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INFLUENCE OF EXPOSURE PATTERNS AND OXIDATION IN UVR-INDUCED CATARACT

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To Ann, Sebastian and Gabriel

Man's goings are of the Lord;

how can a man then understand his own way?

Proverbs: 20: 24

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals

I. Ayala M, Michael R, Söderberg PG.
In Vivo Cataract after Repeated Exposure to Ultraviolet Radiation.
Experimental Eye Research, 2000, 70, 451-456.
(Elsevier Science).

II. Ayala M, Michael R, Söderberg PG.
Influence of Exposure Time for UV Radiation-Induced Cataract
Investigative Ophthalmology & Visual Science, October 2000, 41, 3539-3543. (Association for Research in Vision and Ophthalmology).

III. Ayala M, Söderberg PG.
Vitamin E Can Protect against Ultraviolet Radiation-Induced Cataract in Albino Rats.
Ophthalmic Research, 2004, 36, 264-269.
(S. Karger AG, Basel).

IV. Ayala M, Söderberg PG.
Reversal of reciprocity failure for UVR-induced cataract with vitamin E
Accepted in Ophthalmic Research.

V. Ayala M, Strid H, Jacobsson U, Dong X, Söderberg PG.
p53 in the lens after ultraviolet radiation exposure.
Manuscript

List of abbreviations

ANOVA	Analysis of variance
ATP	Adenosin triphosphate
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
GSH	Reduced glutathione
HPLC	High-Performance Liquid Chromatography.
J/m^2	Physical unit for the exposure dose (1 kJ/m^2=0.1 J/cm^2) (1 J = 1 W * s)
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acids
RCT	Randomized control trials
RDI	Recommended daily intake
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase- PCR
$_tEDC$	Transformed Equivalent Diazelmus Concentration
UVR	Ultraviolet radiation
UVR-A	Ultraviolet radiation between 315 and 400 nm
UVR-B	Ultraviolet radiation between 280 and 315 nm
UVR-C	Ultraviolet radiation between 100 and 280 nm
W/m^2	Physical unit for irradiance (1 W/m^2 = 100 $\mu W/cm^2$)

CONTENTS

1. ACKNOWLEDGMENTS

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2. SUMMARY

Background: Ultraviolet radiation (UVR) is one among a number of possible factors that may lead to the development of cataract. Whereas induction of cataract as the result of onetime exposure to UVR has been studied extensively, the data on lens damage resulting from repeated exposures to UVR are scarce. The Bunsen-Roscoe law of photochemical reaction (also known as the reciprocity law) states that if the products of time of exposure and irradiance are equal, then the quantities of material undergoing change will be equal. Vitamin E is an antioxidant with essentially unknown effects during lens aging and cataractogenesis. The *p53* gene is related to apoptosis, an important phenomenon in cells, incuding the lens cells. Aims: 1.To investigate the additive effects of radiation exposure in UVR-induced cataract. 2. To evaluate the validity of the reciprocity law for UVR-induced cataract. 3. To determine whether vitamin E may protect against UVR-induced cataract. 4. To investigate the influence of vitamin E in the application of the reciprocity law for UVR-induced cataract. 5. To investigate whether *p53* expression increases after UVR exposure. Methods: Common for all five studies was the unilateral use of 300 nm UVR in vivo exposure of Sprague-Dawley rats. The degree of cataract was quantified by measurement of forward light scattering in the lenses. In the first study (additivity), rats received two UVR exposures of 4 kJ/m^2 at increasing intervals (6 hours, 1 day, 3 days, 9 days and 30 days). In the second study (reciprocity), each rat was exposed to 8 kJ/m^2 UVR for a different length of time (5, 7.5, 11, 15, 30, 60, and 120 minutes). In the third study (vitamin E and cataract) rats were fed vitamin E or not and then exposed to UVR. In the fourth study (vitamin E and reciprocity), vitamin E was added and rats were exposed to UVR for 5 or 15 minutes. In the fifth and final study rats were exposed to UVR and *p53* gene expression was measured using reverse transcriptase polymerase chain reaction (RT-PCR). Results: In the first study (additivity), highest light scattering was found in the group with a 3-day interval between exposures. In the second study (reciprocity), the group exposed to UVR for 15 minutes showed the highest level of light scattering. In the third study (vitamin E and cataract), the vitamin E-treated group showed less light scattering than the control group. In the fourth study (vitamin E and reciprocity), no significant difference in forward light scattering was found among the lenses treated with vitamin E and exposed for 5 versus 15 minutes. In the fifth study *p53* expression was 147% higher in the lenses exposed to UVR compared with the nonexposed lenses. Conclusions: Additivity does not always hold true for UVR-induced cataract. The reciprocity law cannot be applied to UVR-induced cataract for exposures between 5 –120 minutes. Vitamin E protects the lens against UVR-induced cataract. The inapplicability of the reciprocity law for shorter periods of time can be attributed to oxidation. Apoptosis in the lens due to UVR exposure may be mediated through increased *p53* expression.

3. INTRODUCTION

3.1 Historical review

The earliest appreciation of ocular anatomy began with a discovery credited to Alcmaeon of Croton (500 B.C.) who believed that the eye has a special humor (*humor of vision*) and that this humor flows into the eye from the brain. He thus provided the first description of the optic nerve.

More detailed understanding of the anatomy of the eye is attributed to Democritus (5th century B.C), who described the organ as a relatively simple structure of two layers-an outer (the corneo-sclera) and inner (retina-choroides)-containing a humor connected to the brain.

Aristotle (384-322 B.C.) described a new anatomy of the eye comprising three layers instead of two, with the inner part filled with an undifferentiated humor. For 300 years (330-30 B.C.) the Hellenistic school (Aristotle's teachings) flourished in Alexandria (in the present-day Egypt), under the patronage of the Ptolomies. After the death of Cleopatra in 30 B.C., Rome seized control of Egypt. The Great Library in Alexandria was burned, and a large part of scholarly papyrus scrolls was destroyed. Nevertheless, Alexandrian tradition was conserved and followed by the Roman writers. Rufos of Ephesus (98-117 A.D.) recognized the conjunctiva as a fourth coating layer of the eye, clearly defined an anterior chamber, and correctly located the lens in its true position.

Galenus (131-201 A.D.) better described the ocular anatomy as consisting of seven layers: the conjunctiva, the muscles, the sclera, the choroid, the retina, the vitreous humor and the crystalline lens (Figure 1) [1].

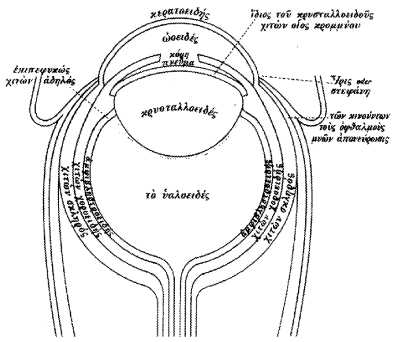


Figure 1. The eye by Galenus. (Reprinted from *System of Ophthalmology* [Duke-Elder 1961] with permission Henry Kimpton, London.)

Late in the 12th century, Ibn Rushd, also known as Averroes (1128-1198 A.D.), suggested that the retina was involved in vision, a view that was accepted by Leonardo da Vinci (1452-1519 A.D.). It was not until the 16th century, however, that Platter showed that the lens was merely a dioptric medium. With the appearance of the "Tractatus de Oculo Visusque Organo" by Fabricius ab Aquapende (1537-1619), the correct position of the lens behind the iris was determined. This view was not generally accepted, however, because cataract was considered to be a change in the humor that accumulated in front of the lens.

The lens was considered a homogeneous humor by the Dutch microscopist Anthony van Leeuwenhoek (1632-1783), who in 1684 described the lens structure as "orbicular scaly parts lying upon one another". His work was followed by John Hunter (1728-1793), who gave a description of the lens structure in the cuttlefish. In the 17th century, when the microscope came into general use, knowledge of the minutae of the lens anatomy developed [2].

3.2 Cataract definition

Etymologically, *cataract* is derived from a Greek term meaning waterfall. The ancient Greeks debated whether diminished vision was due to a release of visual spirit from the eye, as Plato wrote, or to the reception of the stimuli by the eye, as Aristotle believed [3]. There was no mention of the lens until after the founding of Alexandria, Egypt, in 332 BC where scientific anatomy was likely first studied. The Roman physician Claudius Galenus based his observations on animal dissection and described a difference between the anterior and the posterior surfaces of the lens. After the decline of the Roman Empire, Greek and Roman medical concepts were preserved by the Arabs. In the late Middle Ages monks translated the Arabic medical knowledge and founded the first universities in Europe in Bologna, Italy. By this way the term cataract has been preserved till nowadays.

Cataract can be defined as an opacity in the crystalline lens that impairs vision. This opacification is secondary to a disturbed architecture in the lens structure. Disturbed architecture induces increased light scattering throughout the lens. At the same time as an alteration in the regularity creates opacification, a homogenous media is seen as transparent. Transparency is necessary for proper function of the eye. Lens transparency depends on the regular organization of its cells and proteins. Two important types of potential scattering centers in the lens are cytoplasmic proteins and cell membrane proteins. Fluctuation in the cytoplasmic protein density induces small particle scattering, whereas membrane degeneration gives rise to large particle scattering.

Cataracts may occur as a result of a wide variety of factors, including metabolic disorders, exposure to toxic agents, trauma, exposure to radiation, nutritional deficiencies and hereditary factors. Exposure to ultraviolet radiation (UVR) is a proven risk factor for cataract development [4-6]. Changes in global atmospheric conditions, such as depletion of ozone in the stratosphere, lead to increased levels of UVR on the Earth (Figure 2).

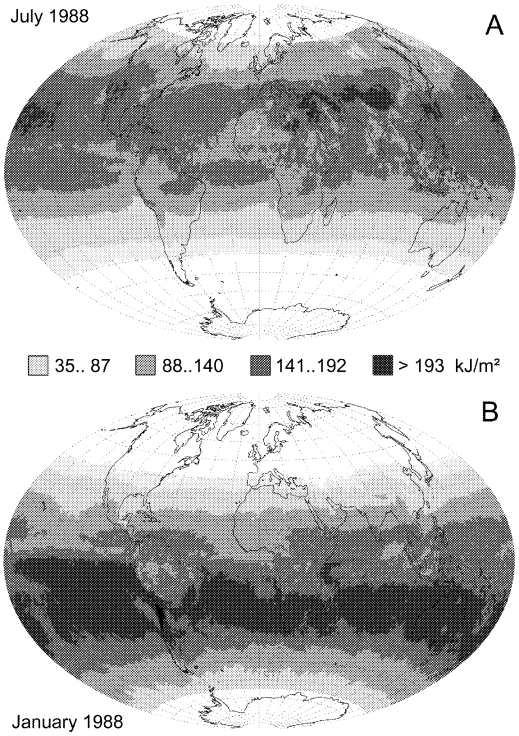


Figure 2. The geographical distribution of monthly integrated UVR from 290 to 400 nm as determined by the CIE. (Adapted from Herman et al, *J. Geophys. Res.* Vol 104-D10, pp 12059-12076, 1999. Copyright by the American Geophysical Union).

3.3 Biological effects of UVR

What is UVR? Electromagnetic radiation can be divided into different categories according to wavelength. Optical radiation is usually subdivided into UVR, visible radiation and infrared radiation (IRR). Ultraviolet radiation is invisible optical radiation located in the wavelength band between 100 and 400 nn; it is subclassified into UV-C (100-280 nm), UV-B (280-315 nm) and UV-A (315-400 nm) (Figure 3). Visible optical radiation comprises wavelengths between 400 and 760 nm. Finally, IRR is invisible optical radiation in the 760-to 3000-nm waveband.

Adverse biological effects occur within all spectral bands from UVR to IRR.

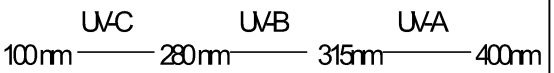


Figure 3. Wavelengths intervals of UVR as defined by the CIE.

