation with different concentrations of H_2O_2 (1, 2, and 4 mM) for 6 h on GSH and GSSG concentrations and GSH/GSSG ratio in the cortical region of the lenses.

Actual individual data of GSH and GSSG concentrations (µmol/g cortex) is in Appendix F (2).

Actual individual data of GSH/GSSG ratio in cortex is in Appendix F (4).

*** = ($P < 0.05$), ** = ($P < 0.01$) and *** = ($P < 0.001$), bars lacking a common letter differ, $P < 0.05$.**

Therefore, the effect of H_2O_2 on the cortical part of the lens can be summarized as; reduced glutathione as well as oxidized glutathione were only affected by the incubation at 2 and 4 mM H_2O_2 whereas 1 mM H_2O_2 did not exert any effect.

5.3.2.3 Nucleus

After 48 h settling period, the nuclear part of lenses contained 14.2 ± 6.3 µmol GSH/g nucleus tissue, 0.25 ± 0.02 µmol GSSG/g nucleus tissue and a GSH/GSSG ratio of about 57. Exposure to H_2O_2 for 6 h did not change the levels of GSH compared with the controls (16.8 ± 1.6, 17.4 ± 1.4 and 19.0 ± 2. 8 µmol GSH/g nucleus tissue for 1, 2 and 4 mM H_2O_2 treated lenses, respectively). Similarly, the concentrations of GSSG were not different ($P >$ 0.05) from the controls (0.20 ± 0.05, 0.24 ± 0.08 and 0.30 ± 0.04 µmol GSSG/g nucleus at 1, 2 and 4 mM H_2O_2, respectively). Thus, the GSH and GSSG levels in the nucleus were not affected by H_2O_2 up to 4 mM (Table 5-2).

Table 5-2 The effect of incubation with different concentrations of H_2O_2 (1, 2, and 4 mM) for 6 h on GSH and GSSG concentrations and GSH/GSSG ratio in the nuclear region of the lenses.

Group	GSH	GSSG	GSH/GSSG Ratio
Control	14.2 ± 6.3	0.25 ± 0.02	56.8 ± 23.8
1 mM	16.8 ± 1.6	0.20 ± 0.05	89.5 ± 17.0
2 mM	17.4 ± 1.4	0.24 ± 0.08	79.1 ± 25.8
4 mM	19.0 ± 2. 8	0.30 ± 0.04	62.6 ± 6.35

Actual individual data of GSH and GSSG concentrations (µmol/g nucleus) are in Appendix F (3).

Actual individual data of GSH/GSSG ratio in nucleus is in Appendix F (4).

5.3.3 Pro-oxidant Assay (DCFH-DA) Result

The mean values for the fluorescence emitted from control and H_2O_2 treated lens were not different ($P >$ 0.05) and no clear trend for pro-oxidant capacity could be observed in either cortex or nucleus parts over 1-4 mM H_2O_2 range (Table 5-3).

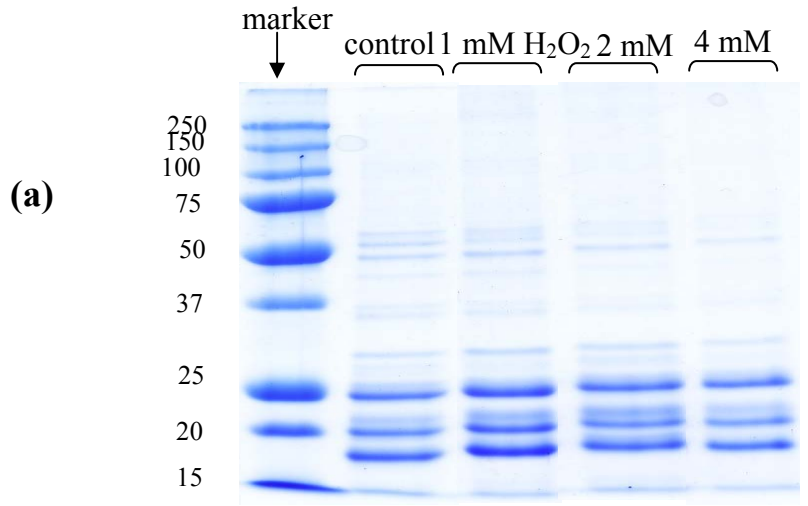
Table 5-3 The effect of incubation with different concentrations of H_2O_2 (1, 2, and 4 mM) for 6 h on the rate of change of DCF fluorescence (ΔDCF/s. g lens fraction; Mean ± SD) in cortical and nuclear regions of ovine lenses.

