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ULTRAVIOLET RADIATION CATARACT DEVELOPMENT AND ASCORBATE SUPPLEMENTATION

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Abstract

Background: Cataract is the major cause of blindness in the world, and long-term solar ultraviolet radiation (UVR) is a major risk factor. The pathogenesis of UVR-induced cataract is studied in various animal models and cell systems. The significance of oxidation processes in cataract and other eye diseases has made the study of protective antioxidants increasingly important. Ascorbate, or vitamin C, is an important dietary antioxidant and essential nutrient in the human and guinea pig, while the rat is capable of synthesizing ascorbate. Ascorbate has been implicated as a protectant in cataract development.

Purpose: The main purpose of this thesis was to establish two new animal models for the investigation of the effect of ascorbate supplementation in UVR cataract development.

Methods: *Paper I:* Albino rats were kept on chow (solid diet) supplemented with ascorbate for four weeks. The animals were then sacrificed, the lenses extracted and homogenized. Ascorbate and other low molecular weight compounds were isolated with ultrafiltration and ascorbate was quantified with subsequent high performance liquid chromatography (HPLC) with detection at 254 nm. *Paper II:* Rats were exposed to five doses of UVR 300 nm and the degree of cataract was quantified after one week by measurement of lens forward light scattering. The safety measure Maximal Tolerable Dose ($MTD_{2.3:16}$) for avoidance of UVR-induced cataract was calculated. Lens ascorbate concentration was measured using HPLC. *Paper III:* Pigmented guinea pigs received ascorbate supplementation via the drinking water for four weeks. Since guinea pigs cannot synthesize ascorbate, a low concentration of essential ascorbate was distributed through the chow to avoid illness in the zero supplementation group. Lens ascorbate was analyzed with HPLC. *Paper IV:* Ascorbate supplemented guinea pigs were exposed to 80 kJ/m^2 UVR 300 nm. One day later, the animals were sacrificed and lenses were extracted, cataract degree quantified and lens ascorbate analyzed.

Results: The ascorbate supplementation did not induce cataract or signs of illness in the animals. Rat lenses contain ascorbate, even without ascorbate in the food, while guinea pig lenses had very low ascorbate level. For both animal models, the lens ascorbate increased significantly, 19% for the rats and 39% for the guinea pigs. The $MTD_{2.3:16}$ in the albino rat was 3.01 kJ/m^2. UVR exposure leads to a significant consumption of ascorbate in the exposed lenses, but also a small decrease in the the non-exposed lenses. UVR-exposed albino rat lenses exhibited mainly cortical cataract while UVR-exposed pigmented guinea pig lenses developed superficial anterior cataract.

Conclusions: The lens sample preparation and subsequent ascorbate analysis by HPLC is easy and feasible for animal lenses. Ascorbate supplementation via food or drinking water both increases lens ascorbate levels. The $MTD_{2.3:16}$ for albino rat is similar to that in the pigmented mouse, rat and rabbit, while pigmented guinea pig has several fold higher tolerance to UVR. UVR exposure leads to a consumption of lens ascorbate in rats and guinea pigs. Ascorbate supplementation does not protect guinea pig lenses against UVR cataract development.

Publications included in the thesis

I. Mody Jr. VC, Kakar M, Elfving Å, Söderberg PG, Löfgren S. Ascorbate In The Rat Lens, Dependence On Dietary Intake. *Ophthalmic Research* 2005; 37:142-149. Published by Karger Publishers.

II. Mody Jr. VC, Kakar M, Elfving Å, Söderberg PG, Löfgren S. Ultraviolet Radiation-B-Induced Cataract In Albino Rats: Maximum Tolerable Dose and Ascorbate Consumption. *Acta Ophthalmologica Scandinavica* 2006;84:390-395. Published by Blackwell Publishing.

III. Mody Jr. VC, Kakar M, Elfving Å, Söderberg PG, Löfgren S. Ascorbate In The Guinea Pig Lens, Dependence On Drinking Water Supplementation. *Acta Ophthalmologica Scandinavica* 2005; 83:228-233. Published by Blackwell Publishing.

IV. Mody Jr. VC, Kakar M, Elfving Å, Löfgren S. Drinking water supplementation with ascorbate is not protective against UVR-B-induced cataract in the guinea pig. *Acta Ophthalmologica Scandinavica* 2008; 86:188-195. Published by Blackwell Publishing.

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To My Parents Vino and Anita and Brother Beijoo

1. INTRODUCTION

1.1 Ascorbate definition, requirement and ocular distribution

Ascorbic acid, or vitamin C (Figure 1)

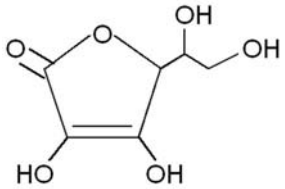


Figure 1 Ascorbic acid

has two ionizable –OH groups with pK_{a1}=4.25 and pK_{a2}=11.8. Ascorbate is the favored form at physiological pH (Figure 2) (Halliwell and Gutteridge; 1999).

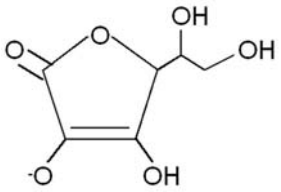


Figure 2 Ascorbate at physiological pH

Therefore, the name ascorbate is used throughout.

Ascorbate is an essential nutrient in the human and guinea pig. The rat, however, is capable of synthesizing ascorbate. Long found the ascorbate concentration in the lens to be much higher in diurnal than in nocturnal animals and speculated that this may be due to a protective effect of ascorbate against eye damage (Long; 1961). In the rat, the lens ascorbate concentration was found to be very low, equal to 0.08 mmol/kg lens wet weight (Varma; 1991). In the guinea pig, the lens ascorbate concentration was found to be on the same order as that in the human, equal to 0.65 mmol/kg lens wet weight (Varma; 1991). A schematic of ocular structures is shown (Figure 3).

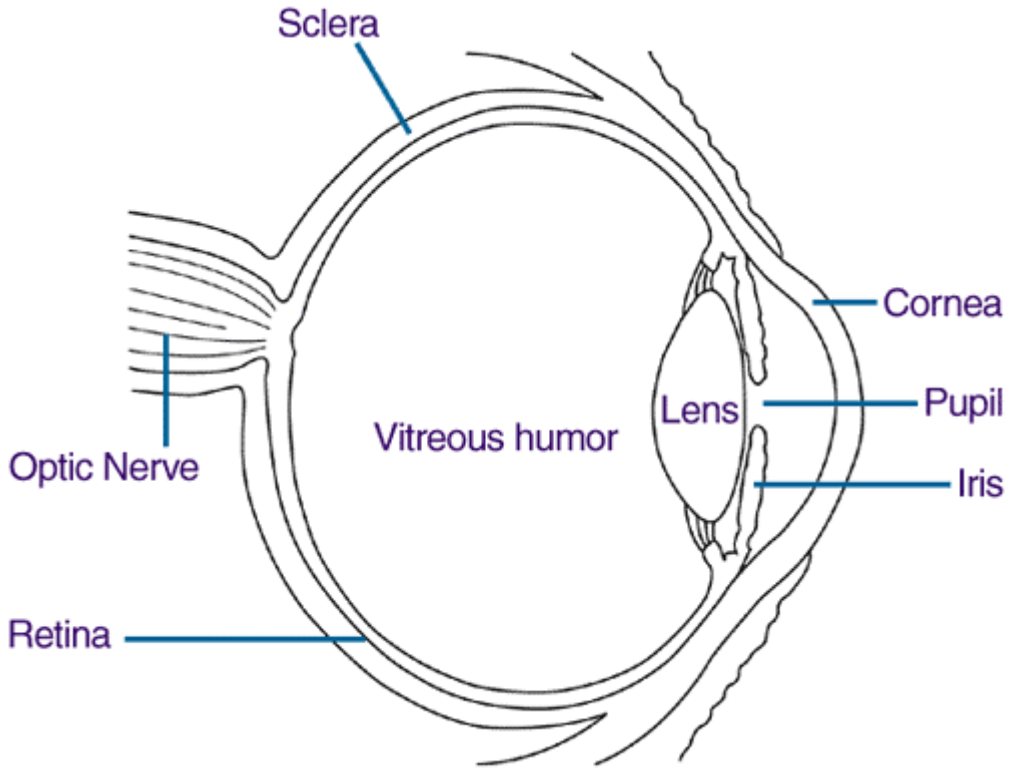


Figure 3 Schematic of ocular structures. The lens is located behind the iris and in front of the vitreous.

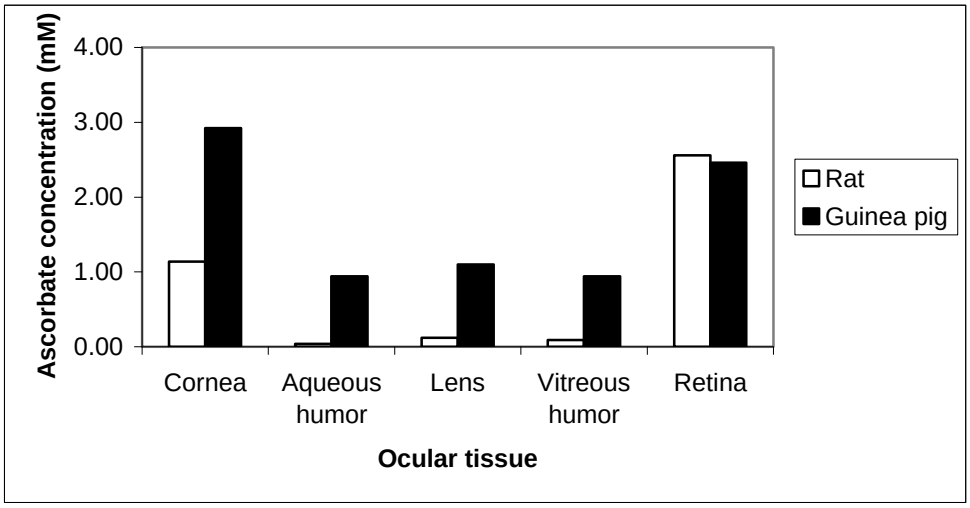


Figure 4 Ascorbate concentration in ocular tissues of the rat and guinea pig

Ascorbate content in the ocular tissues of rat and guinea pig reported in the literature is plotted (Berger, Shepard, et al.; 1989; DiMattio; 1989; Heath, Beck and Rutter; 1961; Reddy, Giblin, et al.; 1998; Ringvold, Anderssen and Kjonniksen; 1998; Ringvold; 1980; Taylor, Jacques, et al.; 1997; Varma; 1991) (Figure 4).

1.2 Ascorbate functions

Ascorbate is of particular interest because of its role as a redox regulator. Ascorbate may serve as an antioxidant or pro-oxidant in living tissues depending on redox status. By functioning as an antioxidant, ascorbate is capable of preventing oxidative damage to protein, lipid, and DNA in a number of tissues including the lens (Reddy and Bhat; 1999). Besides functioning as an antioxidant in the lens, ascorbate in the aqueous humor attenuates UVR by absorption and conversion of the UVR energy into heat or fluorescence, thereby reducing UVR damage to the lens (Ringvold; 1995).