The aim of this thesis is to study the effects of UVR, specifically UV-B, on the optical lens. UV-B was selected due to its toxicity in the lens, despite the low UV-B penetration through the cornea. In 1883, De Chardonnet [7] studied light transmission through the different ocular tissues from humans and a number of animal species. He found that the human lens absorbs all radiation below 375 nm and that the human cornea absorbs all radiation below 300 nm. Later studies [8] estimated that 92% of the radiation at 300 nm is absorbed by the cornea and 6% is absorbed in the anterior chamber (Figure 4). This means that only the 2% of 300 nm UVR incident to the cornea reaches the lens.

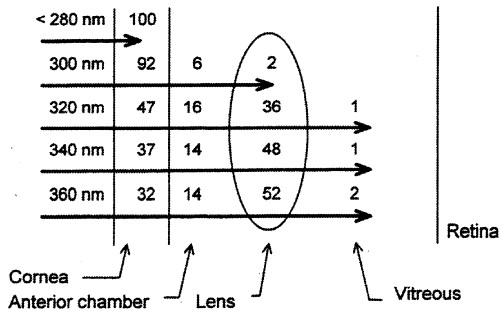


Figure 4. Attenuation of UVR in the human eye for different wavelengths. Numbers are the percentages of incident radiation on the cornea absorbed by each tissue. (From Boettner & Wolter 1962).

All extraterrestrial UV-C is filtered in the atmosphere. Ozone is an effective absorber of both UV-C and UV-B, taking in most radiation below 290 nm. As described previously, the UVR band with the longest wavelengths and lowest energy is the UV-A (315-400 nm). Like UV-B, UV-A radiation may contribute to cataract, albeit to a lesser extent because of its lower absorption in the lens. The focus on UV-B in the present thesis was chosen because of its toxic effects on lens structures [9], despite its low penetration through the cornea.

3.4 Epidemiology UVR cataract

Several epidemiologic studies have been conducted to test the relation between UVR exposure and cataract development. The earlier age of onset of cataract in countries located in equatorial zones has led to a number of theories to explain latitude dependence. Although some studies conclude that exposure of the human eye to UVR plays an etiologic role in the development of ocular disease, including cataract, the role in cataractogenesis has been questioned [10].

The amount and spectral distribution of solar UVR reaching the Earth's surface depends on a number of factors, including the folowing:

a) wavelength of the UVR
b) solar zenith angle, which depends on latitude, date of the year and time of day
c) altitude above the sea
d) ozone column thickness
e) molecular absorption and scattering by the atmosphere
f) absorption, scattering and reflection by the clouds
g) reflectance characteristics (albedo) of the ground
h) shadowing by surrounding objects

Two well-known epidemiological studies merit discussion. In the Chesapeake Bay, Taylor et al [11]examined 800 watermen and estimated the individual UVR dose for each subject. Taylor et al demonstrated a positive correlation between individual UVR dose and cortical cataract but this correlation was not found for nuclear cataracts. In the Beaver Dam study, Cruickshanks et al [6] found a similar correlation between UVR dose and cortical cataract, but only applicable for male subjects.

Conflicting results among epidemiological studies arise as a result of such variables as heterogeneity of the populations studied, as well as differences in methodology and data collection. The highest reliability of information is obtained if individual exposure is estimated as part of the clinical procedure, because retrospective estimation of individual exposure is difficult to determine.

Individual ocular UVR exposure is dependent on the following four factors: 1) ambient level of UVR; 2) amount of time the individual spends outdoors and is exposed to UVR; 3) ocular ambient exposure ratio (the proportion of ambient UVR that actually reaches the eye) and 4) use of ocular protection such as hats and sunglasses [12].

A study by Merriam [3]supports the association between solar exposure and cataract. The investigator measured the UVR dose in different quadrants of a modeled eye and found that the highest dose of UVR was located in the inferonasal quadrant. Merriam correlated these findings with the epidemiological and clinical evidence that cataracts are more often located in the inferonasal quadrant.

3.5 General concepts of photochemistry and photobiology

Photochemistry

Chemical changes caused by radiant energy are known as photochemical reactions. Such reactions can induce cell damage either directly or indirectly. In the direct, *phototoxic reaction*, the absorbed energy produces a toxic molecule. In the indirect, *photosensitized reaction*, the absorbed energy excites a primary molecule, or sensitizer, that transfers its energy to a second molecule, which becomes toxic. In biology, the photosensitized reaction is the most common.

The first and the second laws of photochemistry describe these reactions. The first law, also called Grotthus Draper's Law, postulates that only light which is absorbed can cause photochemical effects. The second law, the Stark-Einstein law, has two aspects. The first says that each photon absorbed by a molecule activates one molecule in the primary step of a photochemical reaction. The second aspect—the Bunsen-Roscoe law, or law of reciprocity—is a consequence of the

first aspect and postulates that time (t) and irradiance (E) are reciprocal for the dose or number of photons received (H). These three quantities are linked as shown in the following equation:

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

In other words, the photochemical effect is determined by the total dose of radiation independently of the time and irradiance as long as the product remains constant [13, 14].

Photobiology

Photobiology is the study of how radiation interacts with living matter. All photobiological effects depend on an initial photochemical event. In a photobiological response, one or several biological events are triggered by a photochemical reaction. For many photobiological responses, the transformation of a photochemical reaction into a biological event is still incompletely understood. Biological responses may be complex reactions with a wide variation among individuals and experiments. The amount of light absorbed by a tissue and its consequent damage depends principally on the number of chromophores the tissue contains. Chromophores can be intrinsic molecules native to the cell, such as aromatic amino acids including tryptophan and tyrosine. Chromophores may alternatively be extrinsic or exogenous to the cell. In the lens, the most important intrinsic chromophore is tryptophan. Other intrinsic amino acids—including phenylalanine, tyrosine, and cysteine, as well as 3-hydroxykynurenine glycoside (3-HKG)—also contribute to UVR absorption. Sensitizers may cause biomolecules that do not primarily absorb radiation to be rendered vulnerable to the exposure of radiation by energy transfer, thus increasing damage. Extrinsic sensitizers have been widely used for treatment of several diseases.

There are two principle pathways by which a sensitizer may act. In the Type I, or radical pathway, the sensitizer interacts directly or indirectly (through a “radical”) with the target molecule. The term *radical* often refers to “free radical” which is defined as a molecule that contains at least one unpaired electron [15]. In the Type II, or singlet oxygen pathway, the sensitizer interacts with oxygen in the tissue with formation of reactive oxygen

species (ROS), such as singlet oxygen, which induces chemical changes in the target molecules (Figure 5). A type II reaction is called a photodynamic reaction. Photodynamic reactions are often used for treatment of diseases. Such treatments are referred to as photodynamic therapy (PDT).

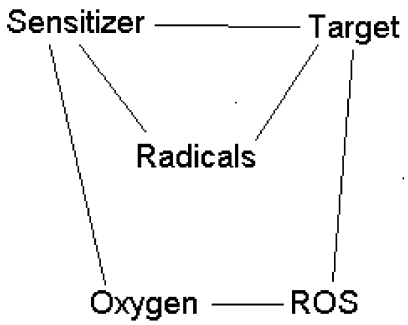


Figure 5. Simplified scheme of oxygen-independent (radical pathway) or oxygen-dependent (ROS pathway) photo-induced damage.

3.6 Mechanisms of lens damage

Multiple mechanisms of lens damage induced by UVR exposure have been postulated. In the lens, UVR exposure elicits a photochemical response that involves formation of photoproducts and free radicals (Figure 6).

Generation of free radicals causes cellular damage. Among detrimental phenomena that have been correlated with free radical generation are protein aggregation, enzyme inactivation (decreased antioxidant protection), membrane damage via lipid peroxidation, and DNA alteration [16]. Protein aggregation produces irregular refractive index gradients that result in increased light scattering. Enzymatic inactivation may result in decreased cellular metabolism; this can lead to decreased synthesis of membrane proteins and subsequently to cellular edema. Lipid peroxidation of cellular membranes may lead to increased membrane permeability, thereby causing cellular edema. Localized cellular edema causes local irregularities in the refractive index, resulting in light scattering. UVR may cause strand breaks or formation of photoproducts from DNA. DNA damage may induce DNA repair but if the damage is extensive, it may lead to necrosis (cell death) or apoptosis (programmed cell death). [17]

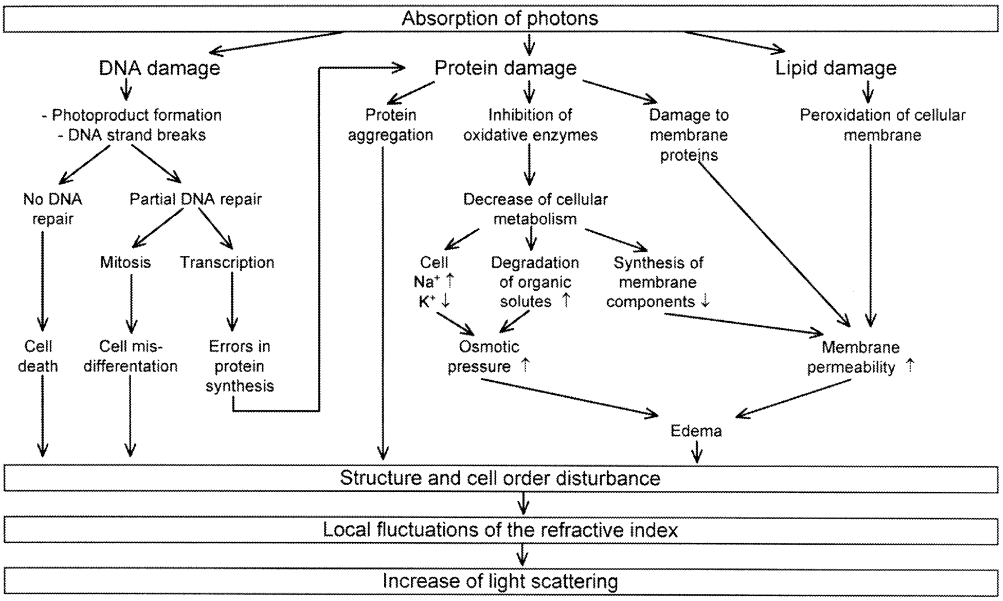


Figure 6: Possible mechanisms of lens damage induced by UVR. (Reprinted with permission from Ralph Michael [Stockholm, Sweden]. Development and Repair of Cataract Induced by Ultraviolet Radiation. *Ophthalmic Research* 2000; February (suppl): 7. Copyright by S. Karger, Basel).

3.7 Importance of time in UVR-induced cataract.

Influence of fractionation of exposure

In a series of studies on the effects of onetime exposure to UVR in the rabbit, Pitts et al. [9], found that the threshold for permanent lenticular damage was reached at a dose of 5 kJ/m^2 with exposure to UVR 300 nm. UVR-induced cataract after single exposure has been extensively studied. However, scarce information was found regarding lens damage after repeated exposure to UVR. Bachem [18] demonstrated that repeated exposure to UVR induces anterior cortical opacities. Duke-Elder concluded that multiple exposures at intervals shorther than 24 hours are additive in effect [19].