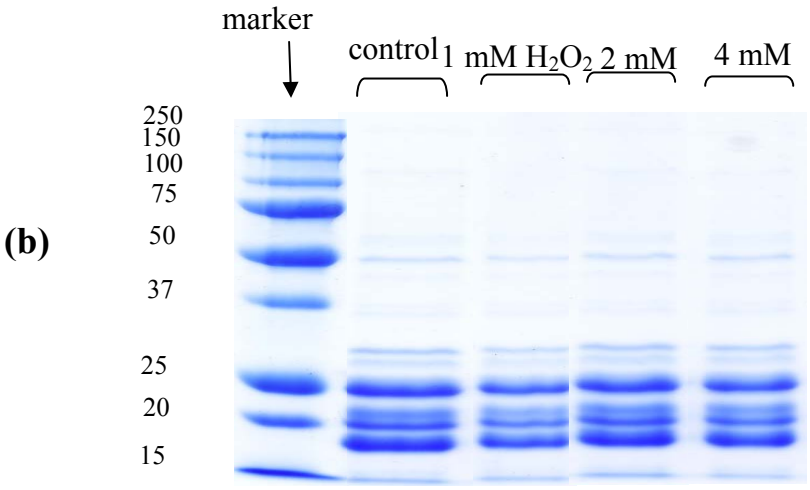
Group	Cortex	Nucleus
control	183.2 ± 42	1258 ± 1540
1 mM	179.2 ± 25.1	287.1 ± 89.5
2 mM	201.9 ± 77.7	177.6 ± 90.4
4 mM	145.0 ± 13.0	404.0 ± 564

Actual individual data of ΔFluorescence of DCF/s. g lens cortex or nucleus is in Appendix G.

5.3.4 SDS-PAGE Result

Figure 5.3 showed no apparent difference or consistent change in the crystallin proteins (size 17-38 KDa) after 6 h exposure to different concentrations of hydrogen peroxide treatment (1, 2 and 4 mM) for ovine epithelial (Figure 5.3a), cortical (Figure 5.3b) and nuclear fractions (Figure 5.3c).





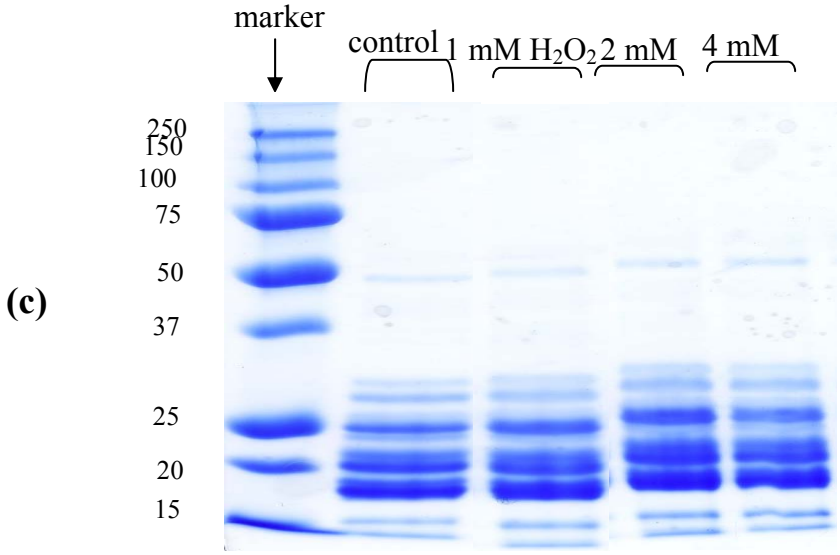


Figure 5.3 12% SDS PAGE of 15 µg soluble proteins extracted from ovine epithelium (a) , cortex (b) and nuclear (c) part of the lenses after cultured at different H_2O_2 level (1, 2, and 4 mM).

5.4 Discussion

The measurement of GSH/GSSG levels in the different fractions of ovine lens (epithelium, cortex and nucleus) showed that oxidative stress by H_2O_2 at 1 mM concentration was not sufficient to exhibit significant differences compared with controls. This probably explains the lack of significance in GSH/GSSG levels observed in lens treated with 1 mM H_2O_2 compared with control lens in chapter 4. However, since clear and distinct differences in the opacity levels were visible between the two groups (1 mM H_2O_2 treated and controls), the opacification of the lens in the current experiment seemed to be *initially* caused by other mechanisms rather than GSH/GSSG redox pathway.

In the epithelial cells, higher concentrations of H_2O_2 in ovine LOCH model (e.g., 2 and 4 mM) caused significant increase in GSSG levels (1.74 and 1.64 µmol GSSG/g epithelium for 2 and 4 mM H_2O_2, respectively) compared with control ($P < 0.05$) although no differences were found for GSH levels (Table 5-1). This might suggest that cellular oxidative stress require a threshold (e.g., 2 mM) and that the oxidation start from the epithelial cells.

Whereas, higher concentrations of H_2O_2 in ovine LOCH model (e.g., 2 and 4 mM) caused significant decrease in cortex GSH levels (16.7 and 14.6 µmol GSH/g cortex for 2 and 4 mM H_2O_2, respectively) compared with control ($P < 0.05$). An increase in the levels of oxidized glutathione corresponded to the decrease in the levels of reduced glutathione (0.53, 1.06 and 1.43 µmol GSSG/g cortex for control, 2 and 4 mM H_2O_2 after 6 h,

respectively; $P < 0.05$). A subsequent increase in opacity was observed at 2 mM H_2O_2 compared with 1 mM H_2O_2, which coincided with an increase in GSSG level. This might suggest a required threshold to observe any role for oxidative stress. However, this hypothesis was not justified at 4 mM H_2O_2, as the opacification level was not different from that at 1 mM H_2O_2 while a higher GSSG level was found in 4 mM H_2O_2. In fact, no differences in GSH levels at the three concentrations of H_2O_2 used in the present study with variable opacification in these treatments and increase in GSSG levels in 4 mM H_2O_2 compared with 1 mM H_2O_2 (Figure 5.2) without any differences in opacification supported our previous contention that GSH/GSSG redox pathway might **not** be the primary regulator for LOCH opacification in the present study.