Ascorbate interacts readily with oxygen, giving rise to oxidation reactions in protein, lipid, and DNA in cells. In the presence of endogenous transition metals and high ascorbate concentration, oxidation of ascorbate occurred in hepatocytes, resulting in glyoxal/advanced glycation endproduct (AGE) formation and cytotoxicity (Shangari, Chan, et al.; 2007). In addition, ascorbate serves as a pro-oxidant in chromium-exposed lung cells (Martin, Schoenhard, et al.; 2006). Chromium is a transition metal which plays a role in the Fenton reaction. Animal cells are protected from ascorbate oxidation by a number of mechanisms. Extracellular dehydroascorbate reduction and ascorbate recycling by plasma membrane redox system is described in erythrocytes (Rizvi, Jha and Maurya; 2006). Intracellular reductants such as NAD, NADP, and glutathione serve as ascorbate protectors (Noctor; 2006).

Supraphysiological ascorbate levels may facilitate the restoration of vascular function in the critically ill patient by protecting the vascular endothelium (McGregor and Biesalski; 2006). In the guinea pig heart, ascorbate supplementation was found to protect against endotoxin-induced oxidative stress. The endotoxin-induced oxidative stress in sepsis depleted the heart ascorbate, but ascorbate supplementation prevented an increase in myocardial uric acid, a marker of ischemia-induced oxidative stress (Rojas, Cadenas, et al.; 1996). In a liver model, dietary ascorbate supplementation protected against endotoxin-induced oxidative injury to the proteins in the guinea pig liver (Cadenas, Rojas and Barja; 1998).

In vitro and in vivo, ascorbate removed key precursors to oxidative damage by cell-free haemoglobin. The multifunctional role of plasma ascorbate is that of increasing redox defenses (Dunne, Caron, et al.; 2006). Thus, oxidative damage causes ascorbate depletion, and increased ascorbate supplementation prevents oxidative damage in plasma, living animal tissues, and human tissues.

1.3 Ocular protection by ascorbate

In vitro studies have demonstrated the protective effect of ascorbate in the rat and guinea pig ocular lens. Ascorbate conferred in vitro protection against UVR-B inactivation of rat lens enzymes, including the glycolytic pathway enzyme hexokinase, the pentose phosphate shunt enzyme glucose-6-phosphate dehydrogenase, and the cation pump Na/K ATPase (Reddy and Bhat; 1999; Tung, Chylack and Andley; 1988). Large quantities of dietary ascorbate in the guinea pig protect lens proteins against heat-induced damage (Tsao, Xu and Young; 1990). Recently, ascorbate was shown to protect against cataract in aldose-reductase deficient mice lenses in vitro (Hegde and Varma; 2004) in the xanthine oxidase model. Physiological levels of combined antioxidants including ascorbate have been found to increase viability of UVR exposed cultured human lens epithelial cells and maintain transparency of rat lenses exposed to UVR in vitro (Sasaki, Hata, et al.; 2000).

To date, very few *in vivo* studies describe the role of ascorbate in preventing cataract in the rat or guinea pig. Reddy (1998) has shown in vivo that acutely increasing ascorbate concentration in the aqueous humor and lens of the rat through intraperitoneal injection confers protection against UVR-B-induced DNA strand breaks in the lens epithelium (Reddy, Giblin, et al.; 1998). The same study showed that ascorbate-deficient guinea pigs

developed UVR-B-induced DNA strand breaks in the lens epithelium in contrast to controls. In vivo, ascorbate has been found to protect the lens against oxidative cataract induced by selenite in rats (Devamanoharan, Henein, et al.; 1991). Another in vivo study looked at the effect of vitamin C deficiency and UVR, on guinea pigs, examining the degree of cataract (Malik, Kojima and Sasaki; 1995). While this study did show that scorbutic guinea pigs developed cataracts, it examined the effect of ascorbate deficiency rather than the effect of ascorbate supplementation.

1.4 Ascorbate and human cataract

There is some evidence that oral intake of ascorbate protects against cataract formation in the human lens (Jacques, Taylor, et al.; 1997) (Leske, Chylac and Suh-Yuh; 1991) (Robertson, Donner and Trevithick; 1991). Further, lenses with increasing degree of cataract and browning secondary to protein oxidation were associated with lower ascorbate content (Tessier, Moreaux, et al.; 1998).

1.5 Ascorbate pharmacodynamics

The pharmacodynamics of ascorbate in the lens is important in order to understand the physiological mechanism of protection against eye damage afforded by ascorbate. In diurnal animals such as the guinea pig, the concentration of ascorbate in the aqueous humor is higher than in the plasma and further higher in the ocular lens than in the aqueous humor (Garland; 1991). The concentration gradient is due primarily to active transport. Recently, the sodium-dependent vitamin C transporter (SVCT 2) has been identified in the human lens epithelial cell line HLE-B3 (Kannan, Stolz, et al.; 2001). The transporter may also exist in the guinea pig lens, although it has not yet been found. In the rat, ascorbate enters the aqueous humor from the plasma and the lens from the aqueous humor primarily by passive diffusion (Garland; 1991).

Ascorbate is rapidly metabolized in both the rat and guinea pig, starting with oxidation to dehydroascorbate (Committee on Animal Nutrition; 1987). This compound is delactonized to diketogulonate and a number of other compounds, which are mainly excreted in the urine.

There are important differences between rat and diurnal species such as the human and the guinea pig; the rat can synthesize ascorbate, the rat has a 10-fold lower baseline concentration of ascorbate, and in the rat ascorbate is believed to enter the lens by passive diffusion (Table 1).

Table 1 Species ascorbate differences

Variables	Species Dark living animals E.g. Rat	 Diurnal animals E.g. Human and guinea pig
Ascorbate synthesis	Yes	No
Baseline concentration (mmol/kg wet weight lens	0.08	0.65
Aqueous – lens transport mechanism	Passive diffusion	Active transport

An equilibrium exists between ascorbate free in solution and ascorbate bound to proteins (Equation 1).

Equation 1

$$[P^+] + [Asc^-] \leftrightarrows [P\text{-}Asc]$$

where $[P^+]$ is concentration of free protein, $[Asc^-]$ is concentration of free ascorbate, [P-Asc] is ascorbate bound to protein. The equilibrium is defined in Equation 2.

Equation 2

$$k = \frac{[P - Asc]}{[P^+][Asc^-]}$$

Here, k is the equilibrium coefficient.

1.6 Ascorbate measurement methods

Biochemists were able to measure ascorbate in lens extracts more than sixty years ago utilizing a color titration method with 2,6-dichlorophenolindophenol (Duke-Elder; 1968). However, the method was not specific for ascorbate since it also assayed for glutathione. In 1979 Omaye et al. determined ascorbate concentration in animal tissues using a spectrophotometric assay with 2,6-dichlorophenolindophenol (Omaye, Turnbull and Sanberlica; 1979). The spectrophotometric assay is more specific for ascorbate than the color titration method. The technique has been applied to measure ascorbate in the rat lens (Reddy, Giblin, et al.; 1998).

Within the last fifteen years, a method of measuring intraocular ascorbate utilizing HPLC with either electrochemical (Blondin, Baragi, et al.; 1986; Taylor, Jahngen-Hodge, et al.; 1995) or UVR (Hallström, Carlsson, et al.; 1989) detection has been described. HPLC provides the advantage over previous methods of better sensitivity and specificity since molecules of interest can be separated from background molecules based on several molecular aspects such as size, solubility and electrical charge.

HPLC with UVR detection is a simple and efficient method for measuring ascorbate in biological samples. UVR detection is possible because of expressed absorption of UVR by ascorbate. The high molar extinction coefficient for ascorbate at 265 nm (neutral pH) or at 256 nm (acidic pH) allows for quantification of the ascorbic acid peak as it elutes from an HPLC column. The method of HPLC with UVR detection has been used for over a decade to analyze ascorbate from a number of tissues and plasma (Washko, Welch, et al.; 1992). HPLC with electrochemical detection has been applied to measure ascorbate in the lens tissue from Emory mice (Taylor, Jahngen-Hodge, et al.; 1995), guinea pigs, and humans (Taylor, Jacques, et al.; 1997).

Electrochemical detection must overcome two hurdles. First, the mobile phase must allow for separation of ascorbate from background molecules and be capable of carrying the electric charge. Second, it is difficult to maintain a constant, electrochemically reactive electrode surface (Washko, Welch, et al.; 1992). UVR detection may provide better specificity for a complex molecule since, when combined with ion-exchange chromatography, HPLC separates based on size, charge, and UVR absorption. Electrochemical detection separates based on only size and charge.

1.7 Cataract

Cataract is defined as opacity of the lens causing visual disturbance, and cataract in humans is primarily age-related. There is no single primary cause of cataract. A number of of secondary causes have been defined, including genetic factors, diabetes, steroids, smoking, UV radiation, ionizing radiation, and trauma. The pathophysiological mechanism of each cause is not entirely known. The metabolism of the lens which affects water ion balance and

protein and cellular organization is responsible for the maintenance of lens transparency. A change in water balance which is manifest by a change in lens wet weight may result in loss of lens transparency. Increased oxidation which changes lens structure secondary to damage to protein, DNA, and lipid may also result in cataract formation. However, it has been shown that a disruption in normal ion balance between Na and K secondary to cellular pump damage leads to lens cell swelling and acute cortical cataract. Other causes of acute experimental cataract in vitro include damage to the cellular lipid bilayer, membrane proteins, enzymes, DNA, intracellular calcium release, decreased ATP production, and necrosis and apoptosis of epithelial and differentiating lens fiber cells. Glycolytic enzymes and Na/K ATPases in the lens are targets of UVR-B, and therefore vulnerable to damage.

Antioxidant systems in the lens, including glutathione, vitamin C, and vitamin E, serve to protect against oxidation. However, the antioxidant systems available in the lens may become overloaded when stressed acutely, resulting in cataract.

1.7.1 Ultraviolet radiation-B and cataract development

Ultraviolet radiation consists of wavelengths 100-280 nm [UVR-C], 280-315 nm [UVR-B], and 315-400 nm [UVR-A]. Visible light is in the range 400-760 nm. Sunlight is responsible for most UVR exposure to humans. The ozone layer in the atmosphere partially blocks sun radiation in the UVR-A and UVR-B wavebands, and a decrease in ozone has occurred over the last twenty years. Decreased ozone results in greater UVR exposure and cataract development. The burden and cost to society is increased visual disability and subsequent increase in necessary cataract operations. To facilitate for the public to take precautions during high ambient solar UVR levels, a UV index has been created. Information about ambient solar UVR levels is important for skin protection measures, while cataract is more closely related to ocular UVR-B fluency rather than ambient levels.

The cornea absorbs most of incident UVR, with increased transmittance with longer wavelength. A specimen of middle age human cornea transmitted 0% of UVR at 290 nm, 10% at 300 nm, and 63% at 380 nm (Dillon, Zheng, et al.; 1999). Increasing transmittance is seen with decreased corneal thickness, when comparing human (10%), rabbit (13%), rat (32%), and mouse (37%) samples at 300 nm (Dillon, Zheng, et al.; 1999). Although only a fraction of solar UVR-B reaches the lens, there is still enough damaging energy to cause cataract. The rat has a maximum sensitivity of the lens to UVR in vivo at around 300 nm (Merriam, Löfgren, et al.; 2000; Pitts, Cullen and Hacker; 1977). The lens transmittance of UVR-A is higher than for UVR-B and this combined with absorption of UVR-A by endogenous chromophores are hypothetized to be the main cause of age-related nuclear cataract in humans (Gaillard; 2000; Taylor, Aquilina, et al.; 2002; Truscott; 2000).

Epidemiological data supports an association between exposure to UVR-B and development of cortical cataract (McCarty and Taylor; 2002). Experimental studies on animals, including rats, mice, and rabbits, link UVR-B exposure to development of and cortical cataract (Cruickshanks, Klein and Klein; 1992; Delcourt, Carrière, et al.; 2000; Hightower and McCready; 1993; Jose and Pitts; 1985; McCarty and Taylor; 2002; Pitts, Cullen and Hacker; 1977; Taylor; 1990; Wegener; 1994).