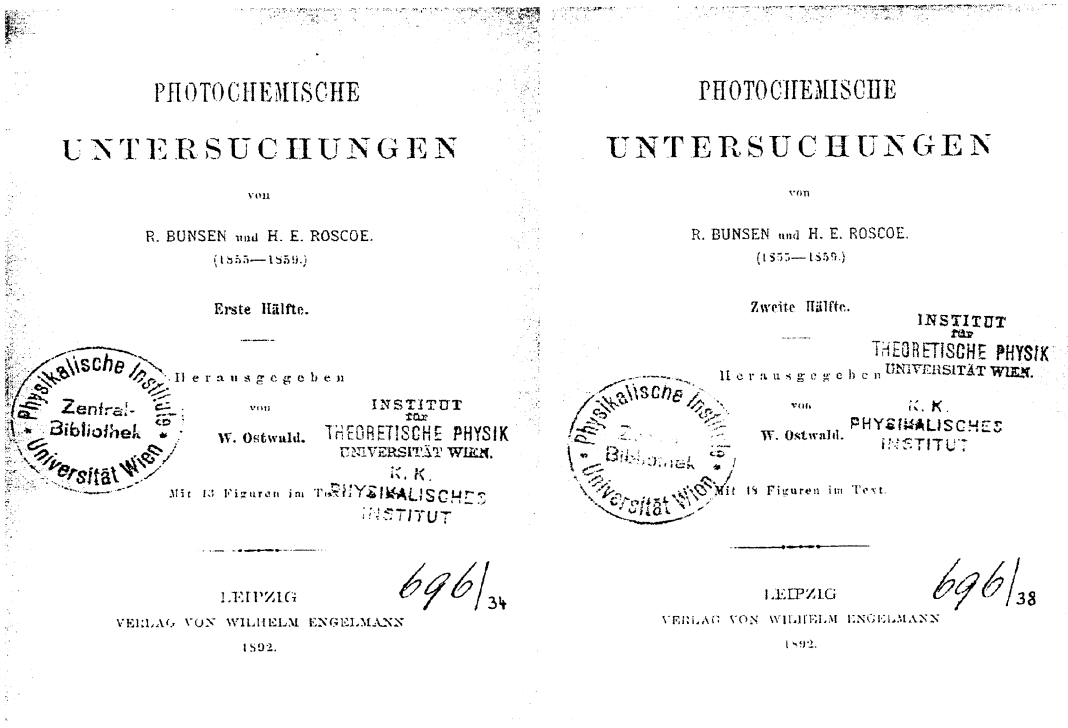
The effect of repeated UVR exposures may be additive in different ways. *Pure additivity* occurs when the resultant effect is the mathematical sum of the effects of the exposures (viz., 1+1=2). *Synergistic additivity* occurs when the resultant effect is greater than the sum of the effect of each exposure (viz., 1+1=3). Finally, *partial additivity* occurs when the resultant effect is less than the sum of the effect of each exposure (viz., 1+1=1.5).

The effects of repeated exposures show *pure additivity* if there is no repair or replacement of the damaged tissue between exposures. The time between exposures in which a damaged tissue undergoes repair is not well established. If repair occurs between two exposures, the exposure will express *partial additivity*. However, when a tissue shows an increased sensitivity after a previous exposure, the response will express *synergistic additivity*. A comprehensive safety standard for the safe use of UVR should include specification of the period of time over which UVR exposures show *pure additivity* [20].

Influence of exposure time

In 1892, Bunsen and Roscoe published the reciprocity law, or second law of photochemistry, stating that irradiance and time exist in a reciprocal relationship to the dose (Figure 7). This has been interpreted to directly apply to some photobiological effects of UVR exposure. Although the biological effect is initiated by a photochemical event that depends on the product of the irradiance and the exposure time, the photobiological effect may not always depend only on the product of the irradiance and the exposure duration. Nevertheless, in many instances if the irradiance and exposure duration changes reciprocally so that the product is constant, the same photobiological effect is expected[21]. In biology, the reciprocity law has been applied to model various photobiological phenomena. Studies were conducted to evaluate the law's validity in UV-A exposure experiments. Cultured human skin fibroblasts suffer lipid peroxidation after UV-A irradiance and this response follows the principle of reciprocity [22]. However, it was shown that the reciprocity law is not valid for in vitro UV-A-induced photohemolysis sensitized by psoralens[23] . In the retina, the

reciprocity law was used to explain the effect of temporal summation of sequential light exposures. To reach a liminal value for retinal stimulation, light of high intensity requires less time than light of low intensity. In the lens, it is believed that for a certain exposure to UVR, the biological effect depends on the product of irradiance and exposure time. The resultant damage remains constant in relationship to a constant cross-product (dose). Ocular safety measures for avoidance of damage from UVR in industrial use assume that the reciprocity law holds true for biological models [24].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UNTERSUCHUNGEN

von

R. BUNSEN und H. E. ROSCOE.

(1855—1859.)

Erste Hälfte.

Herausgegeben

von

W. Ostwald.

INSTITUT für THEORETISCHE PHYSIK UNIVERSITÄT WIEN.

K. K. PHYSIKALISCHES INSTITUT

Mit 13 Figuren im Text.

LEIPZIG

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1892.

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Mit 18 Figuren im Text.

LEIPZIG

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1892.

Figure 7. Reproduction of the cover page of the original thesis in which Bunsen and Roscoe detailed the reciprocity law (1892). The originals are in the Central Library of Physics (Zentralbibliothek für Physik) in Viena, Austria. The reproduction was copied from Hautarzt, 2001, 52: 781. (Copyright by Springer-Verlag).

3.8 Lens biochemistry.

The lens is an avascular transparent tissue enveloped in a basement membrane called the capsule. The principal soluble structural components of the lens are proteins called crystallins. All mammalian lenses have α, β and γ crystallins, although the guinea pig lens contains an additional ζ- crystalline [25]. The lens contains a broad range of electrolytes and low molecular weight solutes, some of which are listed in Table 1. Glutathione is a key component of the lens redox system, with most of the glutathione in the lens in a reduced form (GSH) that plays an important role in protecting lens molecules against oxidation.

Table 1. Major Amino Acids and low molecular weight solutes in lenses of various species

Component[1]	Rat	Cattle	Rabbit	Human
Taurine	19.7	49.9	6.85	0.79
Serine	0.58	6.3	1.42	0.56
Proline	1.23	1.8	1.05	0.16
Glutamic acid	3.83	225.1	5.83	3.42
Glycine	0.31	93.3	1.79	0.79
Glutathione[2]	10.3	4.3	17.3	1.43
Alanine	2.21	26.1	1.27	1.34
Ammonia	2.0	0.5	1.01	----
Urea	2.7	3.8	5.98	4.7
Ascorbic acid	30.0	340.0	150.0	300.0

1) Quantities expressed as micrograms per gram wet weight.
2) Oxidized plus reduced (the major form) glutathione.

Adapted from E. Berman, *Biochemistry of the Eye*, 1991: 204. (Copyright by Plenum Press, New York).

Regarding lens metabolism, it is important to emphasize that the avascular lens relies on the aqueous humor as the major source of oxygen, glucose, and other nutrients. The oxygen level in the lens is very low. Consequently, anaerobic metabolism of glucose versus glycolysis provides at least two-thirds of the lens ATP. The remainder is generated by oxidative metabolism through the tricarboxylic cycle. The highest enzymatic activity in the lens generally is found in the epithelial cells [25].

3.9 Importance of oxidation in the lens

Photooxidative stress results primarily from absorption of radiation by the constituents of the lens (Figure 8). Quantitatively dominant intrinsic UVR-absorbing chromophores in the lens are believed to be tryptophan, 3-HKG and epithelial cell DNA. Certain drugs, such as psoralens, are examples of extrinsic chromophores. The absorption of electromagnetic energy in the primary chromophore produces an excited-state molecule. The excited state molecule may cause direct oxidation or may initiate a complex biochemical chain reaction that leads to secondary oxidation[26].

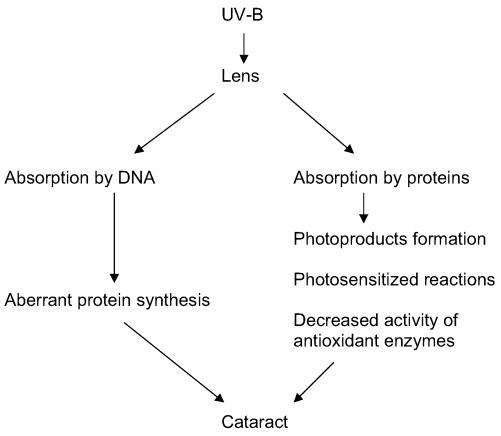


Figure 8: Simplified scheme of photo-oxidative damage in the lens due to UVR exposure.

The principal sites of photochemical oxidation in the lens are believed to be proteins in mitochondrial membranes and the cytoplasm of the cells and the nucleic acids.
Photochemical oxidation of membrane proteins may lead to altered active and passive transport, as well as altered transmembrane signalling. Photooxidation of proteins causes cross-linking, conformational changes, and sometimes aggregation. Damage to intracellular proteins may change

cell structure and intracellular signaling.

Damage to membrane lipids may lead to increased permeability and loss of trans-membrane concentration gradients. Free radicals account for a substantial amount of the oxidative damage in the lens membranes. Important targets of free radical attack are membranes with polyunsaturated fatty acids (PUFA). Lipid peroxidation can be defined as free radical-induced non-enzymatic oxidation of long-chain PUFA. Free radicals react with O_2 and induce the formation of lipid peroxide radical ROO^-, which in turn initiates the propagation of lipid peroxidation, producing a disorganization of cellular membranes. Free radical scavengers carry out the termination of lipid peroxidation. Vitamin E and vitamin C are the most commonly studied free radical scavengers. Membrane-bound vitamin E reacts directly with ROO^- to form a relatively stable phenoxyl radical (tocopheroxy radical). This terminates lipid peroxidation.

Photochemical damage to nucleic acids primarily may disturb DNA replication and transcription and secondarily may affect translation.

Photo-induced changes in the lens that cause aggregation of molecules or disturbs local water balance causes irregular refractive index gradients that result in opacification.

The antioxidant defense mechanisms of the lens protect against photo-oxidative stress. It is generally believed that cataract results when the antioxidant defense mechanisms are overwhelmed[26]. Antioxidant compounds are reducing electron donors, known as free radical quenchers, which often work by providing H^+ and an electron.

Antioxidant compounds in the lens may be divided into extrinsic and intrinsic compounds, although some compounds may bear both properties. Depending on solubility, the antioxidant compounds may be further categorized as water and lipid soluble, with different potential action depending on their solubility.

Glutathione is believed to be the quantitatively dominating intrinsic antioxidant. It is present in high concentration in the lens, predominantly in the reduced form (GSH), thus playing an important role in protecting lens molecules against oxidation. Glutathione is water-soluble and therefore occurs in the cytoplasm of epithelial cells and lens fiber cells.

Pyruvate is also believed to be an intrinsic water-soluble antioxidant[27-29]that plays an

important roll in the antioxidant defense of the lens.

In humans and guinea pigs, ascorbate is an exclusively extrinsic antioxidant but is intrinsically synthesized in some species such as the rat. Ascorbate, is the oxidized form of Vitamin C, or ascorbic acid, and it is the predominant form of ascorbic acid at physiological pH. Ascorbate is water soluble, and it is abundant in the aqueous humor and in the water-soluble fraction in the lens, including cytoplasm of epithelial cells and lens fiber cells.

α-Tocopherol (vitamin E) is an extrinsic antioxidant molecule with reducing action. It is lipid soluble and therefore mainly present in lens membranes.

In addition, there are a number of enzymes that reduce oxidized proteins directly, detoxifies toxic secondary photoproducts, and reduce oxidated antioxidant molecules.

There are several examples of detoxifyng enzymes in the lens. Superoxide dismutase converts the toxic superoxide ion into hydrogen peroxide. Catalase converts hydrogen peroxide into water and oxygen. Glutathione peroxidase reduces hydrogen peroxide with reduced glutathione into oxidized glutathione and water.

There are also several enzymes in the lens that recycle oxidized antioxidants by catalyzing reduction to the reduced form. One of the most important is glutathione reductase, which in combination with NADPH, changes oxidized glutathione into its reduced form (GSH).

3.10 Alfa-tocopherol: a member of the vitamin-E family?

Although *α-tocopherol* and *vitamin E* are used interchangeably in the literature, vitamin E is a functional term that does not refer to a particular chemical structure, whereas the other tocopherols have vitamin E activity. The term *vitamin E* was first used in reference to a fat-soluble factor that, in 1922, was discovered as essential in the diet of rats to facilitate normal reproduction. *Tocopherol* comes from the Greek words *tokos* (childbirth) and *phero* (to bring forth). Eight naturally occurring substances with so-called vitamin E activity have thus far been found in animal tests: d-α-, d-β-, d-γ-, and d-δ- tocopherols and d-α-, d-β-, d-γ-, and d-δ- tocotrienols. Sources of vitamin E in the human diet include

wheat germ, vegetables oils, margarines, nuts, grains and green leafy vegetables [30].

The recommended daily intake (RDI) of α-tocopherol varies in the literature. A common recommendation for humans is a daily intake of 15 international units (IU) for males and 12 IU for females [31, 32] (α-tocopherol 1.49 IU is equivalent to 1 mg α-tocopherol). The RDI for α-tocopherol for rats is around 15 mg/kg[33]. The present investigation used rats weighting 200 g, thus corresponding to an RDI of 3 mg or 4.5 IU. In the studies presented in this thesis the rats were fed 100 IU α-tocopherol daily for 1 month to reach a significant concentration of vitamin E in the lens. Vitamin E is the least toxic of the fat-soluble vitamins. No toxicity in the rat has been observed at doses up to 450 IU/day [32]. The relation between per-oral (PO) intake and intralenticular content remains unknown.