At the nuclear level, GSH concentrations seemed to have numerically, but not significant, higher values within the increasing of H_2O_2 concentration (14.2, 16.8, 17.4 and 19.0 μmol GSH/g nucleus for control, 1, 2 and 4 mM H_2O_2 treatments after 6 h, respectively). There were no significant differences in GSSG levels, either. The above data on the GSH and GSSG in epithelium, cortex and nuclear fractions indicated that the oxidation was initiated from the epithelial cells to the cortex but not to the nucleus in LOCH model, which is consistent with the expected role of extra-lenticular oxidant such as H_2O_2.

Our results are in contrast to those reported by Truscott & Augusteyn (1977) and Truscott (2005) for human advanced nuclear cataractous lens. Truscott & Augusteyn (1977) reported an extensive oxidation of nuclear proteins (90% of the cysteine oxidized and 45% of the methionine present as the sulphoxide), while less damage was found in the cortical proteins during senile cataract formation. The authors concluded that the oxidation spreads from the nucleus to the cortex probably due to simple oxidation of the proteins with O_2 or H_2O_2. Similar contentions were provided by Truscott (2005), who reported that in age-related nuclear (ARN) cataract in human, a large decrease in nuclear concentrations of GSH can occur while the cortical levels of GSH remain well within, if not higher than, the expected normal range. These findings may call into question the postulated role of extra-lenticular oxidants, such as H_2O_2, in cataract development. It should be noted that experimental methods such as the LOCH system and age-related cataract (ARC) are different in the contact period and concentration of oxidant used. Different duration for H_2O_2-induced cataract (short-term) and ARC (long-term) may explain why different GSH responses occurred in cortex and nucleus fractions of lens for LOCH (present study) and ARC (published reports by Truscott & Augusteyn in 1977 as mentioned above). For

LOCH model, extra-ocular H_2O_2 firstly targets the outer cortex region by diffusion in short term (6 h), therefore, reduced glutathione decreased significantly in cortex rather than in the inner region of nucleus. The mechanism of cataract formation in ARC is a long-term accumulation of oxidative stress. As discussed in the literature review, the level of GSH in the nucleus of the lens is relatively low, particularly in the aging lens (Giblin, 2000), which reflects the different capacities of the two fractions (nucleus and cortex) to maintain GSH. With GSH being generated in the cortex, it will be much easier for the cortex region to recover from GSH depletion due to oxidative stress than the nuclear region. This is particularly important as the development of a barrier to the migration of GSH from its site of synthesis and regeneration in the cortex, into the nucleus in older normal lenses, may over time allow oxidative modification of proteins to take place in the nucleus, resulting ultimately in nuclear cataract (Sweeney & Truscott, 1998).This has been demonstrated in various experimental models (e.g. hyperbaric oxygen, UVA light and the glutathione peroxidase knockout mouse) where a relatively low ratio of GSH to protein -SH in the nucleus of the lens, combined with low activity of the glutathione redox cycle in this region, makes the nucleus GSH especially vulnerable to oxidative stress (Giblin, 2000). Cortex, due to its comparatively high level of GSH and high activity of glutathione synthesis and regeneration, is less prone to changes in the GSH levels during long-term oxidative stress. In another word, cortex could adjust to recover to the normal range of GSH by positive feedback in the long term, while, the nucleus is more vulnerable.

No differences were found in DCF fluorescence generated in H_2O_2-treated lenses and controls. The use of DCFH as a molecular probe to detect and monitor the level of cellular oxidative stress has been successful in several studies (Wang *et al.*, 2003; Chen *et al.*, 2004). However, these reports used a human lens epithelial cell line (HLE B3) that was loaded with DCFH-DA directly, unlike direct addition to homogenate as in the present study. This indicates that the method of Driver *et al*. (2000) that uses lysates/homogenates as a source of pro-oxidants is not suitable for the detection of pro-oxidant capacity in lenses. A release of peroxidase or cytochrome *c* during sonication or homogenization in the presence of H_2O_2 traces on the lens surface after the 6 h incubation period means a direct oxidation of DCFH could occur (Wrona & Wardman, 2006). This consequently could lead to fluorescence values not reflecting the variation in the pro-oxidant activity inside the lens. Thus, no conclusions could be made from these results except that the use of

homogenate/lysates is not suitable for lens pro-oxidant studies. Future work is recommended using lens epithelial cells as described by Chen *et al*. (2004).

Similarly, as in Chapter 4, SDS- PAGE results did not show apparent changes in the crystallin protein profile between control and H_2O_2-treated lenses (1, 2, and 4 mM) in three different fractions (epithelium, cortex and nucleus), which confirmed that calpain proteolysis of crystallins was not involved in the mechanism of change on the lens transparency induced by oxidative stress.