Experimentally, our group has shown that acute exposure to UVR-B induces both nuclear and cortical cataract in the rat in vitro and in vivo (Dong, Ayala, et al.; 2003; Löfgren, Michael and Soderberg; 2003; Söderberg, Löfgren, et al.; 2002; Söderberg; 2003), (Dong, Ayala, et al.; 2003; Söderberg; 1990a). Acute development of cataract after in vivo exposure to UVR-B (Söderberg; 1988) is secondary to a sodium potassium shift resulting in lens swelling (Söderberg; 1991).

1.7.2 Photochemistry and Photobiology

Photobiology is the study of how electromagnetic radiation interacts with living matter (Pitts, Cameron and Jose; 1986). When radiant energy causes chemical changes, a photochemical reaction has occurred. Indirect or direct reactions results in cellular damage in vivo. In the direct phototoxic reaction, absorbed photon energy produces a toxic molecule. In the indirect photosensitized reaction, absorbed photon energy excites a primary energy-transmitting molecule, a photosensitizer, which transfers energy to a second molecule, which becomes toxic.

The laws of photochemistry describe photochemical reactions. The first law of photochemistry, Grottus Draper's law, states that only absorbed light can cause photochemical effects. The second law, Stark-Einstein law, has two parts. The first states that each absorbed photon activates one molecule in the primary photochemical reaction step. The second law, Bunsen-Roscoe law, law of reciprocity, holds that time (t) and irradiance (E) are reciprocal for the dose or number of photons received (H), as defined by the following equation:

$$H\ (J/m^2) = E\ (W/m^2) * t\ (seconds)$$

The photochemical effect has been thought to be independent of time and irradiance when the product (total UVR dose) is constant (Encyclopaedia; 2000; Söderberg; 1990a). However, our group has shown that the photochemical reciprocity law does not apply for UVR-B-induced cataract in the albino rat when the UVR-B exposure time is less than sixty minutes (Ayala, Michael and Söderberg; 2000).

The photobiological effect is determined by complex biological mechanisms including defense mechanisms and repair mechanisms. Antioxidants including glutathione, ascorbate, vitamin E, and enzymes serve as antioxidant defense mechanisms. Biological repair mechanisms which become active secondary to UVR-B exposure may cause the second law of reciprocity to not hold true in the animal model.

1.7.3 Dose-response function, threshold dose

A dose-response function represents the relationship between the dose of an agent and the response as a result of the agent. The response is here the difference in forward light scattering between UVR-B-exposed and non-exposed lenses, a measure of degree of cataract.

It is imperative to express a statistically significant response. If the dose-response function is binary, the response is defined only by "event" or "no event" with no gradation. If the dose response function is continuous, the significant response must be defined based on data analysis.

The threshold dose refers to the dose of an agent, in our studies UVR-B, above which significant response occurs and below which significant response does not occur (Finney; 1971).

1.7.4 Maximum Tolerable Dose (MTD), a safety limit measure

Current safety standards for avoidance of UVR-B-induced cataract are based on toxicity estimation, which in turn is based on the assumption that the occurrence of cataract secondary to UVR-B exposure is a binary response event (Pitts, Cullen and Hacker; 1977).

However, our group has shown that UVR-induced lens light scattering follows a continuous dose-response function (Michael, Söderberg and Chen; 1998) in albino rats.

Hence, the concept of Maximum Tolerable Dose (MTD) was recently developed as an index of toxicity for continuous dose-response functions, in this particular case increase in lens light scattering after UVR exposure. The MTD concept provides a statistically well-defined estimate of threshold dose for avoidance of toxicity (Söderberg, Löfgren, et al.; 2002). We have previously reported the MTD for the six-week-old albino rat to be 3.65 kJ/m^2 (Söderberg, Löfgren, et al.; 2002) and for the six-week-old pigmented rat to be 4.20 kJ/m^2 (Kakar et al., 2003 [ARVO Abstract]). Pitts et al. (Pitts, Cullen and Hacker; 1977) determined the threshold for development of permanent lenticular opacity in the pigmented rabbit to be 5.0 kJ/m^2. We have found the pigmented guinea pig to be resistant to developing cataract because UVR-B doses ranging from 5.0 to 20.0 kJ/m^2 were not sufficient to induce cataract formation (data unpublished). The pigmented guinea pig is adapted to high UVR exposure being a diurnal animal and, as in the case of the human, the pigmented guinea pig (Mody et al., 2004 [ARVO Abstract]) is much less sensitive to UVR exposure compared to other species including the pigmented rat (Kakar et al., 2003 [ARVO Abstract]) and pigmented mouse (Meyer et al., 2005 [ARVO Abstract]) (Table 2).

Table 2 Species and UVR-B sensitivity expressed as MTD, Maximum Tolerable Dose

Species	MTD (kJ/m^2)
Pigmented guinea pig	69.0
Pigmented rat	4.2
Pigmented mouse	3.2

1.7.5 Cataract diagnosis, treatment, and costs

Upon presentation to an optometrist or ophthalmologist, a disabling decrease in visual acuity is often the first presenting sign for cataract. Loss of accurate color visual perception and glaring lights are among the earliest signs of cataract. Light absorption and scattering by a cataractous lens disturb vision. According to the WHO, cataract is the leading cause of blindness in the world.

Cataracts may be classified by location, predominantly cortical, nuclear, and posterior subcapsular. Age-related cataract, the most common cataract type, is primary nuclear in location. Slit-lamp examination is the most common method for cataract diagnosis, and Scheimpflug photography serves as a quantitative measurement method for cataract. A number of slit-lamp examination grading systems for cataract exist, including Lens Opacities Classification System (LOCS), Wilmer System, Oxford System, Cooperative Cataract Epidemiology Study Group system (CCESG), and World Health Organization (WHO) System.

There is no proven prophylactic measure or medical treatment for cataract. Avoidance of risk factors including smoking and sunlight should decrease the incidence of cataract. Since there are no medical treatments for cataract, the only method for treatment is surgery, which is a cost-effective intervention. The earliest cataract surgery was simple couching where the lens was depressed downwards in the eye, out of the visual axis. These days vision is restored after phacoemulsification operation in which the lens is dispersed and removed with an ultrasound device, followed by implantation of an artificial lens. In developing countries where ultrasound devices are scarce, whole lens extraction is common. A large

number of people need cataract surgery, with the result being a burden on the health care system. In 2007, more than 70,000 cataract operations were performed in the Swedish 9 million population, for about 1 000 Euros per operation. Cataract surgery has been calculated to be socioeconomically cost-effective in all regions of the world, but the lack of physicians and instruments make cataract surgery a luxury in many countries.

ICNIRP, International Commission on Non-Ionizing Radiation Protection, has derived UVR exposure limits based on wavelengths (IRPA, 1996). Because prophylactic measures and medical treatment for cataract will eventually be discovered, the public gain secondary to UVR cataract research is significant. A delay in cataract onset by ten years would decrease cataract prevalence by 50% (Javitt and Taylor; 1994).

2. AIMS OF THE STUDY

The aims of the study, using the paper numbering, were to:

I. Investigate whether lens ascorbate concentration is dependent on solid dietary ascorbate intake in the albino rat.

II. Determine Maximum Tolerable Dose (MTD) for avoidance of UVR-B cataract in the albino rat, and to investigate whether UVR-B alters lens ascorbate levels.

III. Investigate whether lens ascorbate concentration may be elevated with drinking water supplementation in the guinea pig.

IV. Investigate protective properties of ascorbate supplementation in UVR-B cataract development in the guinea pig.

3. METHODS

Ethical approval was obtained from the Northern Stockholm Animal Experiments Ethics Committee. The animals were kept and treated according to the *ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.*

3.1 Method overview, animals and sample size

In paper I, 12 three-week-old female albino Sprague Dawley rats (B&K Universal AB, Sweden) were supplemented with ascorbate. The age was chosen so that the rats would be seven weeks after four weeks of supplementation. This was selected since the group has extensive experience of the effect of UVR in rats aged around 6 weeks and since UVR exposure experiments were planned as a continuation of this experiment. Lens ascorbate was analyzed with HPLC.

In paper II, 30 seven-week-old female albino Sprague-Dawley rats were exposed to UVR. Lens ascorbate was analyzed with HPLC.

In paper III, 24 five-to-eleven-week-old pigmented guinea pigs (HB Lidköpings Kaninfarm, Lidköping, Sweden) were supplemented with ascorbate. Guinea pigs were chosen because their ascorbate metabolism and ascorbate lens homeostasis resembles that of humans (Table 1). The wide age interval was due to limitations related to the supplier. Lens ascorbate was analyzed with HPLC.

In paper IV, 60 six-to-nine-week-old pigmented guinea pigs were supplemented with ascorbate. Forty of the animals were exposed to UVR, while 20 were kept as controls. Lens ascorbate was analyzed with HPLC.

3.2 Ascorbate analysis

3.2.1 Ascorbate supplementation

Ascorbate quickly oxidizes in aqueous solution (Samocha-Bonet, Lichtenberg and Pinchuk; 2005). Therefore, we initially decided to use ascorbate supplementation to the chow. In paper I, four groups of three rats each were fed ascorbate-free chow (R 36 bas; Analycen, Sweden) supplemented with L-ascorbate, either 0.00, 5.70, 57.0, or 114 mmol/kg for a duration of four weeks. These concentrations were chosen based on a previous study of the effect of megadose ascorbate supplementation on liver damage prevention in rodents, in which the dose used was higher than the highest dose used in the current study (Cadenas, Barja, et al.; 1997). The doses were chosen to maintain a non-toxic concentration. Four weeks of supplementation was chosen to achieve a steady state lens ascorbate concentration.

In paper III, four groups of six guinea pigs were supplemented with 0.00, 2.84, 5.68, or 8.52 mM ascorbate in the drinking water for a duration of four weeks. To minimize auto-oxidation of ascorbate in the drinking water bottle, each bottle was covered in a black plastic bag and was changed with freshly prepared solution twice daily. In addition, the chow fed to all animals contained a low ascorbate concentration (0.125 mol L-ascorbate/kg chow). This was because ascorbate is an essential compound for the guinea pig and necessary for the zero supplementation group. We used high amounts of ascorbate supplementation, up to ten-fold the amount used by Taylor et al. in a study on vitamin C in guinea pig eye tissues in relation to intake (Taylor, Jacques, et al.; 1997). The reason for not choosing to feed guinea pigs ascorbate-deficient diet was that ascorbate deprivation for two to three weeks in two studies caused guinea pigs to become ill and lose weight (Malik, Kojima and Sasaki; 1995; Reddy, Giblin, et al.; 1998). The theory was instead to see whether we could increase lens ascorbate levels through drinking water supplementation.

In paper IV, guinea pigs were again supplemented with ascorbate as described for paper III above. After four weeks of supplementation the animals were exposed to UVR-B.

The rats were sacrificed with carbon dioxide asphyxiation while pentobarbital overdose were used for the guinea pigs.

3.2.2 Lens sample preparation

Each lens was extracted, photographed, wet weight measured, homogenized in 1.0 ml of 0.25% metaphosphoric acid, and centrifuged. Besides protein denaturation, metaphosphoric acid was used to chelate metals, thus preventing metal catalyzed oxidation of ascorbate. (Koshiishi, Mamura, et al.; 1998; Washko, Welch, et al.; 1992). The supernatant was ultrafiltered and the ultrafiltrate injected into an HPLC column.