3.11 Protection of vitamin E against UVR-induced cataract

Several experimental models have been used to test the effects of vitamin E in cataract protection. Creighton et al [34] found that vitamin E reduced cataract in a galactose-induced cataract model. Ohta et al [35] demonstrated beneficial effects of vitamin E in a steroid cataract model. Varma et al[36] showed that lens lipid and protein oxidation developed in Emory mice fed vitamin E-deficient chow. In a chronic UVR exposure model using Brown Norway rats, Wegener et al[37] showed that dietary zinc and vitamin E deficiencies increased the levels of cataract.

Epidemiologic data on the relation between α-tocopherol intake and risk for cataract are controversial. High dietary and plasma levels of α-tocopherol have been found to decrease occurrence of lens opacities in some, [38-41] but not in other, [42-44] studies. As mention previously, comparisons among different epidemiologic studies are difficult because of difference in the populations studied and the methodologies used.

Randomized clinical trials (RCT) of vitamin E protection against cataract show different outcomes. The Roche European American Cataract Trial (REACT)[45] showed that daily use of antioxidants produced a deceleration

in cataract progression. A meta-analysis by Trevithick et al[46] showed support for vitamin E administration in cataract protection. However, the Age-Related Eye Disease Study (AREDS)[47] found no effect of vitamin E on cataract progression. Similarly, in a recently published study directed by McNeil,[48] no effect was found of vitamin E on the progression of age-related cataract.

3.12 Importance of *p53* and apoptosis in the lens

The gene *p53* encodes a phosphoprotein involved in the control of cell growth. Its expression and function have been documented in malignancy, apoptosis, and abnormal cell proliferation processes.

The *p53* gene has been extensively studied for more than 2 decades, mostly in regard to cancer research; it is frequently mutated in human cancer[49]. Early work on *p53* suggested that it might be implicated in the promotion of cell proliferation. Earlier experiments by Reich and Levine[50] showed that arrested mouse cell growth exhibited very low levels of *p53* mRNA and protein.

The *p53* gene plays multiple roles in cells. In the normal cell cycle, the *p53* gene is inactive. It is in damaged cells that *p53* acts to regulate growth. Normally, damage to cellular DNA initiates increased expression of *p53*, which leads to cell-cycle arrest. This interruption permits DNA repair, but if the repair is unsuccessful the cell undergoes apoptosis. There are two types of p53 proteins, the wild-type found in normal cells and the mutant type found in cancer cells. Expression of high levels of wild-type p53 protein has two possible outcomes: cell cycle arrest or apoptosis.

Apoptosis due to UVR is a well-described process in the lens[51, 52]. Apoptosis can be mediated through increased expression of p53 protein or can be independent from p53 protein overexpression. DNA-damaging agents induce higher levels of p53 protein in cells. In response to genotoxic stress, *p53* acts as an "emergency brake" inducing cell cycle arrest or apoptosis, and protecting the genome from accumulating excess mutations. Consistent with this notion, cells lacking *p53* were shown

to be genetically unstable and thus more prone to tumors[53] .

Recently, the p53 protein has been described in normal cells in the murine eye [54]. Association between *p53* activation and UVR exposure has been extensively studied in the skin[55, 56], mostly in relation to malignant melanoma and other skin cancers. However, scarce information was found in the literature regarding an association between p53 protein expression and damage after UVR exposure in the lens.

4. AIMS OF THE THESIS

The aims of the thesis are to:

- Investigate the additive effects of radiation exposure in UVR-induced cataract.
- Evaluate the validity of the reciprocity law for UVR-induced cataract.
- Determine whether α-tocopherol may protect against UVR-induced cataract.
- Investigate the influence of α-tocopherol in the application of the reciprocity law for UVR-induced cataract.
- Investigate whether expression of the *p53* gene increases after UVR exposure.

5. METHODS

All experiments were conducted in vivo in Sprague-Dawley (SD) rats. In each animal, one eye was exposed to UVR while the contralateral eye served as control. Different exposure times and intervals between exposures were used. In two experiments α-tocopherol was added. In all experiments, forward lens light scattering was measured 1 week after the UVR exposure.

5.1 Experimental animals and ethical permissions

A 6-week-old female SD rat was used as the experimental animal. Animals were kept and treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Approved ethical permission was granted by the Stockholm Northern Animal Experiments Ethical Committee. Ethical approval codes were N37/98 (studies I and II); N209/00 (studies III and IV); N135/02 (study V).

Ten minutes preceding the unilateral UVR exposure, the animal was anesthetized with ketamine 94 mg/kg plus xylazine 14 mg/kg intraperitoneally [57]. The mydriatic tropicamide was instilled in both eyes. One week after the last exposure the animals were killed by carbon dioxide asphyxiation followed by cervical dislocation. Michael and colleagues [58]have shown that forward light scattering peaks 1 week after UVR exposure in 6-week-old SD rats. The eyes were enucleated, and both lenses were extracted and placed in balanced salt solution. Vestiges of the ciliary body were removed from the lens equator under a microscope.

5. 2 Source of UVR

The experiments used an exposure system based on the principle of Köhler's illumination (Figure 9). The system was set up in our laboratory by Söderberg [59]. The radiation source was a 200 W high pressure mercury lamp (HBO 200 W, Osram, Germany). A spherical reflector was placed behind the lamp to collect and concentrate the radiation from the backward direction. The radiation passed

through a water filter that absorbed the infrared radiation and then was put through a 300-nm interference filter with a half band width of 10 nm (λ_{max}= 300 nm, $\lambda_{0.5}$=10 nm). Finally the radiation was projected onto the cornea of the exposed eye .

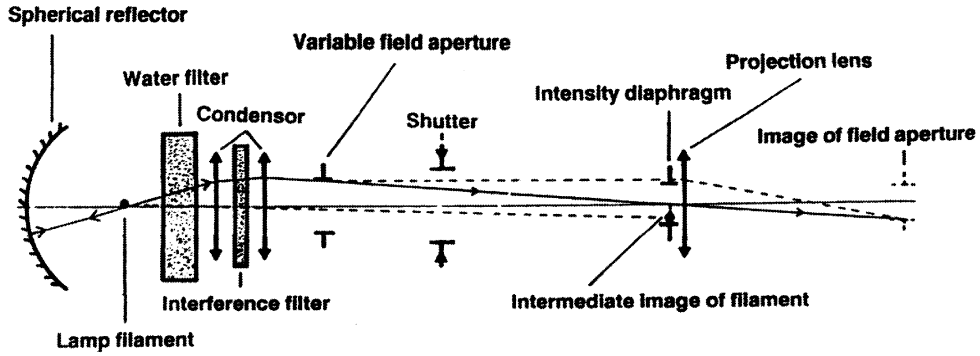


Figure 9: Principle of the interference filter-based exposure source. (From Experimental Cataract Induced by Ultraviolet Radiation, *Acta Ophthalmologica* 1990; 68 (suppl 196): 24. Copyright by Blackwell Publishers).

The relative spectral irradiance behind the filters was recorded (Figure 10) with a spectrometer (Ocean Optics PC 2000; Ocean Optics, Dunedin, Florida, USA) and the total UVR dose at the corneal plane was measured with a thermopile (model 7104; Oriel, Stratford, Connecticut, USA) calibrated regularly by the Swedish National Bureau of Standards to a source traceable by the US National Institute of Standards (NIST). The intensity of light was measured before and after each UVR exposure.

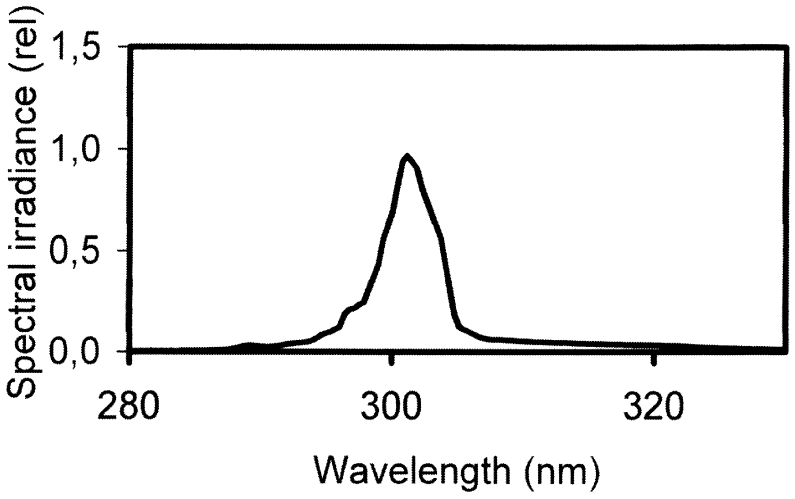


Figure 10: Spectral distribution of the radiation used. Note that the radiation peaks at 300 nm (UV-B).

5.3 Experimental procedure

5.3.1 Exposure to UVR

All animals were exposed to the same total dose of radiation (8 kJ/m^2) and same spectrum of UVR in the 300-nm wavelength region. In all studies the animals were killed 1 week after the last exposure.

5.3.2. Photography

Morphologic changes were photographically recorded and evaluated with a stereomicroscope (model MZ6; Leica Microssystem AG, Wetzlar, Germany). Photographs were taken of each lens against a dark background with a white grid. During photography the anterior surface of the lens faced the camera.

5.3.3.Forward light scattering

The intensity of forward light scattering was measured three times for each lens with the Light Dissemination Meter developed by Söderberg et al [60]. The Light Dissemination Meter uses the principle of the dark-field illumination, in which the illuminating light transilluminates the lens at 45° below the horizontal plane. At this angle, the light cannot enter the objective aperture. If the object scatters light in the forward

direction, a defined fraction of light reaches the objective and is measured by a photodiode (Figure 11).

The scattering standard was a lipid emulsion of diazepam (Diazemuls ®, Kabi Vitrum, Stockholm, Sweden), and the unit was expressed as transformed Equivalent Diazemuls Concentration ($_t$EDC) [60]. A typical value for a normal rat lens is about 0.1 $_t$EDC whereas for a very opaque lens about 1 $_t$EDC. Between 0 and 1 $_t$EDC, the intensity of forward light scattering increases linearly with the concentration of standard in the measurement cuvette.

Figure 11. Photographic view of the light dissemination meter with basement.

5.3.4 High-Perfomance Liquid Chromatography (HPLC) Procedures

In the third and fourth studies, α-tocopherol was measured with small molecule separation using high-performance liquid chromatography (HPLC) with an HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, California, USA) and fluorescence detection with an HP 1100 fluorescence detector. The same technique was used for measurement of GSH concentration in the lens in the third study.

Immediately after forward light scattering measurement, each lens was weighed, placed in Ringer

solution (1 ml) and frozen at –70 °C. The frozen lenses were transported on dry ice to AS Vitas (Research Park, Gaustadalleen 21, N-0349 Oslo, Norway) for analysis.

α-Tocopherol analysis

Excess Ringer solution was removed from each rat lens by transferring the lens to facial tissue paper. The lens was then transferred to a new Eppendorf vial containing 150 µL of the original Ringer solution for homogenization with a motorized pellet grinder (Kimble/Kontes, Vineland, New Jersey, USA). The homogenate was then diluted with 300 µL 2-propanol containing the internal standard tocol and the reductant butlylated hydroxytoluene (BHT). After mixing for 15 min and centrifugation at 4000 g at 10 °C for 10 min, an aliquot of 25 µL was injected from the supernatant into the HPLC system. Tocopherol isomers were separated on a 2.1 x 50 mm reversed phase column. The column temperature was 40 °C. Detector characteristics were excitation at 295 nm and emission at 330 nm. A calibration curve was made from analysis of a 3 % albumin solution enriched with known α-tocopherol concentration.

Glutathione (GSH) determination

The same lenses that were measured for α-tocopherol were measured for GSH in the third study. Total GSH in each rat lens was measured with an HPLC kit with fluorescence detection (catolog no. 195-4075; Bio-Rad Laboratories, Hercules, California, USA). The sample was protected from light.