Several reports (Jedziniak *et al.*, 1972; Duncan *et al.*, 1988; Delamere *et al.*, 1993; Klonowski-Stumpe *et al.*, 1997; Ahuja *et al.*, 1999; Sanderson *et al.*, 1999) have highlighted different mechanisms that can lead to cataract formation with Ca^{2+} being the major catalyst for cataract formation such as inactivation of calcium pump and direct oxidation of the sulfhydryl groups of the Ca^{2+}-ATPase pump (Ahuja *et al.*, 1999); calcium binding to cytoplasmic proteins (Jedziniak *et al.*, 1972); activation of proteases or lipases (Ahuja *et al.*, 1999); apoptosis and necrosis (Herson *et al.*, 1999; Zhang *et al.*, 2003). It has been demonstrated that the use of H_2O_2 in LOCH system caused increase in the influx of Ca^{2+} in cultured lenses (Sanderson *et al.*, 1999) and led to loss in the cell viability as determined by LDH (chapter 4). Taken together, these factors along with the lack of major changes in GSH/GSSG redox or lens proteins in the present study may indicate that apoptosis may play a role in cataract formation.

5.5 Conclusions

Hydrogen peroxide decreased the amount of reduced glutathione in the lens cortex and increased the levels of oxidised glutathione in the epithelial and cortex fractions but only at levels of 2 mM and above. The nuclear levels of GSH remained well within, if not higher than, the normal range; even at H_2O_2 concentration as high as 4 mM. This suggested that the oxidation initiated by the diffusion of H_2O_2 from the lens surface to the epithelial cells then to the cortex but not to the nucleus in LOCH model during 6 h incubation time, which is consistent with the postulated role of extra-lenticular oxidant such as H_2O_2 *in vitro* culture system. However, *in vivo*, the lack of GSH transportation across barrier between cortex and nucleus/ or the ability of cortex to replenish itself by GSH seems to be the cause of ARN cataract. The use of lens homogenate/lysates for the measurement of oxidative stress is not viable and cultured cells should be used.

The use of LOCH system in the present study did not lead to increased proteolysis or changes in the GSH/GSSG redox system, whereas opacity occurred at 1 mM H_2O_2 in ovine lens suggesting that other mechanisms may be involved in LOCH system opacification.

Chapter 6
Overall Discussion and Future Work

When Cui & Lou (1993) cultured rat lens in 0.5 mM H_2O_2 for 24 h, they found that GSH progressively decreased with time of H_2O_2 exposure and 40% was lost by 24 h. However, the lenses had only patchy opacity at the equator after 24 h. Thus, in that experiment, rat lens showed extensive biochemical changes but very mild morphological damage. These results led Cui & Lou to extend the experiment to a long term period (for up to 96 h) H_2O_2 of exposure for the purpose of monitoring the dynamic changes in GSH level and the progression in lens opacity.

In the current experiment, exposing ovine lenses to1 mM H_2O_2 for 6 h caused substantial opacification, with no significant changes seen in either GSH or GSSG.

Thus, in rats, GSH had been extensively depleted without significant opacity; while in sheep, the lenses had become significantly opaque without significant GSH depletion.This led to the following conclusions:

Glutathione reduction capacity of rodent lens should be much less than that of ovine lens. For the rodent lens, probably because of its comparably poorer glutathione reduction capacity, GSH decreased significantly even when there was no opacity progress caused by oxidative stress. For the ovine lens, GSH levels were maintained even when there was significant opacity progress, which probably reflected a higher glutathione reduction capacity.

To confirm this, future work can be undertaken to measure the ratio of GPDH **:**GR (as an indicator of NADPH turnover with respect to glutathione reduction) in both rodent lens and ovine lens for testing the glutathione reduction capacity in different species.

In addition, these opposing results do not support GSH depletion as a direct mechanism for H_2O_2 cataract formation. Thus, the mechanism of opacification induced by H_2O_2 should not be attributed exclusively to GSH depletion, which is consistent with David & Shearer's conclusion (1984).

Based on earlier research (David & Shearer, 1984; Calvin *et al.*, 1986; Padgaonkar *et al.*, 1989), Truscott (2005) concluded that "a decrease in the levels of GSH is also a typical finding associated with nearly all experimental cataract". However, our findings do not

support GSH decrease as the mechanism for cataract formation and other mechanisms other than GSH/GSSG can be important in ovine LOCH model systems.

In supporting of our findings, David & Shearer (1984) measured reduced glutathione (GSH) and oxidised glutathione (GSSG) in the selenite cataract in rat *in vivo*. They found that while selenite caused a 44% decrease in lens GSH by 6 days post-injection, there was no concurrent increase in GSSG. To determine if GSH loss were the cause of the selenite cataracts, they injected normal rats with the specific glutathione synthesis inhibitor L-buthionine sulfoximine. Lens GSH dropped more than 96% by 4 days post- L-buthionine sulfoximine injection; however, no cataracts formed. Thus, selenite cataract cannot be attributed exclusively to GSH loss.