3.2.3 Measurement of lens ascorbate

The selected method for ascorbate measurement (Hallström, Carlsson, et al.; 1989) allows efficient separation of small molecules with higher sensitivity (less than 0.06 μM in the injected sample) and better specificity than other HPLC methods (Omaye, Turnbull and Sanberlica; 1979). Nuclear magnetic resonance (NMR) is another accurate method for measurement of lens ascorbate, albeit expensive (Tessem, Bathen, et al.; 2006). The column was an ion-exchange, reversed phase column. Sulphuric acid (2 mM) with pH 2.4 was used as mobile phase to prevent ascorbate oxidation during the passage through the HPLC column.

Ascorbate was detected as absorption at 254 nm at expected elution time, checking for the pattern of surrounding peaks in the chromatogram. Many wavelengths within the UVR-C region provide efficient detection with high sensitivity for ascorbate in biological tissues (Hallström, Carlsson, et al.; 1989) (Johnsen, Ringvold and Blika; 1985). We chose 254 nm to maximally accept ascorbate signal and reject dehydroascorbate signal, respectively (Figure 5).

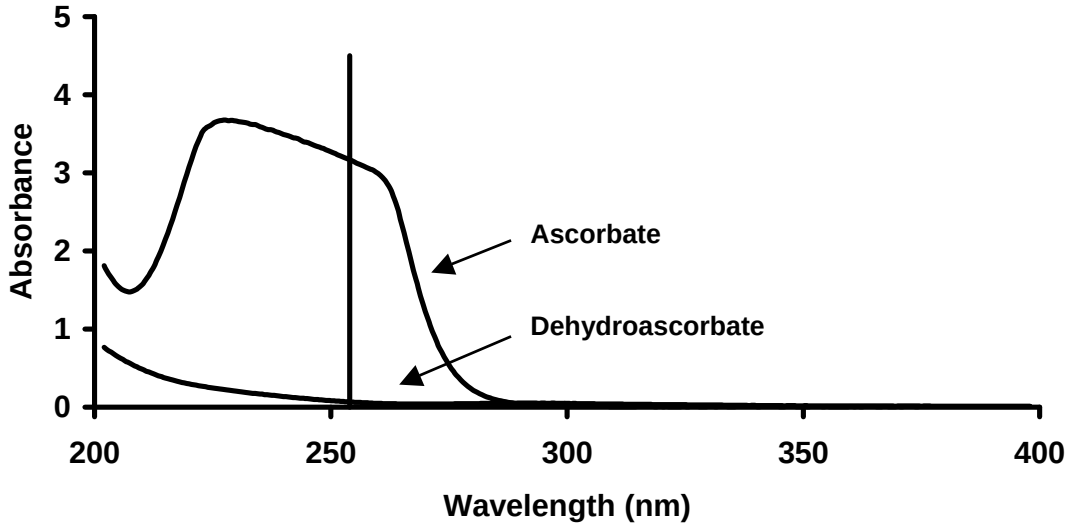


Figure 5 Absorption spectra for 1 mM ascorbate and 1 mM dehydroascorbate in 2.5% metaphosphoric acid. The vertical line denotes absorbance at 254 nm.

The absorbance ratio for the oxidized form of ascorbate, dehydroascorbate, and ascorbate was found to be 4.1% at 254 nm (Figure 5). This implies that 96% of the signal at 254 nm is ascorbate.

Figure 6 shows a typical chromatogram for ultrafiltered lens homogenate. Retention time for ascorbate was 5.7 minutes under the HPLC conditions used in the experiment.

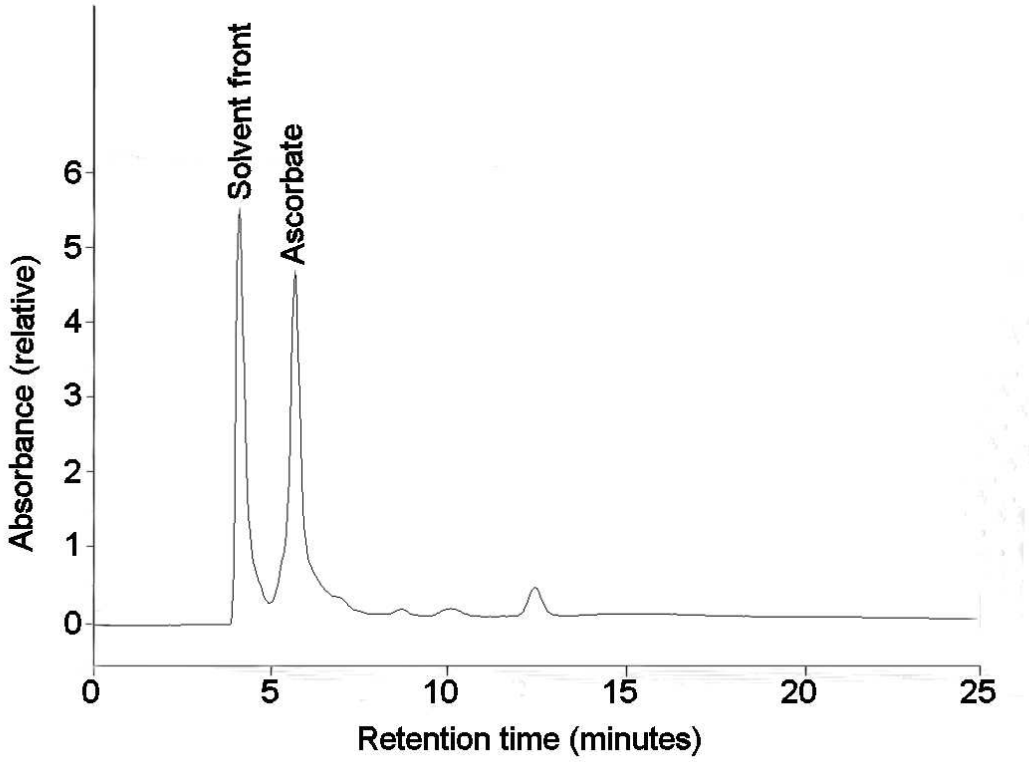


Figure 6 Chromatogram for ultrafiltered whole lens homogenate. Ultraviolet radiation detection at 254 nm.

The peak shape and symmetry allowed for resolution of the ascorbate peak. For analysis of sequential samples, we used chromatography software that was programmed to identify the ascorbate peak based on retention time and shape. The ascorbate concentration was calculated based on calibration against an external 10 μM L-ascorbate standard prepared from a commercially available ascorbate standard stock solution (Merck, Germany). Diluted standard solutions were prepared by mixing weighed amounts of stock solution and distilled water. There is always a risk that external calibration causes an error both in sensitivity and level of the calibration. We estimated this error by comparing external and internal calibration on a pooled ultrafiltrate of rat lenses. External standard with ascorbate solutions ranging from 0 μM to 20 μM were prepared and measured with HPLC. Absorbance increased linearly as a function of concentration (r^2>0.99; data not shown). Internal standard addition samples were created by adding known amounts of ascorbate ranging from 0 - 20 μM to samples of pooled rat lens ultrafiltrate solution. As for the external standard, the increase of absorbance as a function of concentration of standard added was linear (r^2>0.98; data not shown). The ascorbate concentration in the rat lens ultrafiltrate was with the internal standard addition technique estimated to 1.87 μM and with external calibration technique to 1.95 μM. Considering the insignificant difference between the two methods, it was decided to use external calibration for the experiments.

The recovery of ascorbate was determined. The pellet after ultracentrifugation of the lens homogenate was re-extracted and the solution again ultrafiltered. The ascorbate concentration in the ultrafiltrate was 46% of that in the first ultrafiltrate (data not shown).

Our method measures a fraction of the total ascorbate concentration, which is proportional to both the total ascorbate concentration and free ascorbate concentration. Therefore, an increase in ascorbate concentration as measured with the utilized method corresponds to an increase in free ascorbate concentration.

3.3 Ultraviolet radiation-B exposure

The rats in paper II were anesthetized with an intraperitoneal injection of xylazine (14 mg/kg) and ketamine (94 mg/kg) ten minutes prior to exposure. Both eyes were dilated with 1% tropicamide five minutes prior to exposure. The rats were unilaterally exposed to UVR around 300 nm for 15 minutes. The four rats in the first group, zero dose, were sham irradiated. The other groups received 0.25, 3.5, 4.3, and 4.9 kJ/m^2. The UVR was generated with a high-pressure mercury arc source equipped with a water filter and a double monochromator. The radiation from a high-pressure mercury lamp (model 6828, Oriel, USA) was passed through a water filter and double monochromator (model 77250, Oriel, USA), set at 300 nm and 10 nm theoretical full width at half maximum, and finally projected on the cornea of the exposed eye (Michael, Söderberg and Chen; 1996). The spectrum of radiation is shown in Figure 7.

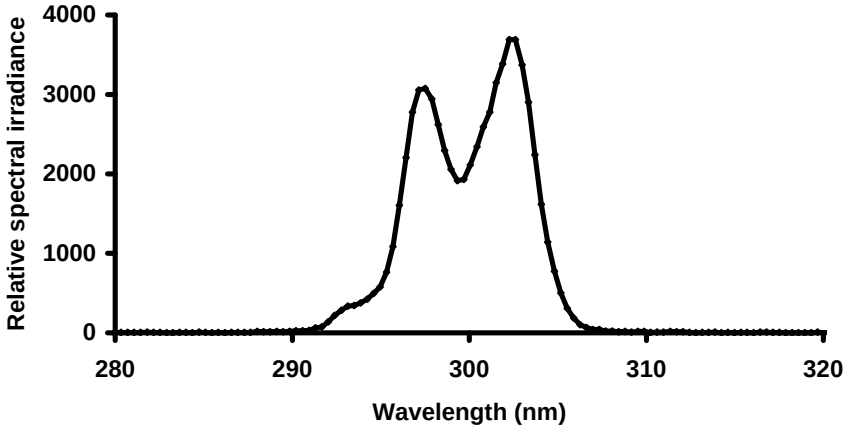


Figure 7 Relative spectral UVR-B irradiance from the monochromator-filtered Mercury high-pressure arc lamp output.

Forty of the ascorbate supplemented guinea pigs in paper IV were exposed to UVR as described for the rats above. However the anesthetic dose was lower (40 mg/kg ketamine and 5 mg/kg xylazine), the UVR-B dose higher (80 kJ/m^2) and exposure time was 1 hour.

Forty of the animals in paper IV were exposed to 80 kJ/m^2 UVR-B after ascorbate supplementation and sacrificed one day thereafter. Twenty of the guinea pigs served as controls and were not exposed to UVR-B, and the animals were sacrificed after ascorbate supplementation. Lens ascorbate concentration was measured in all sixty animals. Lens forward light scattering was measured in the forty UVR-B-exposed animals using a light dissemination measurement (LDM) meter modified for guinea pigs.

3.4 Measurement of degree of cataract

The rats in paper II were sacrificed one week after exposure in order to allow maximum intensity of light scattering to develop (Michael, Söderberg and Chen; 1996; Söderberg; 1990b). Both eyes were enucleated and the isolated lenses were transferred to a cuvette containing a balanced salt solution (BSS, Alcon, USA.). The degree of cataract was

quantified by measurement of the intensity of lens forward light scattering with a Light Dissemination Meter (LDM) (Söderberg, Chen and Lindström; 1990). The lenses were then kept frozen in -80°C before homogenization and subsequent ascorbate measurement. The LDM uses the probing light from a dark-field ring illumination system. The light transilluminates the lens at 45 degrees against the horizontal plane. At this angle the light cannot enter the objective aperture in the photodetector unit. A cataractous lens will scatter light in the forward direction, of which a fraction reaches the objective and is detected by a photodiode (Figure 8).