Each rat lens was homogenized with 60 µL Ringers solution and 200 µL phosphate-buffered saline (PBS) using a motorized pellet grinder (Klimbe/Kontes) in an Eppendorf vial. Following centrifugation at 3500 g, at 10°C for 15 min., 50 µL of the supernatant was transferred to an empty vial. To this was added 100µL Internal Standard, 50 µL Reduction Reagent and 100 µL thiol specific reagent (BioRad kit). After mixing and incubation for 5 min at 50 °C, the mixture was cooled for 5 min. Precipitation reagent (100 µL) was added, and the vial was centrifuged at 3500 g, at 10°C for 5 minutes. The supernatant was transferred to an empty vial, and 20 µL was injected into the HPLC column.

Thiols were separated on a 3.2 mm x 70 mm reversed phase column (BioRad). The column temperature was 45 °C. The detector characteristics were excitation at

385 nm, emission at 515 nm. A calibration curve was made from analysis of PBS enriched with known GSH concentrations.

5.3.5 *p53* gene analysis

In all 10 SD albino rats were unilaterally exposed to UVR, with the other eye serving as a control. 1 week after exposure the rats were killed, lenses were extracted, and 8 lenses (4 exposed and 4 not exposed) were analysed by immunohistochemistry to localize *p53*. The remaining 6 exposed and 6 nonexposed lenses were analysed by real-time reverse transcription polymerase chain reaction (RT-PCR) to estimate *p53* expression. Immediately after the forward light scattering measurements, lenses analyzed by RT-PCR were, weighted and placed in Ringer solution 1 mL and frozen in –70ºC. The frozen lenses were transported on dry ice to the Clinical Research Center at the Örebro University Hospital. Total RNA from lenses was extracted by using TRIZOL (Invitrogen Corporation, Carlsbad, California, USA) according to the manufacturer's guidelines. Synthesis of cDNA was performed using an oligo-p(dT)15 primer and avian myeloblastosis virus reverse transcriptase (Roche Diagnostics Corporation, Indianapolis, Indiana, USA). The cDNA was amplified by RT- PCR in a LightCycler (Roche). The mathematical model used for calculation of differentiated mRNA expression is based on PCR efficiencies and the mean crossing point.

Lenses analyzed by immunohistochemistry were fixated in paraformaldehyde and embedded in paraffin. Paraffin lens sections were incubated for 2 hours at room temperature with a fluorescein isothiocyanate conjugated (FITC) monoclonal p53 antibody (mouse monoclonal [B20.1 (BP 53.122)] antibody to p53 FITC), washed in PBS, and finally mounted with mounting medium containing 4',6-Diamidino-2-phenylindole (DAPI) (Vectrashield, Vector Laboratories, Inc, California, USA).

5.4 Experimental design

5.4.1 Additivity of UVR doses

In the study on additivity of UVR doses with increasing interdose intervals, 100 rats were randomly divided into 5 interdose interval groups (6 hours, 1, 3, 9 or 30 days). In each rat, one eye was exposed to

UVR while the contralateral eye was left unexposed. In each lens, intensity of forward light scattering was measured three times.

5.4.2 Reciprocity of intensity and exposure time

The investigation of intensity and exposure time reciprocity for induced photobiological damage comprised two experiments. First, 100 rats were randomly divided into 5 exposure time groups (7.5, 15, 30, 60 and 120 minutes). In the second experiment, 80 rats were divided into 4 exposure time groups (5, 7.5, 11, and 15 minutes). All rats were exposed to UVR in one eye while the contralateral eye was left unexposed. In each lens, intensity of light scattering was measured three times.

5.4.3. α-Tocopherol protection of the lens from UVR induced cataract

In the study on α-tocopherol protection of the lens from UVR-induced cataract, 40 rats were divided into two supplementation groups. One group was supplemented with α-tocopherol and solvent while the other group received solvent only. All rats were unilaterally exposed in vivo to UVR. All lenses were measured three times for intensity of forward light scattering.
Three samples were taken from each lens and put on the HPLC column for α-tocopherol detection. Three samples were taken from each lens and put on the HPLC column for GSH detection.

5.4.4 α-Tocopherol protection against increased sensitivity with exposure times around 15 minutes

For investigation of a possible protection of α-tocopherol against increased sensitivity with exposure times around 15 minutes, 80 rats were divided into 4 groups (n= 20 per group). The 4 groups respectively received 5 minutes of UVR plus vitamin E and corn oil; 5 minutes of UVR plus corn oil; 15 minutes of UVR plus vitamin E and corn oil; or 15 minutes of UVR plus corn oil. All rats were unilaterally exposed to the same dose of UVR in one eye. Intensity of forward light scattering was measured three times for each lens.

5.4.5. *p53* mRNA and protein expression

For analysis of *p53* mRNA and protein expression, 10 rats were unilaterally exposed to UVR. Intensity of forward light scattering was measured three times in both lenses.

The lenses from four of the animals were processed for immunohistochemistry of p53 protein. For each lens, four sagittal sections were recovered and stained for p53 protein.

The lenses from six of the animals were analyzed for p53 mRNA content. From each lens, two samples were taken. For each sample the mRNA was reverse transcribed into cDNA. From the cDNA solution two samples were taken and submitted for PCR with p53-specific primer for amplification of *p53* genetic message. Each amplified sample was read twice for total content of amplified cDNA message.

5.5 Statistics

Intensities of forward light scattering in the lens were analyzed as the difference between the exposed and the contralateral (nonexposed) eye for each animal. The difference was used as primary data to reduce the effect of interindividual variation and to increase statistical power. Confidence intervals (CI) for means in light scattering were estimated and plotted. Use of CI was preferred to Student t-test, because the CI more intuitively provides variation.
Contrasts of difference of intensities of forward light scattering with regard to the explanatory variable studied among groups were frequently determined by analysis of variance (ANOVA) after initial testing for homogeneity of variances. If ANOVA was significant, subsequent comparison tests were performed. When the initial testing indicated inhomogeneity of variance or departure from normality, nonparametric ANOVA and comparison test were used.
The significance level and the confidence coefficient were set to 0.05 and 0.95, respectively.

6. RESULTS AND DISCUSSIONS

6.1 Additivity study (paper I)

Lenses exposed twice to UVR showed different cataract patterns with varying time intervals between exposures. A nonexposed lens is shown in Figure 12A. Subcapsular and equatorial cataract were found in the 6-hour and 1-day interval groups (Figure 12B). Complete cataract was found in the 3-day interval group. The background grid is obscured by the opacity (Figure 12C). Mild cortical cataract was observed in the 9-day interval group (Figure 12D). Finally, silky subcapsular opacities were present in the 1-month interval group (Figure 12E).

The densest lens opacities (complete cataract) were found when lenses were exposed twice to UVR at an interval of 3 days. The increased lens opacity may be considered to express *synergistic additivity*. Lenses exposed at an interval of 30 days between UVR exposures showed silky opacities. These may be considered to express *partial additivity*.

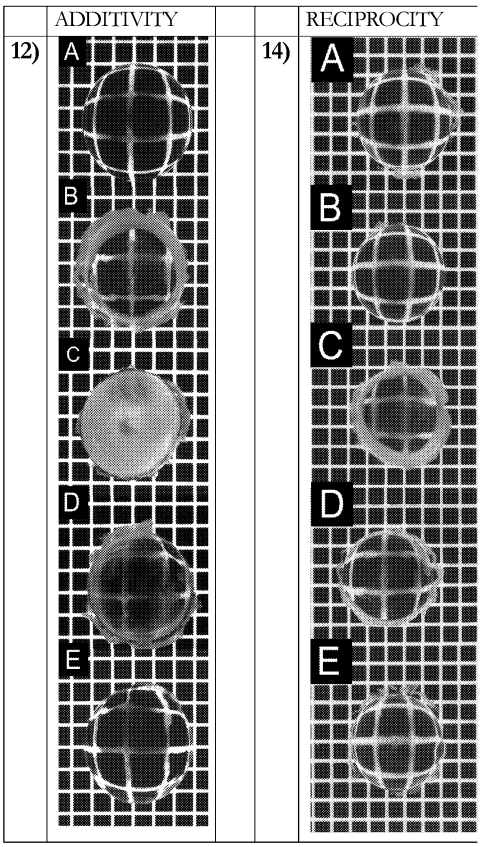


Figure 12 & 14: Microphotographs of isolated rat lenses 1 week after the last exposure to UVR. The distance between the white wires is 0.79 mm. Fig 12 (on the left), (A) nonexposed lens; and lenses exposed at intervals of (B) 6 hours, (C) 3 days, (D) 9 days, and (E) 30 days. Fig 14 (on the right), (A) non-exposed lens; and lenses exposed at intervals of (B) 5 and 7.5 minutes, (C) 15 minutes, (D) 11 and 30 minutes, and (E) 60 and 120 minutes.

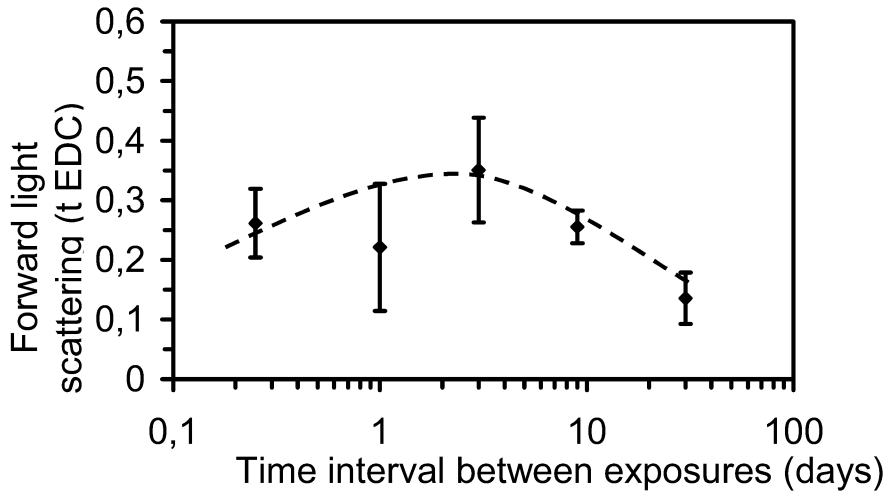


Figure 13: Difference in intensity of forward light scattering between exposed and nonexposed lenses after 4 kJ/m² UVR exposure. Two exposures were performed in each case at different intervals (6 hr, 1 day, 3 days, 9 days and 30 days). Bars indicate 95% CIs for the means. The approximate fitted curve (---) is shown.

The difference in intensity of forward light scattering between exposed and nonexposed lens was measured (Figure 13). There was no difference in forward light scattering between the 6-hour and 1 day interval groups. The highest amount of forward light scattering was seen in the 3-day interval group. However, the intensity decreased as the interval between exposures increased. The lowest level of light scattering was detected in the 1-month interval group.

The approximate fitted curve shows the general trend among the data [Figure 13 (---)]. Light scattering increases at lower time intervals, peaks at 3-day interval, and then decreases at greater intervals. The first part of the curve shows *increasing synergistic additivity* followed by a peak. The last part of the curve reveals *decreasing synergistic additivity. Increasing synergistic additivity* in the time interval between UVR exposures of 6 hours and 3 days may be due to increased photosensitization. The increased photosensitization can be explained by formation of photoproducts between the two

exposures. Photoproducts may increase UVR absorption and, thus, cellular damage increases after a second exposure to UVR.
Another explanation might be that, after UVR exposure, DNA undergoes partial repair in the period of 1 to 9 days after exposure. Partial repair creates instability in the DNA structure. A second exposure in this period may produce greater damage to the DNA structure. *Decreasing synergistic additivity* in the time interval between UVR exposures of 3 and 30 days may be attributed to repair mechanisms occurring in the lens. After UVR exposure, cellular and DNA repair occurs and diminishes the damage inflicted on the lens.

6.2 Reciprocity study (paper II)

Lenses were exposed to the same doses of UVR at varying exposure times. A nonexposed lens is shown in Figure 14A. Very mild superficial cataract was found in the groups exposed for 5 and 7.5 minutes (Figure 14B). The group exposed to UVR for 15 minutes showed the densest opacities of any group. Cortical, equatorial opacities (localized cortical cataract), and vacuoles were seen in lenses exposed for 15 minutes (Figure 14C). Lenses that were exposed for 11 and 30 minutes developed silky equatorial cataract and vacuoles (Figure 14D). The groups exposed for 60 and 120 minutes showed mild superficial cataract (Figure 14E). No group developed nuclear cataracts.