Similarly, Calvin *et al*. (1986) found that L-buthionine sulfoximine induces severe glutathione depletion and age-specific pathological changes when repeatedly administered to male suckling mice. Dense cataracts developed when mice aged 9 to 12 days were given a series of injections of L-buthionine sulfoximine, despite excellent survival and the absence of other significant long-term effects. By contrast, similar treatment of mice aged 14 to 17 days, although slightly less effective in reducing glutathione levels, resulted frequently in death, hind-leg paralysis, or impaired spermatogenesis, but did not produce cataracts. Furthermore, in adult mice, L-buthionine sulfoximine was relatively non-toxic. This suggests that for older mice, GSH depletion did not cause lens opacification, whereas for preweanling mice, cataract formation was more sensitive to the depletion of lens glutathione.

Moreover, Padgaonkar *et al*. (1989) treated cultured rabbit lenses with 100 atmospheres of 100% O_2 for 24 h and found that the level of reduced glutathione was depleted by greater than 95% in both lens cortex and nucleus. However, the lenses were hazy in appearance but not opaque. This also suggests that the GSH depletion not be the mechanism in the model of hyperbaric O_2-induced opacification for rabbit lens.

The above studies suggest that cataract patients have significant GSH depletion only associated with cataract formation, but does not support GSH as a cause of lens opacification.

If H_2O_2 caused the opacification without affecting the GSH levels, what caused the opacification? Duncan *et al*. (1988) found that modification of lens membrane channel proteins by diamide, which oxidize a sulphydryl (SH-) group on the surface of the channel,

led to an influx of Ca^{2+} (calcium may enter the lens through the activated non-specific cation channels) and Na^{+}, a loss of ionic homeostasis and loss of lens transparency. Hydrogen peroxide might cause the opacification in the same way of oxidation of lens membrane channel proteins thus leading to the changes in lens membrane permeability and transparency. Sanderson *et al*. (1999) confirmed it in their rat LOCH model: H_2O_2 (1 mM) induced a 235% increase in Na^{+} influx and a 58% increase in $^{45}Ca^{2+}$ influx. TRPM2, a member of the transient receptor potential (TRP) protein superfamily, has been reported by Zhang *et al*. (2003) as a widely expressed Ca^{2+} -permeable channel that was activated by micromolar levels of H_2O_2 and conferred susceptibility to cell death. Hence, oxidative stress, through the production of oxygen metabolites including H_2O_2, can cause an increase in the intracellular calcium concentration $[Ca^{2+}]_i$, which would result in cell injury, apoptosis and necrosis (Herson *et al.*, 1999). In addition, Klonowski-Stumpe *et al*. (1997) found that a rapid and sustained increase in $[Ca^{2+}]_i$ preceded all other morphological and functional alterations investigated.

Regulation of $[Ca^{2+}]_i$ is of critical importance in determination of cell fate. On one side, the loss of ionic homeostasis may cause the lens cell death (which has been confirmed in the current experiment –LDH leakage), thus leading to the loss of transparency. On the other side, linking Ca^{2+} rise to "protease hypothesis", the mechanism of H_2O_2-induced opacification may also involve the calcium activated neutral enzymes, calpains, which could induce proteolysis and truncate crystallins to precipitate and scatter light to form cataract. However, the current work did not show evidence of protein profile (crystallins) changes (i.e., SDS-PAGE). The theory of "phase separation phenomena" thus could explain the mechanism of lens opacification without affecting protein profile on the SDS-PAGE. This indicated that three hypotheses regarding the aetiology of cataract formation (oxidation, calpain protease, phase separation phenomena) were not working individually. There should be some inter-links among these three theories. For example, protein crosslinking required an oxidative environment and oxidative stress might result in proteasome activity.

The current research showed that resveratrol prevented oxidative damage induced by H_2O_2 (e.g., LDH leakage) whereas it did not prevent lens opacification. In contrast, quercetin showed prevention both on oxidative damage and on lens opacification. Resveratrol seemed much more susceptible to change to other forms in EMEM than quercetin, which might affect resveratrol's capacity in preventing lens opacification.

Although the present experiment had tested that quercetin and resveratrol almost had no inhibitory effect on calpain activity *in vitro*, Sanderson *et al*. (1999) found that quercetin significantly inhibited the H_2O_2-induced Ca^{2+} and Na^{+} influx by 80% and 45% respectively after 4 h with the protective role in the rat LOCH assay, and thus stopped the pathway to calpain activation. However, no differences in the protein profiles were detected in the trials carried out in the present work and this was probably resulted from the activity of the calpain system being the same in all treatments and controls.

In the future, experiments can be carried out on Lincoln cataract sheep to test whether the dietary antioxidant supplements (quercetin) will slow cataract formation in the ovine heritable cataract as quercetin has been successfully shown to slow down lens opacification in lens culture during the current work. Also, a comparison of pro-oxidant/antioxidant system in cataract and control sheep at Lincoln University could shed some light on the importance of this system in the development of cataract.

The data presented in this thesis using the LOCH assay showed that quercetin reduced lens opacification. Meanwhile, epidemiology study showed that a decreased risk of cataract was associated with a daily tea consumption of greater than 500 mL (Robertson *et al.*, 1991) and tea is a major source of quercetin (Hertog *et al.*, 1993). Therefore, these evidences suggest that dietary quercetin or resveratrol may reduce the risk of certain types of cataract. Since cataract is the major cause of blindness worldwide and cataractous lens removal is the most common surgical procedures performed in the elderly, optimization of dietary intake of protective nutrients could be an effective approach towards reducing the incidence of this disease.