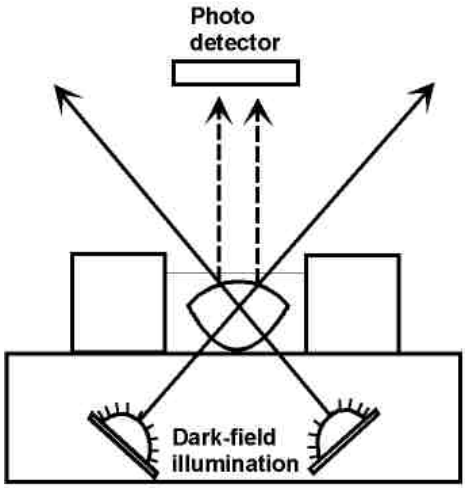


Figure 8 Schematic of forward lens light scattering measurement using the Light Dissemination Meter

The scattering standard is a lipid emulsion of diazepam (Stesolid Novum, Kabi Pharmacia, Sweden) and the unit is expressed as transformed equivalent diazepam concentration ($_t$EDC) (Söderberg; 1990a).

The ascorbate supplemented guinea pigs in paper IV were sacrificed at one day after UVR-B exposure, and the lenses taken for cataract measurement. We have previously determined that maximum degree of cataract develops at one day after exposure in the pigmented guinea pig and remains constant till eight days after exposure (Mody VC, et al. 2006 [EVER Abstract]). The original meter apparatus was adapted to the larger size of guinea pig lenses. We used a cuvette with a larger (15 mm) perforating hole and deeper (10 mm) well, and a photodetector equipped with a larger (3.9 x 3.9 mm) photodiode (S1226-44BK, Hamamatsu Photonics K.K., Japan).

3.5 Statistical methods and parameters

Throughout the thesis, the significance levels and confidence coefficients were set to 0.05 and 0.95, respectively. All mean estimates were expressed with a 95% confidence interval (CI). The use of the term significant in the text indicates *statistically* significant.

4. RESULTS AND DISCUSSION

4.1 Paper I, ascorbate in the rat lens after solid diet supplementation

To exclude that the ascorbate supplementation per se affects lens light scattering, all lenses were monitored macroscopically in incident light with a grid background. All lenses were devoid of cataract (Figure 9).

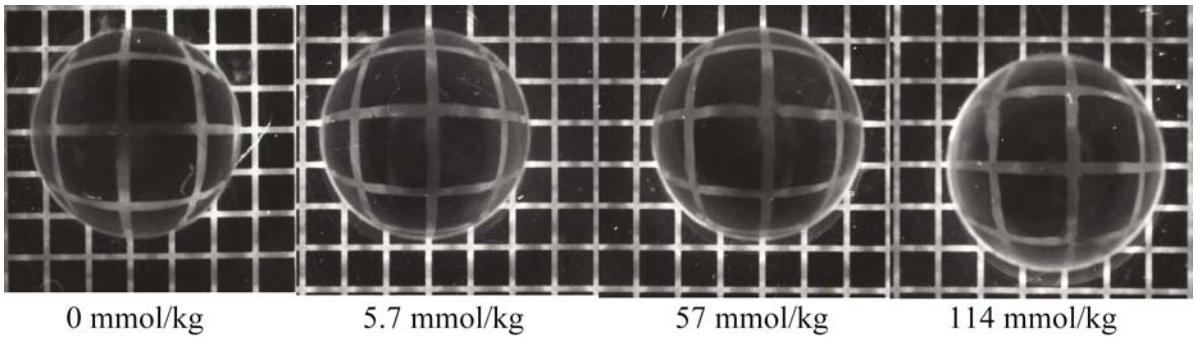


Figure 9 Clear lenses from rats fed chow supplemented with varying amounts of ascorbate. Grid square diameter is 0.79

Lens ascorbate concentration, expressed as μmol ascorbate/g wet weight lens, increased linearly with dietary ascorbate intake (mol/kg chow) (Figure 10).

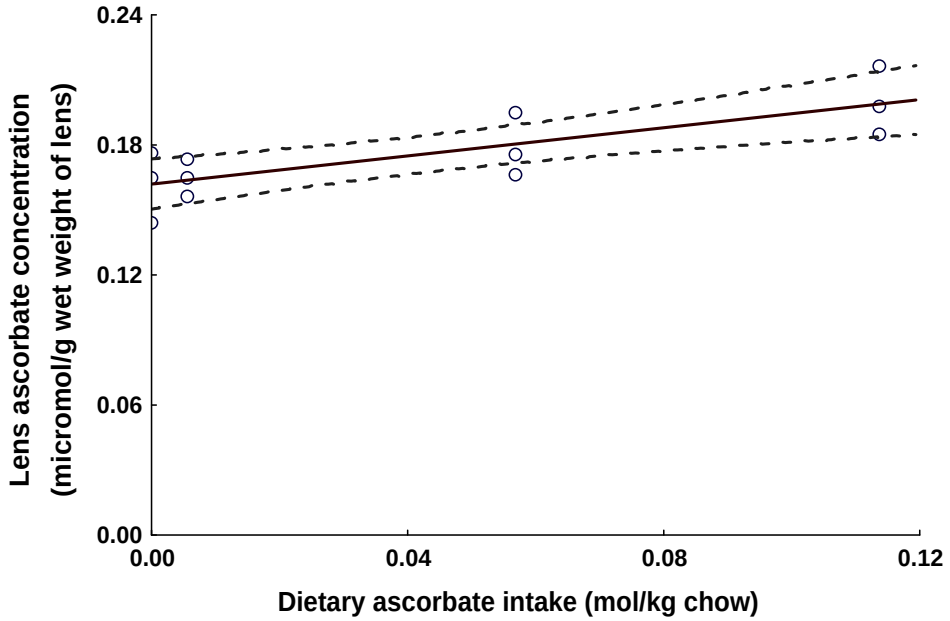


Figure 10 Rat lens ascorbate concentration as a function of dietary ascorbate intake. Dotted lines demark the 95% confidence intervals for the solid regression line.

The data were fitted to a first order polynomial assuming a baseline content of ascorbate without any dietary intake, A_0 (μmol/g lens wet weight), and that the content of ascorbate in the lens per lens wet weight, A_{lens} (μmol/g lens wet weight), is directly proportional to the

dietary intake, *I* (mol/kg chow), with the proportionality constant *k* (μmol ascorbate)(g lens)$^{-1}$(mol ascorbate)$^{-1}$ (kg chow) (Equation 3).

Equation 3

$$[A_{lens}] = A_0 + k\ I + \varepsilon$$

The baseline lenticular content of ascorbate (A_0) was significantly higher than zero, even without oral ascorbate supplementation (0.16 ± 0.01 μmol/g lens wet weight). The value is consistent with that reported in the literature, 0.078 μmol/g lens wet weight (Reddy, Giblin, et al.; 1998). The baseline lenticular content of ascorbate is in agreement with the fact that the rat is capable of synthesizing ascorbate.

There was a significant increase of lenticular ascorbate per ascorbate intake (k = 0.33 ± 0.18 (μmol ascorbate) (g lens)$^{-1}$ (mol ascorbate)$^{-1}$ (kg chow)$^{-1}$, r^2=0.62). The linear relationship between lenticular content of ascorbate and dietary intake supports previous observations that, in the rat, ascorbate enters the aqueous humor from the plasma and the lens from the aqueous humor by passive diffusion (Garland; 1991).

The increase in lenticular ascorbate concentration with dietary supplementation in the rat was prominent, approximately 25% from group 1 to group 4. The increase is important since the dependence of lens ascorbate concentration on dietary intake in the rat has not been studied previously. However, our data do correspond with an experiment analyzing the effect of a single intraperitoneal injection of ascorbate into the rat (Reddy, Giblin, et al.; 1998). The observed increase of lenticular ascorbate is important since ascorbate has been shown to decrease after oxidative stress. The results show that this rat model can be used to study the preventive effect of lenticular ascorbate against oxidatively- and photochemically-induced cataract in vivo.

4.2 Paper II, UVR-B-exposed albino rats

4.2.1 Maximum Tolerable Dose (MTD) estimation

No lenses in the control group or the 0.25 kJ/m^2 UVR-B dose group developed cataract. Lenses in the 3.5 kJ/m^2 or higher dose groups developed prominent anterior subcapsular and equatorial cortical opacities (Figure 11).

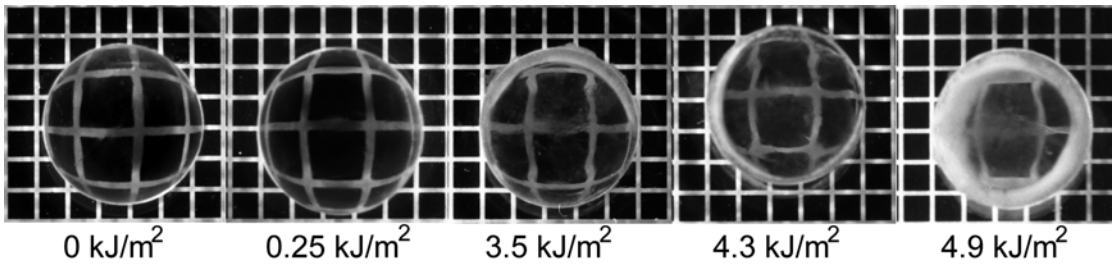


Figure 11 Rat lenses one week after in vivo UVR-B exposure. Grid square diameter is 0.79 mm.

The difference of intensity of light scattering between exposed and contralateral non-exposed lenses increased with increasing UVR dose (Figure 12).

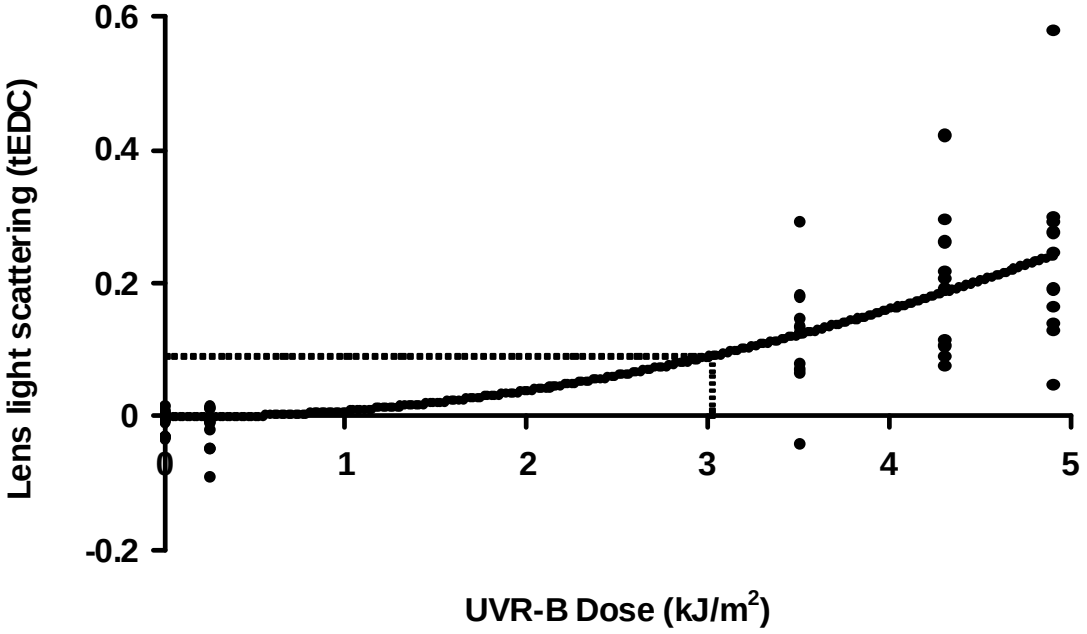


Figure 12 Paired difference in forward lens light scattering between exposed and contralateral non-exposed rat lens as a function of UVR-B dose. Curved line is best least square fit. Vertical line illustrates 3.01 kJ/m^2 Maximum Tolerable Dose ($MTD_{2.3:16}$).