All lenses were exposed to the same dose of UVR. According to the reciprocity law, the same level of damage in all lenses was expected. However, cortical and equatorial opacities and vacuoles were seen only in lenses irradiated for 15 minutes and not in lenses irradiated for shorter or longer periods. These findings contradict the expected applicability of the reciprocity law in the UVR-induced cataract model. Thus, the photochemical reciprocity law does not necessarily hold true for biological models.

The groups were compared by calculating the difference in intensity of forward light scattering between the exposed and the nonexposed lenses (Figure 15). Intensity of forward light scattering was lowest in lenses exposed to UVR for 5 minutes. Forward light scattering increased gradually in all groups from 5 to 15 minutes, with lenses exposed to 15 minutes showing the

highest intensity of forward light scattering. At all exposure times greater than 15 minutes, forward light scattering decreased. There was no difference in forward light scattering in lenses exposed to UVR for 60 and 120 minutes.

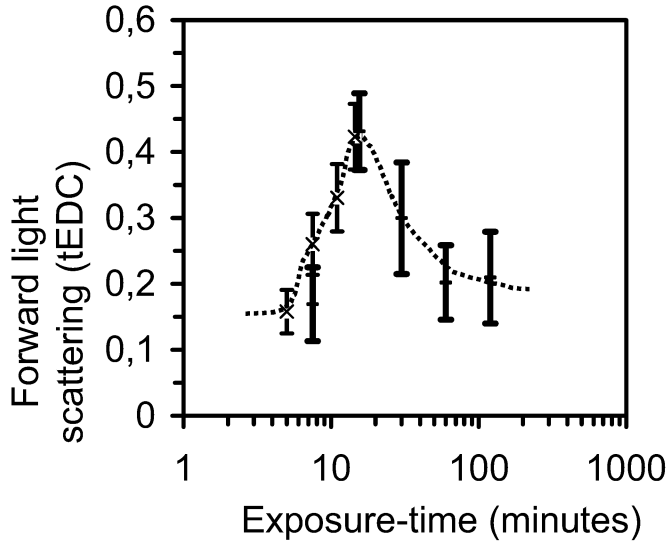


Figure 15: Difference in intensity of forward light scattering between nonexposed lenses and lenses exposed to UVR at various periods (5, 7.5, 11, 15, 30, 60 and 120 minutes). Bars indicate 95% CIs for the means. The approximate fitted curve (---) is shown.

An approximate fitted curve shows the general trend among the data Figure 15 (---). Light scattering increases, peaks and decreases with increasing exposure time. It is possible to say that the first part of the curve shows an *increasing sensitivity*, the middle part a peak and the final part shows *decreasing sensitivity*. *Increasing sensitivity* may be secondary to protection by antioxidant mechanisms. Antioxidants may protect the lens during short exposure times (5 minutes). The antioxidants are then consumed and lens protection decreases with longer exposure times (15 minutes). *Decreasing sensitivity* when lenses are exposed for longer than 15 minutes may be attributed to a decrease in photosensitization.

Photosensitization in the lens is principally attributed to the chromophores, which have the capability to increase the absorption of UVR and, thus, increase damage. A decreased level or decreased activity of UVR-absorbing chromophores may account for the finding of less damage in lenses exposed for longer than 15 minutes. The *decreasing sensitivity* might also be attributed to a reduced lens UVR dose in the 60- and 120- minute exposure groups, caused by corneal opacities. These opacities result both from the desiccation during anesthesia and as well as from the UVR itself. Another explanation for the *decreasing sensitivity* could be attributed to the lower irradiance with prolonged exposure time. Irradiance may possibly be more important than the total dose administrated as an explanation for decreased damage in the lens.

6.3 Vitamin E and UVR-induced cataract (paper III)

Treatment with α-tocopherol 100 IU /day PO for 4 weeks protects the rat lens from expressing forward light scattering after exposure to 8 kJ/m^2 UVR. Significant differences in the amount of cataract was found between α-tocopherol-treated and untreated lenses. Very mild superficial cataract developed in the UVR-exposed lenses in the α-tocopherol-supplemented group, whereas the corresponding control group developed lens cortical and equatorial opacities and vacuoles (Figure 16).

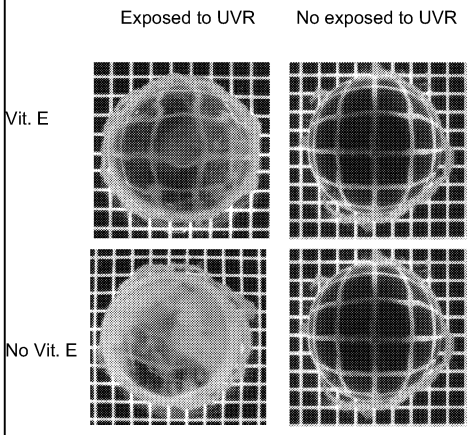


Figure 16: Photographic images of rat lenses treated or not treated with vitamin E that were either exposed or not exposed to UVR.
Top row: lenses of the rats treated with vitamin E. Bottom row: lenses of the rats not treated with vitamin E.

α-Tocopherol certainly protects the lens against cataract in experimental models. Whether vitamin E derivatives can protect against cataract in humans remains a subject of debate. Numerous observational studies have examined the association between antioxidants and cataract[38-44]. The results of the

observational studies are inconsistent, and in any case, should be interpreted with caution, because the characteristics of the UVR-exposed and UVR-unexposed participants may not be similar.

Evidence from RCT that tested the protective effects of vitamin E against cataract showed different results. The AREDS study [47] demonstrated no impact of antioxidants in cataract progression, whereas the REACT study [45] showed a deceleration in cataract progression due to antioxidants, including vitamin E. The different antioxidant concentrations and different follow-up periods make comparisons difficult. Recently, Chylack [61] pointed out that high doses of antioxidants may not prevent against cataract, although lower doses may have protective effects.

According to the Cochrane Database of Systematic Reviews, a review of the role of antioxidant supplementation in preventing and slowing the progression of age-related cataract is now in progress [62]. The aim of this systematic review is to examine evidence regarding the effectiveness of antioxidants in preventing and delaying the progression of different types of cataract. At the time of this writing, the reviewers had not reached a conclusion.

Several compounds have antioxidant properties in the lens. Vitamin C is a well-known antioxidant that seems to protect the lens against oxidative damage and cataract[63]. The most prevalent antioxidant in the lens is glutathione[64], which also promotes the antioxidant properties of vitamin C and vitamin E by maintaining these nutrients in a reduced state. It seems that supplementation with vitamin C or vitamin E enhances the resistance of glutathione-depleted lens epithelial cells to oxidative cell death [65]. Because vitamin C is a water-soluble vitamin, PO administration can increase its concentration in the rat lens [66]. However, whereas rats are capable of synthesizing vitamin C, humans cannot, making vitamin C an essential nutrient in the human diet. Although the rat model has been appropriate for several vitamin C studies, the dissimilarity in rat and human vitamin C metabolism is a disadvantage. The guinea pig seems to be a closer model to the human, because guinea pigs also lack the ability to synthesize vitamin C. Lens vitamin C concentration in guinea

pigs can be increased by PO vitamin C administration. Shang et al [65]showed that glutathione, vitamin C and vitamin E have common targets and work synergistically to protect the cells from oxidative damage. Further investigations to test synergistic effects of vitamin E and C are needed.

The lens glutathione concentration increased in the rats treated with α-tocopherol. α-Tocopherol may indirectly inhibit the consumption of glutathione through oxidation of α-tocopherol leaving the –SH groups on glutahione intact. Alternatively, α-tocopherol may directly stimulate glutathione synthesis. Seth [67] postulated that such a direct stimulation could be due to a modulating effect on some of the glutathione-related enzymes in the lens. Masaki et al[68] demonstrated a direct increase of glutathione levels in the HaCaT keratinocytes by α-tocopherol application, probably due to up-regulation of γ-GCS-HS mRNA.

6.4 Reversal of lack of reciprocity in UVR-induced cataract treated with vitamin E (paper IV).

Lenses exposed to UVR for 5 minutes showed a lower intensity of forward light scattering than lenses exposed to UVR for 15 minutes (Figure 17). In the 5-minute exposure group, no significant difference in light scattering measurements was detected between the α-tocopherol plus solvent group and the solvent-only groups. However, when the lens was exposed to UVR for 15 minutes, a significant difference in light scattering was detected between the groups.

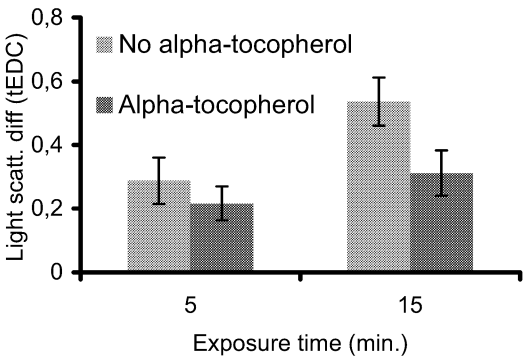


Fig 17: Difference in intensity of forward light scattering between lenses that were exposed or not exposed to UVR, treated or not treated with vitamin E, for exposure periods of 5 or 15 minutes. Bars indicate 95 % CIs for the mean. Light scattering difference was measured as ₜEDC.

Because the primary event ─the photochemical reaction─ is known to obey the reciprocity law, a hypothesis of this study was that the observed increased efficiency in causing a photobiological effect in the 5- to 15- minute exposure time domain, could be due to increased photosensitization, decreased quenching or both. Regardless of the process, the kinetics must be biologically driven and independent of the number of photons. A hypothesis in the paper II was that lack of the applicability of the reciprocity law in UVR-induced cataract in the lens in the exposure time domain of 5- to 15- minute exposure domain was probably due to increased oxidative damage. UVR exposure for 5 minutes is not as harmful as exposure for 15 minutes, and protective antioxidant mechanisms in the lens probably are only effective for short periods.

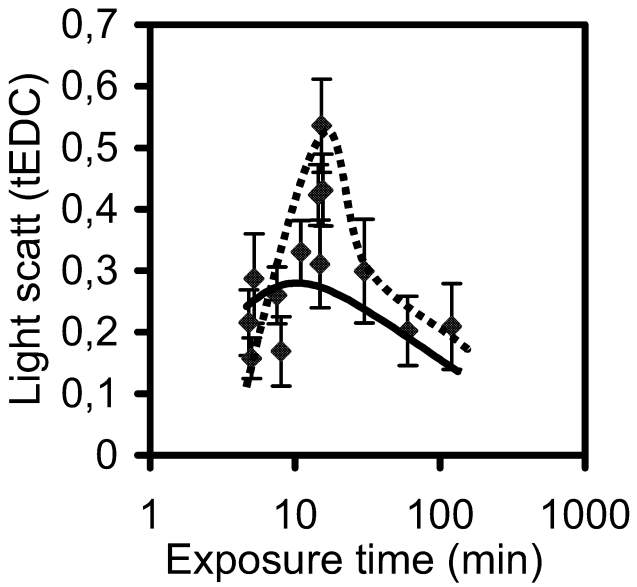


Figure 18. Difference in intensity of forward light scattering between exposed and nonexposed lenses, which were either treated or not treated with vitamin E, and exposed to UVR at intervals for 5, 7.5, 11, 15, 30, 60 and 120 minutes. Approximate fitted curves are shown for lenses treated (──), or not treated (---) with vitamin E.

Forward light scattering data from paper II and paper IV were put together in Figure 18. An approximate fitted curve was plotted for lenses with and without α-tocopherol . In lenses not treated with α-tocopherol the photochemical reciprocity seems not to apply for shorter periods of exposure. *Increasing sensitivity* was found between 5 and 15 minutes. However, when lenses were treated with α-tocopherol, forward light scattering seems to obey the reciprocity law. No *increasing sensitivity* was found between 5 and 15 minutes. *Increasing sensitivity* might be due to consumption of antioxidant mechanisms, and α-tocopherol seems to reverse this *increasing sensitivity*.