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Appendices

Appendix A

		0 h	2 h	4 h	6 h	2 h-0 h	4 h-0 h	6 h-0 h
Control	C1	3.45	3.19	3.42	3.40	-0.26	-0.03	-0.05
	C2	3.90	3.82	3.95	4.11	-0.08	0.05	0.21
	C3	3.37	3.39	3.05	2.94	0.02	-0.32	-0.43
	C4	3.52	3.64	4.37	3.47	0.12	0.85	-0.05
	C5	3.65	3.50	3.37	3.48	-0.15	-0.28	-0.17
	C6	3.42	3.33	3.73	3.66	-0.09	0.31	0.24
	Mean ± SD	3.55± 0.20	3.48± 0.23	3.65± 0.47	3.51± 0.38	-0.07 ± 0.13	0.10± 0.44	-0.04 ± 0.25
H_2O_2	H1	3.36	5.96	7.64	9.23	2.60	4.28	5.87
	H2	3.89	6.22	7.59	8.83	2.33	3.70	4.94
	H3	3.59	5.77	7.97	9.41	2.18	4.38	5.82
	H4	3.33	7.90	8.69	8.96	4.57	5.36	5.63
	H5	3.67	5.84	7.93	9.41	2.17	4.26	5.74
	H6	5.22	8.03	8.75	9.20	2.81	3.53	3.98
	Mean ± SD	3.84± 0.71	6.62± 1.05	8.10± 0.51	9.17± 0.24	2.78± 0.91	4.25± 0.64	5.33± 0.74
Quercetin	Q1	3.50	7.95	8.28	8.79	4.45	4.78	5.29
	Q2	3.23	5.49	6.43	5.21	2.26	3.20	1.98
	Q3	3.83	5.70	7.37	7.94	1.87	3.54	4.11
	Q4	3.64	5.68	6.37	5.47	2.04	2.73	1.83
	Q5	3.47	6.11	7.22	7.20	2.64	3.75	3.73
	Q6	2.92	7.12	7.16	8.50	4.20	4.24	5.58
	Mean ± SD	3.43± 0.32	6.34± 0.98	7.14± 0.70 *	7.19± 1.53 *	2.91± 1.13	3.71± 0.73	3.75± 1.59
	R1	3.37	5.43	7.83	9.1	2.06	4.46	5.73
	R2	4.16	6.90	7.87	8.54	2.74	3.71	4.38

	R3	3.76	7.10	8.25	8.64	3.34	4.49	4.88
	R4	4.21	7.15	8.35	9.26	2.94	4.14	5.05
	R5	3.16	5.92	5.50	4.92	2.76	2.34	1.76
	R6	3.17	7.07	7.78	9.15	3.90	4.61	5.98
	Mean ± SD	3.64± 0.48	6.60± 0.73	7.60± 1.05	8.27± 1.67	2.96± 0.62	3.96± 0.86	4.63± 1.52

Effect of pre-treatment of lenses with quercetin (or resveratrol) on lens opacity in the first LOCH assay was shown by their opacification score at 0, 2, 4, 6 h individually. Lenses were treated with quercetin or resveratrol (30 μM) in EMEM for 24 h, washed with AAH alone and then incubated in AAH (with no quercetin or resveratrol) for 6 h with 1mM H_2O_2. The opacification was measured and graded by a digital image analysis system. * Indicates opacities that are significantly different from those of H_2O_2-treated lenses using the two-tailed Student's *t*-test ($P < .05$, n=6).

Appendix B

		0 h	2 h	4 h	6 h	6 h - 0 h
Q (n=5)	Q1	3.29	5.75	6.38	6.92	3.63
	Q3	3.91	4.18	7.32	7.76	3.85
	Q4	3.94	5.24	7.73	7.92	3.98
	Q5	5.66	6.65	8.03	7.8	2.14
	Q6	3.95	5.66	8.1	8.55	4.6
	Mean ± SD	4.15±0.89	5.50±0.90	7.51±0.70	7.79±0.58*	3.64±0.91**
Hq (n=5)	Hq1	3.54	5.1	7.32	8.92	5.38
	Hq3	3.92	5.49	7.46	8.93	5.01
	Hq4	3.4	6.2	7.71	8.98	5.58
	Hq5	3.76	5.68	7.78	8.18	4.42
	Hq6	3.92	6.07	8.66	9.13	5.21
	Mean ± SD	3.71±0.23	5.71±0.44	7.79±0.52	8.83±0.37	5.12± 0.44
R (n=5)	R2	4.89	6.51	7.78	8.49	3.6
	R3	4.6	6.4	7.42	8.01	3.41
	R4	4.54	5.53	6.9	7.61	3.07
	R5	4.08	5.89	8.17	8.96	4.88
	R6	5.27	7.17	7.82	8.64	3.37
	Mean ± SD	4.68±0.44*	6.30±0.63	7.62±0.48	8.34±0.53	3.67±0.70*
Hr (n=5)	Hr2	4.13	6.78	8.56	8.85	4.72
	Hr3	3.57	6.51	8.14	9.18	5.61
	Hr4	3.56	6.06	7.77	8.54	4.98
	Hr5	3.75	6.35	7.95	8.85	5.1
	Hr6	3.08	6.73	7.17	8.03	4.95
	Mean± SD	3.62±0.38	6.49±0.29	7.92±0.51	8.69±0.43	5.07±0.33
C (n=4)	C1	4.37	3.28	3.48	3.61	-0.76
	C3	2.6	3.14	2.96	3.14	0.54
	C4	3.39	3.61	3.74	3.81	0.42
	C6	3.2	3.24	3.08	3.11	-0.09
	Mean ± SD	3.39±0.73	3.32±0.20	3.32±0.36	3.42±0.35	0.03±0.59