The MTD strategy (Söderberg, Löfgren, et al.; 2002) was used for estimation of threshold for in vivo UVR-300 nm toxicity in the rat lens. The data were fitted with linear regression to a second order polynomial omitting the zero order term. The sensitivity, k, was 1.02 ±0.19 x10^{-2} tEDC/(kJ/m^2)^2 and the residual standard deviation was 9.20 x 10^{-2} tEDC. $MTD_{2.3:16}$ was therefore estimated to 3.01 kJ/m^2 (n=46) (Figure 12).

The $MTD_{2.3:16}$ of 3.01 kJ/m^2 agrees with a previously published value (Söderberg, Löfgren, et al.; 2002) of 3.65 kJ/m^2 for MTD for the six-week-old albino rat. The two albino rat MTDs are both in the same order as the qualitatively estimated threshold limit for permanent lenticular damage of 5.0 kJ/m^2 in the pigmented rabbit, published by Pitts et al. (Pitts, Cullen and Hacker; 1977) and based on a binary dose-response model. The albino rat MTDs are in addition similar to that of the pigmented rat, 4.2 kJ/m^2 (Kakar et al., 2003 [ARVO Abstract]), and pigmented mouse, 3.2 kJ/m^2 (Meyer, Söderberg, et al.; 2005), based on a continuous dose-response model.

4.2.2 UVR-B-induced ascorbate oxidation

The ascorbate in the non-exposed lenses was not significantly altered by UVR-B exposure to the other eye as judged by analysis of variance (ANOVA) (test statistic=1.77, $F_{3;19;0.95}$=3.90, p-value=0.15). Heterogeneity of group variance was excluded according to a Bartlett's test (test statistic=5.23, $\xi^2_{0.05,4}$=9.49, p-value=0.26). The mean ascorbate concentration in the non-exposed lenses, estimated with a 95% CI, was 0.149 ±0.005 µmol/g lens wet weight (Figure 13).

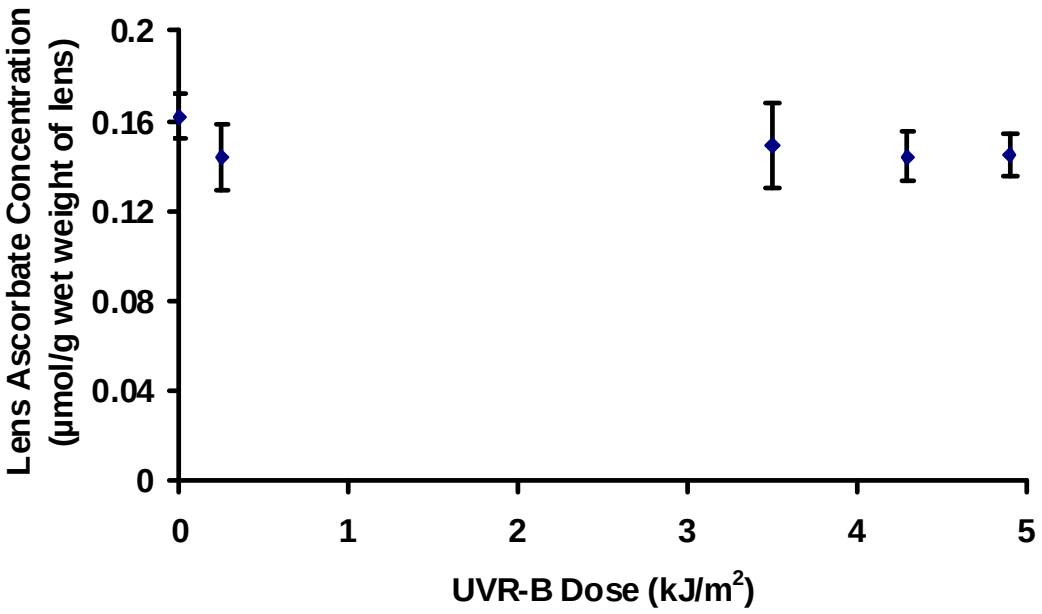


Figure 13 Ascorbate concentration in contralateral non-exposed rat lenses as a function of UVR-B exposure to the other eye. Bars represent 95% confidence intervals.

Since there was no significant change in ascorbate concentration in the contralateral lens upon unilateral exposure to UVR-B, a model was developed (Equation 4).

Equation 4

$C_d=C_{Co}(e^{-kHe}-1)+\varepsilon$

Assuming that the lens contains one pool of UVR consumable ascorbate, C_{Co}, and another pool of non-consumable ascorbate, C_{NonCo}, the differences of total ascorbate content between exposed and contralateral lens, C_d (µmol/g lens wet weight), as a function of exposure to UVR, H_e (kJ/m^2), for each animal, were fitted with non-linear regression considering the rate k (m^2/kJ). The consumable ascorbate was then 0.11 µmol/g lens wet weight and the inverse of the rate constant was 0.86 kJ/m^2 indicating that after a dose of 0.86 kJ/m^2 UVR-B, 63% of the consumable ascorbate will be lost. UVR-B exposure increased the difference in ascorbate concentration between the exposed lens and the contralateral non-exposed lens. (Figure 14).

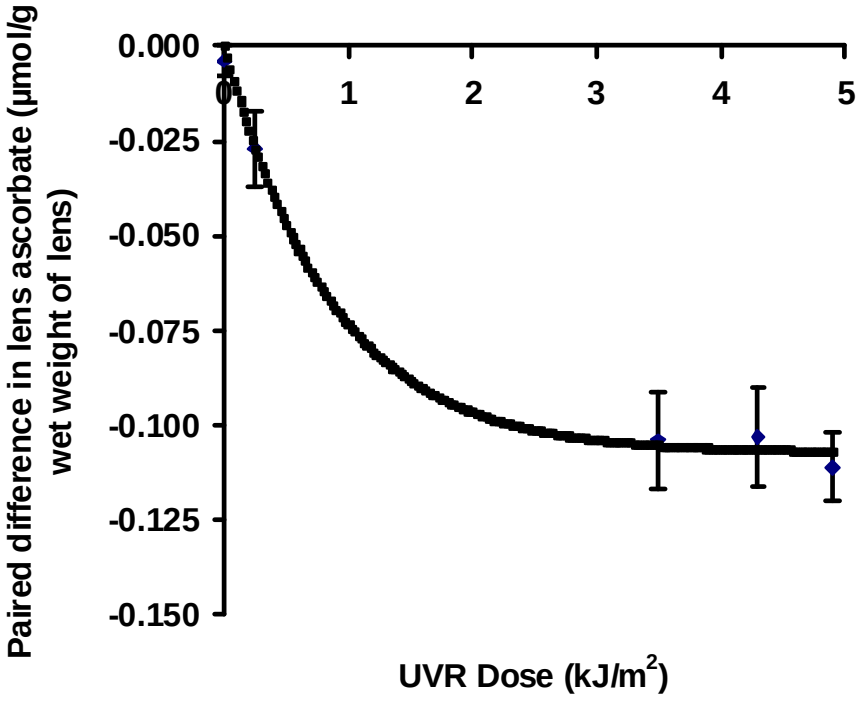


Figure 14 Mean paired difference in ascorbate concentration between UVR-B-exposed and non-exposed rat lenses. Bars represent 95% confidence intervals; r^2=0.98.

By using the data from exposed lenses only, making same assumptions as in Equation 4, and adding a new component, UVR non-consumable ascorbate, C_{NonCo} (µmol/g lens wet weight), a new model was developed (Equation 5).

Equation 5

$$C=C_{NonCo}+C_{Co}e^{-kHe}+\varepsilon$$

The equation models that the concentration of ascorbate at increasing UVR doses decreases exponentially towards the concentration of non-consumable ascorbate.

The data were fitted to the model outlined in Equation 5 with non-linear regression. The C_{Co} was 0.11 µmol/g lens wet weight, the C_{NonCo} was 0.04 µmol/g lens wet weight, and the inverse of the rate constant was 0.60 kJ/m^2. The 0.15 µmol/g lens wet weight baseline ascorbate content at zero UVR-B dose is consistent with the baseline concentration of 0.16 µmol/g lens wet weight in the rat shown in paper I (Mody, Kakar, et al.; 2005), and the baseline 0.08 µmol/g lens wet weight in 20 lenses reported by Reddy et al. (Reddy, Giblin, et al.; 1998). UVR-B exposure decreased ascorbate concentration in the exposed lens. (Figure 15).

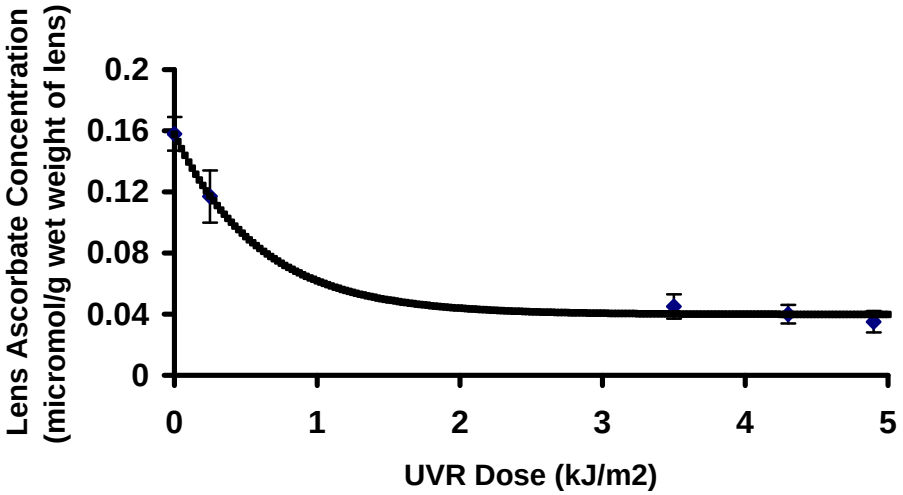
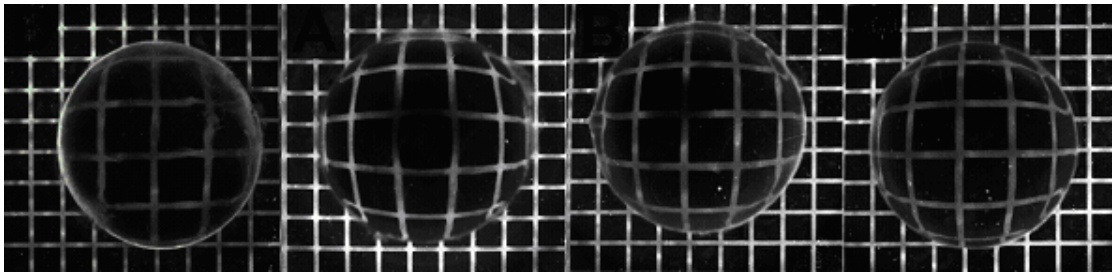


Figure 15 Ascorbate concentration in UVR-B-exposed rat lenses versus UVR-B dose. Bars represent 95% confidence intervals, r^2=0.98.