The photochemical reciprocity law has been demonstrated to not hold true for photobiological changes in the skin. Photocarcinogenesis represents the sum of a complex of simultaneous biochemical events that ultimately lead to the occurrence of skin cancer. These events, initiated by UVR, include the formation of DNA products, DNA repair, mutation of proto-oncogenes and tumor suppressor genes. Other factors that can influence skin cancer development include immunologic responses, antioxidant defenses, and dietary factors [69]. For photocarcinogenesis it has been shown that at any given level of total UVR, radiation intensity is a major determinant for the quality and quantity of the response. Therefore, the photochemical reciprocity law does not apply for photocarcinogenesis [70, 71].

The photochemical reciprocity law provides understanding of certain photobiological phenomena. The Bunsen-Roscoe law applies to certain photobiological effects that are caused by a single event, in most instances it is a cascade of effects, and not a single incident, that modifies the final response. Therefore, the reciprocity law must be applied with caution when considering complex photobiological reactions. Application of this law often results in an oversimplification that ignores the totality of important factors that have an impact on the final response.

6.5 *p53* expression and UVR-induced cataract (paper V)

The fifth study in this thesis showed that *p53* mRNA expression measured after p53-specific amplification of *p53* mRNA code by RT-PCR was significantly higher (147%) in the UVR-exposed lenses than in the nonexposed lenses (Figure 19).

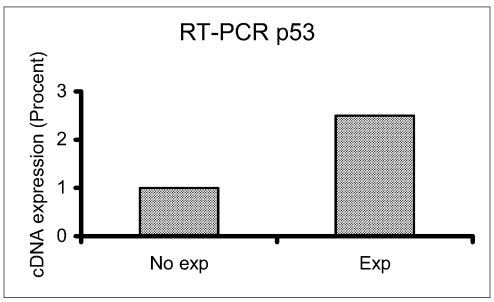
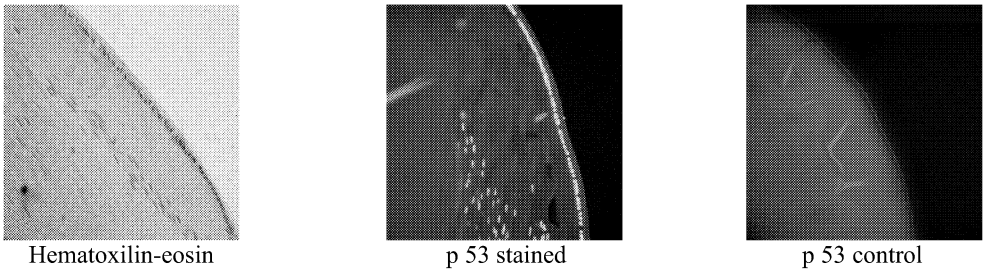


Figure 19. Difference in relative values of cDNA expression of *p53* gene measured with RT-PCR for lenses exposed or not exposed to UVR.

Immunohistochemistry showed that the p53 protein was localized in the lens epithelial cells (Figure 20). All exposed lenses showed cataract.

Lens cell apoptosis is known to occur after UVR exposure [52]. Apoptosis in the lens may or may not be mediated through the *p53* gene. Takamura et al[72] stated that apoptosis certainly occurs in the lens epithelium in the sugar-induced cataract model, and they believed it to be mediated via *p53*. Further studies are needed to corroborate the relation between increased expression of the *p53* gene and apoptosis in the lens.



Hematoxilin-eosin | p 53 stained | p 53 control

Figure 20. Photomicrographs of rat lenses showing immunostaining. (*Left*) Hematoxilin & eosin stain. (*Center*) Immunostaining of lens sections with monoclonal p53 antibody (FITC). (*Right*) Control stained lens (without antibodies against p53).

The *p53* gene has been described as the most studied gene related to cancer. Because it is a cell-cycle regulator gene, mutations in *p53* induce cell proliferations that can lead to cancer. Mutant types of *p53* have been extensively studied in cancer research, but not in normal or cataractous lenses. The question whether cancer patients are more prone to develop cataract than healthy subjects remains an unsolved topic for future research.

As described previously, cataract may occur as a result of a wide variety of factors, including metabolic disorders, exposure to toxic agents, trauma, exposure to radiation, nutritional deficiencies, and hereditary factors. The role of environmental factors, such as UVR exposure, has been extensively documented [4-6]. Differences in *p53* gene activity can probably explain differences in cataract prevalence among subjects who are exposed to the same environment.

It appears that the antioxidant nutrients can inhibit, prevent, or regress experimental cancer through *p53* regulation. Antioxidant nutrients have been found to enhance the expression of the cancer suppressor wild-type *p53* gene, [73]and to diminish the expression of the mutant *p53*, the oncogene expressed in a large number of tumors [74]. Schwartz et al [75] showed that vitamin E inhibits the development of *p53*-mediated squamous cell cancer of hamsters. The present study has demonstrated that vitamin E protects against cataract in the UVR-induced cataract model. Vitamin E protection against cataract was attributed to its antioxidant properties. It is possible that vitamin E alters *p53* gene activity in the lens, thereby offering protection against cataract. Further investigations to test the involvement of the *p53* pathway in cataract protection with vitamin E are needed.

6.6 UVR and the skin

Although, the primary aim of this thesis is to focus on the effects of UVR on the eye, some considerations about the effects of UVR on the skin must be addressed. Logically, the skin is the part of the body that is most extensively exposed to the sun and, consequently, to UVR. Acute UVR exposure of normal skin results in several clinical effects, including sunburn inflammation (erythema) and tanning, histological changes,

(e.g., thickening of the epidermis), and local or systemic immunosuppression. Chronic UVR exposure leads to photoaging, sustained immunosupresion and photocarcinogenesis[76]. All of these changes appear to be caused by both UV-A and UV-B[77-79].
Shindo et al[80] have shown that multiple exposure of the skin to UV-A, even when separated by an interval of time, produces damage in the skin that they attributed to consumption of antioxidant enzymes. Similar results were described in the present thesis with regard to fractioning and addition of UVR doses in the lens.

No direct relation was found between UVR exposure time and photocarcinogenesis in the skin. It was concluded that the reciprocity law seems to not apply to photocarcinogenesis in the skin[69, 71]. This is consistent with the lack of reciprocity found in the present thesis for certain exposure time domains.

Antioxidants have long been used to treat skin diseases. Several reports [81-84] support the use of topical vitamin E to reduce the risk of skin cancer. Vitamin E provides protection against UVR-induced damage through a combination of antioxidant and UVR absorptive properties in the skin. However, evidence supporting oral administration of vitamin E as a strategy for skin cancer risk reduction is still controversial. In 2000, Sthal et al[85] published a study in *The American Journal of Clinical Nutrition* in which they concluded that oral supplementation with carotenoids and vitamin E protects against erythema in humans. However, 4 years later, Mc Ardle et al[86] published an article in the same journal in which they concluded that vitamin E or beta-carotene supplementation had not effect on skin sensitivity to UVR. This is consistent with the contradictive findings with regard to vitamin E protection against cataract.

Apoptosis in the skin after UVR exposure has been also demonstrated [87, 88]. Apoptosis in epidermal keratinocytes after UV-B exposure is thought to be mediated through increased *p53* activity [55, 56]. Exposure of the lens to UVR appears to elicit a similar apoptotic response to radiation exposure in the skin, and similar paths of mediation seem to apply.

6.7 Future developments

Although the results of this thesis provide important insights into UVR-induced cataract, further research is needed to fully elucidate the relationship between the variables studied. Specifically:

6.7.1 Additivity study (paper I):

Future research should investigate the role of addition of various levels of UVR when the lens has been exposed to UVR on more than two occasions. The roles and mechanisms of photoproduct formation should also be explored.

6.7.2 Reciprocity studies (paper II and IV)

Applicability of the reciprocity law to UVR levels other than those used in the present experiment should be investigated. Further biochemical studies are needed to elucidate the role of antioxidants at shorter and longer UVR exposure times. The role of DNA damage and repair for different UVR exposure times also should be explored.

6.7.3. Vitamin E and cataract (paper III)

The protective effect of vitamin E against cataract was demonstrated using an experimental UVR model, but the challenge remains to determine whether vitamin E can protect against cataract in humans. Several RCTs of oral vitamin E supplementation and lens protection have been conducted, but they show inconsistent results. An RCT on topically administered vitamin E and cataract protection might yield useful information.

6.7.4. P53 and cataract (paper V)

The activity of p53 protein in human lens epithelial cells remain to be determined, and the relation between increased expression of p53 and apoptosis in the lens after UVR exposure needs to be corroborated. In addition, the relation between p53 expression, antioxidants and cataract protection in the lens should be investigated.

7. CONCLUSIONS

The principle of additivity does not hold true for UVR-induced cataract. Whereas *synergistic additivity* occurred when UVR exposures were separated by 3 days, *partial additivity* was found when UVR exposures were separated by a 30-day interval.

The photochemical Bunsen-Roscoe reciprocity law should be applied to UVR-induced cataract with caution. Different amounts of lens damage were found when the lenses were exposed to the same dose of UVR delivered at different exposure times.

Oral administration of vitamin E was found to protect against UVR-induced cataract in albino SD rats.

Inapplicability of the photochemical reciprocity law at shorter exposure times in UVR-induced cataract may be due to antioxidant consumption, but vitamin E was found to reverse this failure.

Lens UVR exposure was found to induce *p53* gene overexpression.

8. SAFETY IMPLICATIONS

8.1 Exposure patterns

The data presented in this thesis were obtained from experiments performed on animals (SD rats). The ultimate goal is to extrapolate this information to its possible application in treatment of humans.

Is it more dangerous to be exposed to the sun (i.e.,sunbathe) every day versus once a week? The data from the additivity study indicate that a 3-day interval between UVR exposures is actually more dangerous than a 1-day interval. This is an important point for consideration. Many people spend time outdoors during their weekends off from work, meaning that they may have extensive sun exposure once or twice a week. Although we have not investigated the effect of a 6-7 day interval between UVR exposures, the trend indicated by the additivity study raises a warning flag for such exposure patterns.

In the second study, same total dose was delivered with different exposure time (Table 2).

Exp. time (min)	Irradiance (W/m²)
7.5	17.77
15	8.88
30	4.44
60	2.22
120	1.11

Table 2. Exposure patterns applied when investigating the dependence of damage on exposure time at equivalent doses.
(1 J = 1 W * s)

The measured terrestrial solar irradiance varies for different latitudes, altitudes and environmental conditions. Measurement taken under a clear sky at solar noon on La Palma, Canary Islands, latitude 28°N, altitude 2350 m, solar elevation angle 85° (air mass 1.0) for the wavelengths 280-315 nm was 3.2 W/m². In Stockholm, Sweden, latitude 59.3°N, altitude 0 m, solar elevation angle 50° (air mass 1.3) the irradiance was 0.9 W/m² [89]. These results show that neither in the Canary Islands nor Stockholm is it possible to reach an irradiance of 8.8 W/m² (the level of intensity used in the 15-minutes experiments).

In order for a rat to reach 8 kJ/m² in La Palma it would have to stare continuously at the sun with dilated pupils for 41 minutes; the same outcome whereas it would take 133 minutes in Stockholm. Because the reciprocity law does not hold for UVR-induced cataract, the degree of lens damage would not be the same. Presumably the rat exposed in La Palma for 41 minutes will show more lens opacities than the rat exposed in Stockholm for 133 minutes.

8.2 Vitamin E and cataract protection

It is still unclear whether vitamin E protects the lens against cataract in humans. Trevithick et al [46] performed a meta-analysis on seven different studies and found protective effects of vitamin supplementation. Supplementation with vitamin E alone (400 IU/day) for at least 5 years yield a reduction in risk for cataracts progression of 56%, whereas supplementation in combination with vitamin C resulted in a risk reduction of 72%. The authors estimated that, in the United States, the use of vitamin supplementation would annually

save $2.1 billion in cataract surgery costs.

Other groups have concluded that the available data do not support a strategy of population-wide vitamin administration. In 1997 the Swedish Council on Technology Assessment in Health Care (SBU), a public office affiliated with the Department of Health, presented a systematic literature review on antioxidant supplementation. The report concluded that a relation between antioxidants and rate of disease could not be identified. Therefore, antioxidants would seem to have no effect in cataract prevention, and antioxidant supplementation could not be recommended [90].