Effect of pre-treatment of lenses with quercetin (or resveratrol) on lens opacity in the second paired LOCH assay was shown by their opacification score at 0, 2, 4, 6 h individually. Lenses were treated with quercetin or resveratrol (30 µM) in EMEM for 24 h, washed with AAH alone and then incubated in AAH (with no quercetin or resveratrol) for 6 h with 1mM H_2O_2. The opacification was measured and graded by a digital image analysis system. * Indicates opacities at time intervals (or opacity progressions over 6 h) that are significantly different from their paired lenses treated with H_2O_2 using the Student's paired *t*-test ($p < .05$, n=5). ** Indicates opacity progressions (over 6 h) that are of highly significant difference from those of H_2O_2-treated lenses using the Student's paired *t*-test ($p < .01$, n=5).

Appendix C

Group		GSH	GSH	GSSG	GSSG	
Q	Q1	6685.1	14.4	176.6	0.38	37.85
	Q2	7510.7	15.2	170.3	0.34	44.1
	Q3	7289.9	13.5	195.0	0.36	37.38
	Q4	6993.4	14.6	190.5	0.40	36.71
	Q5	7911.3	17.0	164.4	0.35	48.12
	Q6	6331.2	15.1	264.3	0.63	23.95
Hq	Hq1	7050.8	14.4	110.0	0.22	64.1
	Hq2	7747.9	15.3	195.9	0.39	39.55
	Hq3	8332.7	14.9	214.8	0.38	38.79
	Hq4	7363.3	14.4	208.3	0.41	35.35
	Hq5	10080.3	20.2	162.0	0.32	62.22
	Hq6	10052.5	20.3	166.6	0.34	60.34
R	R1	10003.4	21.1	140.9	0.30	71
	R2	6973.9	15.5	156.6	0.35	44.53
	R3	7533.2	15.7	180.9	0.38	41.64
	R4	8539.8	15.5	228.1	0.41	37.44
	R5	8606.7	17.6	231.1	0.47	37.24
	R6	7988.9	16.5	200.2	0.41	39.9
Hr	Hr1	8640.4	17.8	262.0	0.54	32.98
	Hr2	6789.9	14.8	184.4	0.40	36.82
	Hr3	8584.8	18.9	152.8	0.34	56.18
	Hr4	7913.7	15.5	141.2	0.28	56.05
	Hr5	8308.4	16.6	205.6	0.41	40.41
	Hr6	7951.6	15.9	310.5	0.62	25.61
C	C1	7390.5	14.5	177.1	0.35	41.73
	C2	7551.5	15.3	152.1	0.31	49.65
	C3	8686.6	17.9	172.7	0.36	50.3
	C4	6949.6	15.6	259.7	0.58	26.76
	C5	10147.2	21.1	242.4	0.51	41.86
	C6	6894.6	13.5	215.5	0.42	31.99

Appendix D

		LDH leakage (mU/100 µl)
Q (n=6)	Q1	15.16
	Q2	33.36
	Q3	13.92
	Q4	26.16
	Q5	44.22
	Q6	5.89
	Mean ± SD	23.1 ± 14.2
Hq (n=6)	Hq1	64.44
	Hq2	86.29
	Hq3	45.91
	Hq4	129.87
	Hq5	62.37
	Hq6	37.88
	Mean ± SD	71.1 ± 33.3
R (n=6)	R1	65
	R2	80.05
	R3	34.60
	R4	16.88
	R5	40.11
	R6	35.81
	Mean ± SD	45.4 ± 23.0
Hr (n=6)	Hr1	69.33
	Hr2	137.23
	Hr3	51.10
	Hr4	53.92
	Hr5	42.70
	Hr6	84.22
	Mean ± SD	73.1 ± 34.7
C (n=5)	C1	9.89
	C2	37.08
	C3	2.46
	C4	-3.83 (excluded)
	C5	38.69
	C6	33.54
	Mean ± SD	24.3 ± 16.9

Appendix E

		0 h	2 h	4 h	6 h	6 h-0 h
control	*1*	3.58	3.03	2.90	2.72	-0.86
	2	2.74	2.79	2.73	2.81	0.07
	3	3.09	2.85	3.12	2.55	-0.54
1mM H_2O_2	*1*	4.45	7.29	7.65	8.05	3.6
	2	3.72	7.20	8.07	8.62	4.9
	3	3.56	6.39	7.30	7.90	4.34
2mM H_2O_2	*1*	3.05	5.03	7.64	9.08	6.03
	2	3.07	6.13	7.40	9.16	6.09
	3	3.35	5.42	7.12	8.65	5.3
4mM H_2O_2	*1*	3.25	4.78	5.57	6.13	2.88
	2	3.39	5.08	5.93	8.58	5.19
	3	3.21	3.66	5.23	8.27	5.06

Effect of pre-treatment of lenses with different concentrations of H_2O_2 on lens opacity in the third LOCH experiment was shown by their opacification score at 0, 2, 4, 6 h individually. Lenses were treated with 1, 2 and 4 mM H_2O_2 for 6hrs after 48 h EMEM culture period. The opacification was measured and graded by a digital image analysis system.