The decrease of lens ascorbate after UVR-B exposure (Figures 14 and 15) is corroborated by two reports, one in vitro study on direct lens UVR-B exposure (Reddy, Nayak, et al.; 2001) and the other a study on ascorbate in cataractous human lenses (Tessier, Moreaux, et al.; 1998). Both figure 14 and 15 show curves decreasing towards an asymptote, indicating there is one pool of UVR-consumable ascorbate and another pool of non-consumable ascorbate. The fact that 63% of the UVR-consumable ascorbate has been consumed after only 0.86 kJ/m^2 (Figure 18) while $MTD_{2.3:16}$ is 3.01 kJ/m^2 (Figure 12) indicates that the ascorbate decrease is on the order of 3.5 times more sensitive to detect UVR damage in the lens than forward light scattering. Ascorbate depletion is demonstrated to occur at the same time or even before depletion of alpha-tocopherol and glutathione (Kamegawa, Nakanishi-Ueda, et al.; 2007)

4.3 Paper III, ascorbate in the guinea pig lens after drinking water supplementation

To investigate if ascorbate supplementation per se alters lens light scattering, all lenses were monitored macroscopically in incident light with a grid background. All lenses were devoid of cataract (Figure 16).



0 mM **2.84 mM** **5.68 mM** **8.52 mM**

Figure 16 Lenses from guinea pigs receiving drinking water supplemented with ascorbate. Grid square diameter is 0.79 mm.

4.3.1 Drinking water ascorbate content

There was a 42% decrease in ascorbate content in the drinking water flasks during the 12 hour interval between refilling of fresh content. The mean initial ascorbate concentration before 12 hours was 8.29 ± 0.73 mM (n=45) and final value after 12 hours was 5.96 ± 0.77 mM (n=45). The mean difference of ascorbate content between newly prepared flask and flask before renewal of content was 2.33 ± 0.24 mM (n= 45).

4.3.2 Lens ascorbate

All lenses contained a detectable concentration of ascorbate (Figure 17).

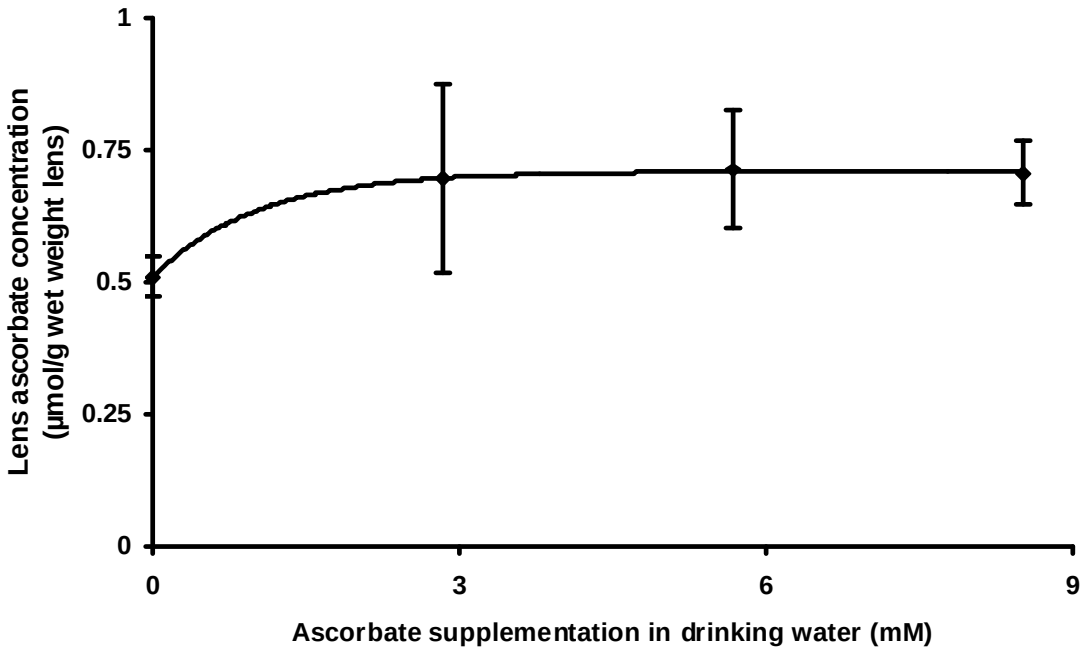


Figure 17 Guinea pig lens ascorbate concentration after drinking water ascorbate supplementation. Bars represent 95% confidence intervals. Line is best fit to a model expressing exponentially declining increase.

The means for animal-averaged lens ascorbate concentrations (μmol ascorbate/g lens wet weight) per group were 0.51 ± 0.04 [0 mM], 0.70 ± 0.18 [2.84 mM], 0.71 ± 0.11 [5.68 mM], and 0.71 ± 0.06 [8.52 mM] (n=23). One animal in the 5.68 mM group was excluded from data analysis because the reading of ascorbate concentration in one of its lenses was more than twice that of all other measurements in the group. A considerable concentration of ascorbate was found in lenses from the 0 mM supplementation group, only receiving 0.125 mol L-Ascorbate/kg in the chow. Inspection of the data suggests that animal-averaged lens ascorbate concentration, $[Asc_{lens}]$, increases with ascorbate supplementation in drinking water, Asc_{water}. The increase in lens ascorbate concentration reached a saturation level at higher drinking water concentrations (Figure 17).

Lens ascorbate concentration increased from a baseline level, and at some point, the lens became saturated. Therefore, the data were fit assuming the model in Equation 6.

Equation 6

$$[Asc_{lens}] = A - Be^{-k\,Asc_{water}}$$

Here, A is the saturation level of ascorbate concentration in the lens with drinking water supplementation, B is the increase of lens ascorbate from baseline to saturation, and k is a measure of the increase in lens ascorbate concentration as a function of ascorbate concentration in supplemented drinking water.

Non-linear regression according to the assumed model provided a good fit ($r^2 = 0.98$). The estimates of the parameters and their estimated standard deviations were as follows. The saturation level was 0.71 ±0.03 μmol ascorbate/g lens wet weight and the increase of lens ascorbate from baseline to saturation was 0.20 ±0.05 μmol ascorbate/g lens wet weight. The baseline lenticular content of ascorbate was 0.51 μmol/g lens wet weight. The baseline lenticular content is on the same order as that reported in the literature for guinea pigs receiving a standard diet, 0.65 μmol/g lens wet weight (Varma; 1991). The saturation rate was 0.98 ±1.49 M^{-1} (1/k = 1.02 M).

In the guinea pig, uptake of ascorbate by the lens from the aqueous humor, and by the aqueous humor from the plasma, occurs by active transport (Garland; 1991). The saturation of lenticular ascorbate secondary to drinking water supplementation (Figure 13) agrees with a previous finding in the guinea pig where lower ascorbate doses were given (Berger, Shepard, et al.; 1988). These two findings indicate that the active transport is concentration dependent and saturable.

The increase in lenticular ascorbate concentration with dietary supplementation in the guinea pig was approximately 40% from group 1 to group 4, and significantly higher than zero. The increase is important since it can be used to study the preventive effect of lenticular ascorbate against oxidatively- and photochemically-induced cataract in vivo.

4.4 Paper IV, UVR-B-exposed ascorbate supplemented guinea pigs

Some animals were excluded due to presence of congenital cataract. Final sample size was 35 in the principal UVR-B experiment and 16 in the verification experiment.

4.4.1 Lens ascorbate content

In both UVR-exposed animals and non-exposed animals, the animal-averaged lens ascorbate concentration was significantly higher in the 5.5 mM supplemented group than in the non-supplemented group (2-way ANOVA, factor Ascorbate $p<1x10^{-6}$, interaction factor Ascorbate vs UVR animal p=0.24; Figure 18). Animal-averaged lens ascorbate was significantly lower in UVR-exposed animals than in non-exposed animals, in either of the 0

mM or the 5.5 mM supplementation groups (2-way ANOVA, factor UVR animal p=0.006, interaction factor Ascorbate vs UVR animal p=0.24; Figure 18). Lens ascorbate in UVR-exposed lenses did not differ significantly from that in non-exposed lenses, in either of the supplementation groups (ANOVA, factor UVR lens p=0.73, interaction factor Ascorbate vs UVR lens p=0.38; Figure 18).

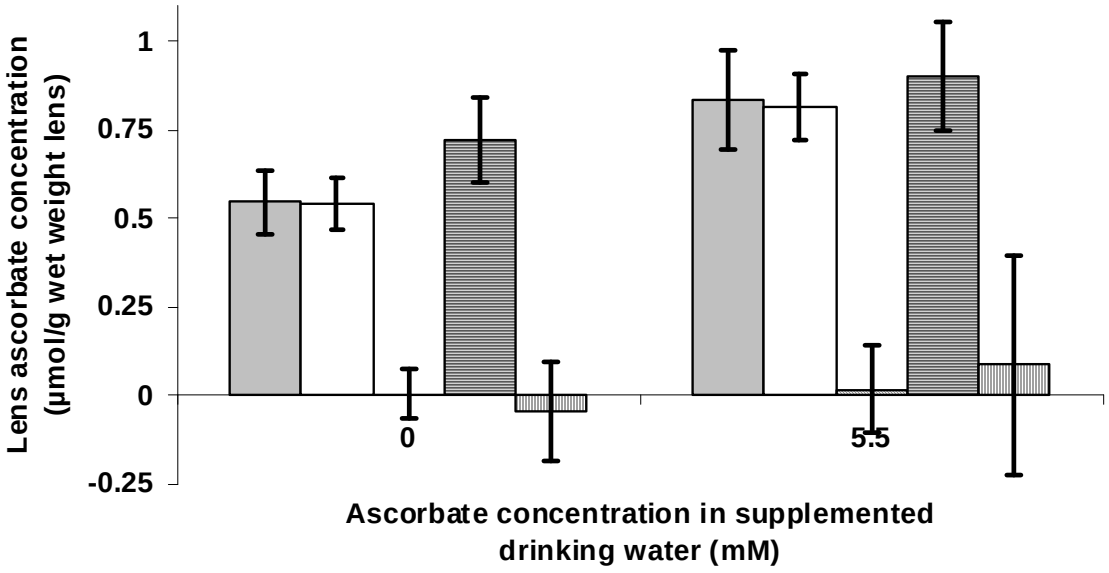


Figure 18 Lens ascorbate in ascorbate supplemented guinea pigs, with and without unilateral UVR-B exposure. There are five bars for each of the 0 mM and 5.5 mM ascorbate supplementation groups, from left to right: 1) Lens ascorbate in UVR-B-exposed lenses from UVR-B-exposed animals; 2) Lens ascorbate in non-exposed lenses from UVR-B-exposed animals; 3) Paired difference in lens ascorbate between UVR-B-exposed and non-exposed lenses from UVR-B-exposed animals; 4) Animal-averaged lens ascorbate in non-exposed animals; 5) Paired difference in lens ascorbate in non-exposed animals. Error bars represent 95% confidence intervals.

UVR-B exposed animals contained 24% (0 mM group) and 8% (5.5 mM group) lower animal-averaged lens ascorbate concentration than corresponding non-exposed animals (Figure 18). The finding is consistent with the results in rats in paper II, and with a ^{1}H NMR spectroscopy study on UVR-exposed rabbits (Midelfart; 2005). In contrast, there was no difference in ascorbate concentration in UVR-exposed lenses compared to non-exposed lenses from the same UVR-exposed animals (Figure 18). This is intriguing because it indicates a systemic signalling effect to both lenses, despite the strictly unilateral UVR exposure. The reason for the bilateral effect is unknown, although the bilaterality may be the result of ascorbate requirement and high ocular concentration in the guinea pig in contrast to the rat and most species. In the mouse, Meyer et al. (2005) found that bilateral cataracts developed secondary to UVR-B exposure in ten out of eighty animals in C57 mice (Meyer, Söderberg, et al.; 2005), consistent with the hypothesis that bilaterality to UVR-induced cataract development does occur in some animals, secondary to enhanced physiological susceptibility.