Appendix F (1)

Sample	GSH (μmol/g epithelium)	GSSG (μmol/g epithelium)
C1	1.60	0.18
C2	3.98	0.25
C3	5.52	0.25
1mM (1)	0.66	0.28
1mM (2)	1.55	0.38
1mM (3)	6.51	0.61
2mM (1)	1.95	1.43
2mM (2)	1.51	1.79
2mM (3)	4.78	2.01
4mM (1)	8.55	1.67
4mM (2)	1.03	1.10
4mM (3)	8.52	2.15

Appendix F (2)

Sample	GSH (μmol/g cortex)	GSSG (μmol/g cortex)
C1	20.43	0.50
C2	23.01	0.54
C3	22.10	0.56
1 mM (1)	20.38	0.46
1 mM (2)	17.78	0.51
1 mM (3)	17.26	0.41
2 mM (1)	16.03	1.13
2 mM (2)	15.84	0.86
2 mM (3)	18.35	1.20
4 mM (1)	12.92	1.31
4 mM (2)	15.26	1.32
4 mM (3)	15.70	1.67

Appendix F (3)

Sample	GSH (µmol/g nucleus)	GSSG (µmol/g nucleus)
C1	8.52	0.23
C2	13.12	0.26
C3	20.88	0.25
1 mM (1)	18.14	0.22
1 mM (2)	17.22	0.23
1 mM (3)	15.07	0.14
2 mM (1)	16.11	0.15
2 mM (2)	18.91	0.31
2 mM (3)	17.13	0.25
4 mM (1)	21.21	0.30
4 mM (2)	19.91	0.34
4 mM (3)	15.87	0.27

Appendix F (4)

Sample	GSH/GSSG Ratio in epithelium	GSH/GSSG Ratio in cortex	GSH/GSSG Ratio in nucleus
C1	8.81	41.24	36.69
C2	15.63	42.55	50.57
C3	21.78	39.53	83.06
1 mM (1)	2.38	44.32	84.36
1 mM (2)	4.04	34.99	75.71
1 mM (3)	10.61	41.62	108.53
2 mM (1)	1.36	14.15	108.51
2 mM (2)	0.84	18.45	60.35
2 mM (3)	2.38	15.28	68.56
4 mM (1)	5.12	9.90	69.94
4 mM (2)	0.94	11.56	58.40
4 mM (3)	3.95	9.41	59.56

Appendix G

Sample	change of fluorescence /s. g cortex	change of fluorescence/s. g nucleus
C1		3028.92
C2	153.54	514.93
C3	212.91	229.63
1 mM (1)	198.91	240.80
1 mM (2)	150.96	230.16
1 mM (3)	187.80	390.24
2 mM (1)	173.08	281.59
2 mM (2)	142.67	118.30
2 mM (3)	289.83	132.83
4 mM (1)	155.17	1054.21
4 mM (2)	130.27	69.84
4 mM (3)	149.46	86.77

Appendix H

Bulk Calpain II Extraction (Adapted from Koohmaraie, 1990)

Trimmed 150 g Ovine lung tissue.

⇓

Homogenised lung tissue in 300 mL extraction buffer (100 mM Tris, pH 8.3; 10 mM EDTA, 0.05% B-MCE) using a Waring blender on low speed for 30 s intervals until smooth.

⇓

Homogenate was centrifuged (9500 rpm for 1 h at 4°C).

⇓

Supernatant was filter through damp cheesecloth.

⇓

pH was adjusted to 7.5 with 1 M Acetic acid.

⇓

Supernatant was diluted to 500 mL with distilled water until conductivity was approx 4 mS

⇓

Pump diluted supernatant onto 300x25 mm BioRad column containing approx 150 mL DEAE sepharose (fast flow) beads (Pharmacia, Uppsala, Sweden) and equilibrated in elution buffer (40 mM Tris, pH 7.4; 0.5 mM EDTA; 10 mM β-mercaptoethanol), at 5 mL/min using P-1 peristaltic pump (Pharmacia, Uppsala, Sweden).

⇓

Using an FPLC system (Pharmacia, Uppsala, Sweden) column was washed with 300 mL of elution buffer. NaCl gradient (in Elution buffer) started from 0 -125 mM over 400 mL, then 125 – 500 mM over 800 mL. Fractions were collected at a rate of 3 mL/min and assayed for m-calpain activity using the BODIPY-FL Casein assay.

Publications/presentations arising from this thesis

Conference poster presentations:

Lei, J., Morton, J. D., Bekhit, A. E. D., Lee, H. Y. Y. and Robertson, L. J. G. (2006). Quercetin and resveratrol protect cultured sheep lenses from oxidation. ComBio 2006, Brisbane, Australia.

Presentations:

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