4.4.2 Cataract development

Superficial anterior cataract developed in UVR-B-exposed lenses from both the 0 mM and 5.5 mM drinking water supplementation groups (Figure 19). All non-exposed lenses were devoid of cataract (Figure 19).

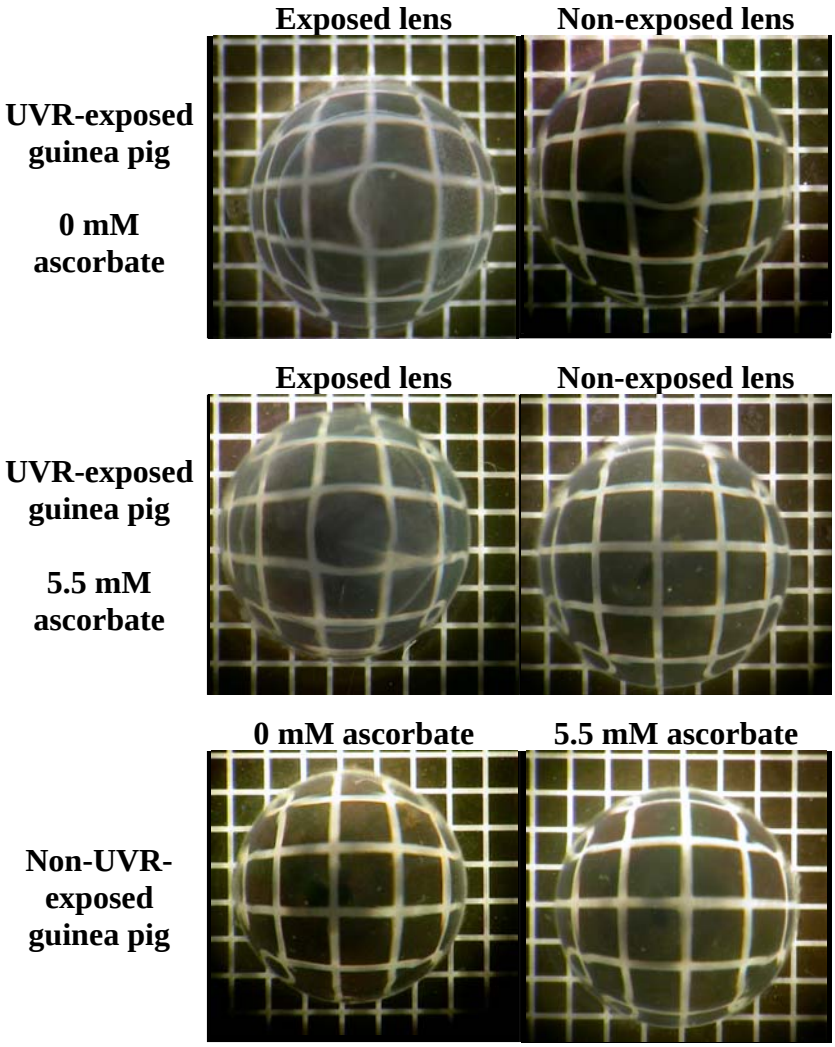


Figure 19 Lenses from 0 mM and 5.5 mM ascorbate supplemented guinea pigs exposed and not exposed to UV-B radiation.

Superficial anterior cataract developed in all lenses one day after exposure to the supra-threshold UVR-B dose 80 kJ/m^2. The cataract type is consistent with that found by Wu et al. (2004) in which guinea pigs were exposed to 82 kJ/m^2 (Wu, Kojima, et al.; 2004). The finding is also consistent with a previous guinea pig UVR-B study from our group (Mody Jr VM, et al. *Acta Ophthalmol Scand* 2006;84;S239: EVER Poster Abstract 227). The previous studies show that the induction of cortical cataract in the pigmented guinea pig requires a much higher UVR dose and is of less severity than that of the pigmented rat (Kakar MK, et al. *IOVS* 2003;44: Arvo E-Abstract 296) and pigmented mouse (Meyer, Söderberg, et al.; 2005). We chose a dose of 80 kJ/m^2 because, although it is a high dose in other species, the dose is not exorbitant for the guinea pig and a lower dose does not induce reliable cataract development. The mechanism for this extraordinary tolerance to UVR in the guinea pig is unknown. The loss of ascorbate secondary to UVR may result in a concomitant reduction of oxidation to other molecules in the lens, thus reducing the effectiveness of the radiation. Since there are more ascorbate molecules available per lens in the guinea pig than in the rat, the direct UVR scavenging effect of ascorbate may be higher in the guinea pig. A concentration as high as 10% of the unique ζ-crystallin has been reported in the guinea pig in contrast to the rat (Rao, Gonzalez, et al.; 1997). ζ-crystallin through its function as a NADPH:quinone oxidoreductase may increase the concentrations of pyridine nucleotides in

the lens, therefore protecting the lens from oxidative stress (Rao, Gonzalez, et al.; 1997; Rao and Zigler Jr; 1992).

4.4.3 Lens light scattering

In both supplemented and non-supplemented animals, the lens light scattering was significantly higher in UVR-exposed lenses than in contralateral non-exposed lenses (2-way ANOVA, factor UVR $p<10^{-6}$, interaction factor Ascorbate vs UVR p=0.74; Figure 20).

Ascorbate supplementation did not significantly influence lens light scattering in either UVR-exposed lenses or non-exposed lenses (2-way ANOVA, factor UVR p=0.06, interaction factor Ascorbate vs UVR p=0.74; Figure 20).

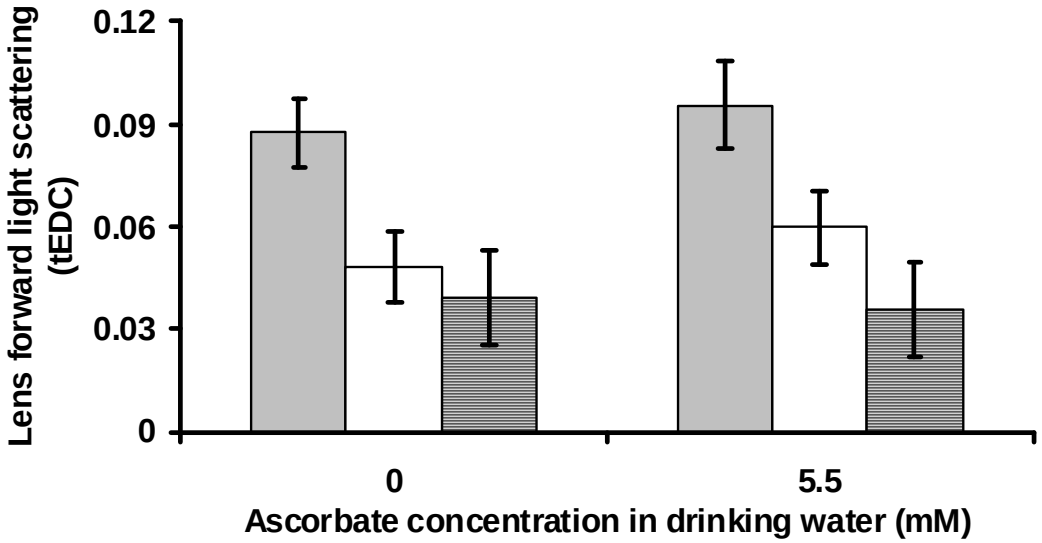


Figure 20 Lens light scattering after UVR-B exposure in ascorbate supplemented guinea pigs. There are three bars for each of the 0 mM and 5.5 mM groups, from left to right: 1) Lens forward light scattering in UVR-B-exposed lenses from UVR-B-exposed animals; 2) Lens forward light scattering in non-exposed lenses from UVR-B-exposed animals; 3) Paired difference in lens forward light scattering between UVR-B-exposed lenses and non-exposed lenses from UVR-B-exposed animals. Bars represent 95% confidence intervals.

The non-significant difference in lens light scattering between the UVR-B-exposed 0 mM and 5.5 mM supplementation groups indicates that there is no photosensitizing effect of ascorbate supplementation in the lens. Further, the cataract-devoid non-exposed lenses demonstrate that permanent cataract does not develop from the anesthesia and that ascorbate supplementation in the drinking water is non-toxic to the guinea pig lens.

Glutathione is the major antioxidant in the lens (Bernat and Bombicki; 1968; Giblin; 2000). Combinative interactions of the water soluble ascorbate and the lipid soluble vitamin E play a role in the antioxidant defense of the lens cell and help reduce glutathione (Kutlu, Naziroglu, et al.; 2005; Shang, Lu, et al.; 2003). Vitamin E requires ascorbate in order to function, and the effect between the two vitamins may be greater than additive (Beyer; 1994). The specific interaction of ascorbate with vitamin E is that ascorbate reduces vitamin E utilizing GSH. The interaction of both ascorbate with vitamin E may be required for a protective effect. The additive effect of ascorbate and vitamin E may explain why there was no significant reduction in lens light scattering induced by UVR-B in the guinea pig in this study.

Biochemically, there are several possible mechanisms for depletion of lens ascorbate secondary to UVR exposure. Firstly, the consumable ascorbate may be directly oxidized by UVR (Reddy; 1996). Alternatively, ascorbate may be consumed while serving its function as an antioxidant in the lens. The ascorbate recycling enzyme systems might be inactivated by UVR, thus allowing for more binding to proteins and other cellular constituents. Finally, consumable ascorbate may be lost by leakage from lens cells damaged by UVR.

Significant lens light scattering developed in ascorbate supplemented and non-supplemeted guinea pigs exposed to the supra-threshold UVR-B dose of 80 kJ/m^2. Although ascorbate serves as an antioxidant in the lens, simple modulation of one factor in the lens may not result in significant protection.

5. CONCLUSIONS

The rat lens has a baseline ascorbate content even without any ascorbate intake. In the rat, lens ascorbate concentration increases, following a linear dependence on dietary ascorbate intake without cataract development.

The $MTD_{2.3:16}$ for avoidance of UVR-B-induced cataract in the seven-week-old Sprague-Dawley albino rat was estimated to be 3.01 kJ/m^2, consistent with the UVR-B cataract safety measures reported for that in the pigmented rabbit, pigmented rat, and pigmented mouse. In vivo UVR-B exposure of the rat eye decreases lens ascorbate concentration with an exponential decline, with a suprathreshold dose having a greater effect than a subthreshold dose.

The guinea pig lens has a detectable quantity of ascorbate with standard low dose dietary ascorbate intake. In the guinea pig, lens ascorbate concentration increases with drinking water supplementation in an exponentially declining fashion to a saturation level, without cataract development.

Drinking water supplementation with ascorbate increases lens ascorbate concentration in guinea pigs. Lens ascorbate concentration is greater in non-exposed animals than in UVR-B-exposed animals, while there is no difference in lens ascorbate concentration between non-exposed and UVR-B-exposed lenses from the same exposed animal. Superficial anterior cataract develops in lenses exposed to UVR-B, regardless of ascorbate supplementation in the drinking water. Ascorbate supplementation does not protect against UVR cataract development in the pigmented guinea pig.

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