8.3. Considerations regarding the future of lens research

At the most recent meeting of the European Association for Vision Eye Research (EVER), Dr. Sheila West [91] emphasized the importance of cataract prevention in a growing global population with increased life expectancy, especially in heavily populated countries such as China and India. Although the US National Eye Institute has proclaimed the availability of simple and effective treatments mean that cataract is no longer a problem in United States, this is not true worldwide. Efforts must be made to enhance both prevention and treatment, but to date the focus has been primarily put on treatment (surgery) with very little investment made in preventive actions. Research leading to cataract prevention should receive higher priority. The future of lens research rests on interdisciplinary efforts that improve our knowledge and expand our frontiers. Research in combination with geriatrics, as well as genetics and genetic-environmental interactions are key areas for further investigation. Genetical treatments undoubtedly will play an important role in future research. Although our understanding of genetics has increased tremendously over last 2 decades, there is still a large gap between basic research and clinical application. Feasible strategies for cataract treatment and prevention must be based on the conjunction of basic science and clinical efforts.

9. REFERENCES

1. Sir Stewart Duke-Elder KW. System of ophthalmology. London: Henry Kimpton 1961, , pp7-17.
2. Sir Stewart Duke-Elder KW. System of ophthalmology. London: Henry Kimpton 1961, , pp309-11.
3. Merriam J. The concentration of light in the human lens. *Trans Am Ophthalmol Soc* 1996;XCIV:803-918.
4. Dolin PJ. Ultraviolet radiation and cataract: a review of the epidemiological evidence. *Br J Ophthalmol* 1994;78:478-82.
5. Hiller R, Sperduto RD, Ederer F. Epidemiologic associations with cataract in the 1971-1972 National Health and Nutrition Examination Survey. *Am J Epidemiol* 1983;118:239-49.
6. Cruickshanks KJ, Klein BE, Klein R. Ultraviolet light exposure and lens opacities: the Beaver Dam Eye Study. *Am J Public Health* 1992;82:1658-62.
7. DeChardonnet M. Sur la penetration des radiations actiniques dans l'oeil de l'homme et des animaux vertebres, et sur la vison des radiations ultra-violettes. *Journal de physique theorique et appliquee* 1883;II. t. II:219-25.
8. Boettner EA, Wolter JR. Transmission of ocular media. *Invest Ophthalmol* 1962;1:776-83.
9. Pitts DG, Cullen AP, Hacker PD. Ocular effects of ultraviolet radiation from 295 to 365 nm. *Invest Ophthalmol Vis Sci* 1977;16:932-9.
10. Sliney DH. Ocular exposure to environmental light and ultraviolet-the impact of lid opening and sky conditions. Cataract Epidemiology. Devlopments in Ophthalmology. Basel: Karger; 1997:27, pp 63-75.
11. Taylor HR, West SK, Rosenthal FS, Munoz B, Newland HS, Abbey H et al. Effect of ultraviolet radiation on cataract formation. *N Engl J Med* 1988;319:1429-33.
12. McCarty Cea. Assessment of lifetime ocular exposure to UV-B: the Melbourne visual impairment project. Developments in Ophthalmology, Cataract Epidemiology. Basel: Karger; 1997:27, pp 9-13.
13. Söderberg PG. Experimental cataract induced by ultraviolet radiation. *Acta Ophthalmol (Copenh)* 1990;68(SUPPL. 196):1-77.
14. Encyclopaedia B. Robert Bunsen. In Encyclopaedia Britannica, Online http://www.eb.co.uk. London: Encyclopaedia Britannica 2000
15. E,B. Radical. *Encyclopædia Britannica* from Encyclopædia Britannica Premium Service. <http://www.britannica.com/eb/article? 2004.
16. Pitts D, Cameron LL, Jose JG. Optical radiation and cataracts. In Optical radiation and visual health. Waxler M. Boca Raton, Florida: CRC Press 1986, pp5-41.
17. Michael R, Vrensen G, Löfgren S, Söderberg PG. Ultrastructural changes in the rat lens after in vivo threshold dose irradiation. *manuscript* 1998;.
18. Bachem A. Ophthalmic ultraviolet action spectra. *Am J Ophthalmol* 1956;41:969-75.
19. Duke-Elder S, MacFaul PA. Radiational injuries. In System of ophthalmology, Vol. XIV Injuries, Part 2 Non-mechanical injuries. Duke-Elder S. London: Henry Kimpton 1972, pp912-33.
20. Zuclich JA. Cumulative effects of near-ultraviolet induced corneal damage. *Health Phys* 1980;38:833-8.

21. Duke-Elder S, MacFaul PA. The sensation of light. System of ophthalmology, Vol. IV The physiology of the eye and of vision. Duke-Elder S. London: Henry Kimpton 1968, pp553-6.
22. Morliere P, Moysan A, Tirache I. Action spectrum for UV-induced lipid peroxidation in cultured human skin fibroblasts. *Free Radic Biol Med* 1995;19:365-71.
23. Kyagova Aea. Suppression of delayed-type hypersensitivity and hemolysis induced by previously photooxidized psoralen: effect of fluence rate and psoralen concentration. *Photochem Photobiol* 1997;65:694-700.
24. ACGIH. 1995-1996 Threshold Limit Values for chemical substances and physical agents and Biological Exposure Indices. Cincinnati: American Conference of Governmental Industrial Hygienists; 1996.
25. Berman E. Biochemistry of the eye. Colin Blakemore. New York and London: Plenum Press 1991, pp201-20.
26. Albert DM, Jakobiec FA. Principles and practice of ophthalmology. Principles and practice of ophthalmology. London: W. B. Saunders; 2000:102, pp 575-7.
27. Varma SD, Devamanoharan PS, Morris SM. Photoinduction of cataracts in rat lens in vitro. Preventive effect of pyruvate. *Exp Eye Res* 1990;50:805-12.
28. Varma S, Hedge,K, Henein,M. Oxidative damage to mouse lens in culture. Protective effect of pyruvate. *Biochim Biophys Acta* 2003;1621(3):246-52.
29. Varma S, Et a. Attenuation of galactose-induced cataract by pyruvate. *Free Radic Res* 1999;30(4):253-63.
30. Halliwell B, Gutteridge J. Free radicals in biology and medicine. Free radicals in biology and medicine. Oxford: Claredon Press; 1998, pp 208-9.
31. Halliwell G. Free radicals in biology and medicine. Free radicals in biology and medicine. Oxford University Press; 1998.
32. Champe P, Harvey R. Biochemistry. Lippincott's. 1987, pp330-1.
33. Institute for Laboratory Animal Research. Nutrient requirements of the laboratory rat. In Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995 (1995). Institute for Laboratory Animal Research. . 1995, pp11-79.
34. Creighton M, Ross W, Stewardt P, Sanwal M, Trevithick J. Modelling cortical cataractogenesis. Effects of vitamin E treatment on galactose-induced cataracts. *Exp Eye Res* 1985;40:213-22.
35. Ohta Y, Okada H, Majima Y, Ishiguro I. Anticataract action of vitamin E: its estimation using an in vitro steroid cataract model. *Ophthalmic Res* 1996;28(SUPPL 2):16-25.
36. Varma,S, et al. Studies on Emory mouse catarcts: oxidative factors. *Ophthalmic Res* 1994;26(3):141-8.
37. Wegener A, Heinitz M, Dwinger M. Experimental evidence for interactive effects of chronic UV radiation and nutritional deficiencies in the lens. *Dev Ophthalmol* 2002;35:113-24.
38. Seddon J, Christen W, Manson J, LaMotte F, Glynn R, Buring J et al. The use of vitamin supplements and the risk of cataract among US male physicians. *Am J Public Health* 1994;84(5):788-92.
39. Leske M, Chylack LJ, He Q, Wu S, Schoenfeld E, Friend J et al. Antioxidant vitamins and nuclear opacities: the longitudinal study of cataract. *Ophthalmology* 1998;105(5):831-6.
40. Vitale S, West S, Hallfrisch J, Alston C, Wang F, Moorman C et al. Plasma antioxidants and risk of cortical and nuclear cataract. *Epidemiology* 1993;4(3):191-3.
41. Jacques P, Chylack LJ, Hankinson S, et al. Long-term nutrient intake and early age-related nuclear lens opacities. *Arch Ophthalmol* 2001;119(7):1009-19.

42. Mares-Perlman JA, Brady WE, Klein BE, Klein R, Palta M, Bowen P et al. Serum carotenoids and tocopherols and severity of nuclear and cortical opacities. *Invest Ophthalmol Vis Sci* 1995;36:276-88.
43. Gale C, Hall N, Phillips D, Martyn C. Plasma antioxidant vitamins and carotenoids and age-related cataract. *Ophthalmology* 2001;108(11):1992-8.
44. Teikari J, Virtamo J, Rautalahti M, Palmgren J, Liesto K, Heinonen O. Long-term supplementation with alpha-tocopherol and beta-carotene and age-related cataract. *Acta Ophthalmol Scand* 1997;75(6):634-40.
45. Chylack LJ, Brown N, Bron A, Hurst M, Kopcke W, Thien U et al. The Roche European American Cataract Trial (REACT): a randomized clinical trial to investigate the efficacy of an oral antioxidant micronutrient mixture to slow progression of age-related cataract. *Opthalmic Epidemiol* 2002;9(1):49-80.
46. Trevithick J, Mitton K. Vitamins C and E in cataract risk reduction. *Int Ophthalmol Clin* 2000;40(4):59-69.
47. Age Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E and beta carotene for age-related cataract and vision loss: AREDS report no 9. *Arch Ophthalmol* 2001;119(10):1439-52.
48. McNeil,J, et al. Vitamin E supplementation and cataract: randomized control trial. *Ophthalmology* 2004;111(1):75-84.
49. Prives C, Hall P. The p53 pathway. *J Pathol* 1999;187(1):112-26.
50. Reich N, Levine A. Growth regulation of a cellular tumor antigen, p53, in nontransformed cells. *Nature* 1984;308(5955):199-201.
51. Li WC, Spector A. Lens epithelial cell apoptosis is an early event in the development of UVB-induced cataract. *Free Radic Biol Med* 1996;20:301-11.
52. Michael R, Vrensen G, van Marle J, Gan L, Söderberg PG. Apoptosis in the rat lens after in vivo threshold dose ultraviolet irradiation. *Invest Ophthalmol Vis Sci* 1998;13:2681-7.
53. Ko L, Prives C. p53: puzzle and paradigm. *Genes Dev* 1996;10(9):1054-72.
54. Pokroy R, Tendler Y, Pollack A, Zinder O, Weisinger G. p53 expression in the normal murine eye. *Invest Ophthalmol Vis Sci* 2002;43(6):1736-41.
55. Kawagishi N, Hashimoto Y, Takahashi H, Ishida-Yamamoto A, Iizuka H. Epidermal cell kinetics of pig skin in vivo following UVB irradiation: apoptosis induced by UVB is enhanced in hyperproliferative skin condition. *J Dermatol Sci* 1998;18:43-53.
56. Healy E, Reynolds NJ, Smith MD, Campbell C, Farr PM, Rees JL. Dissociation of erythema and p53 protein expression in human skin following UVB irradiation, and induction of p53 protein and mRNA following application of skin irritants. *J Invest Dermatol* 1994;103:493-9.
57. Wixson SK, White WJ, Hughes HC. A comparison of Pentobarbital, Fentanyl-Droperidol, Ketamine-Diazepam anesthesia in adult male rats. *Lab Anim Sci* 1987;37:726-30.
58. Michael R, Söderberg PG, Chen E. Long-term development of lens opacities after exposure to ultraviolet radiation at 300 nm. *Ophthalmic Res* 1996;28:209-18.
59. Söderberg PG. Development of light dissemination in the rat lens after exposure to radiation in the 300 nm wavelength region. *Ophthalmic Res* 1990;22:271-9.
60. Söderberg PG, Chen E. An objective and rapid method for the determination of light dissemination in the lens. *Acta Ophthalmol (Copenh)* 1990;68:44-52.
61. Chylack L. Effectiveness of antioxidants against human cataract. *Ophthalmic Res Suppl* 2004;36(1).

62. Tao,JP, Davis R, Navaneethan,SD, Mathew,MC. Antioxidant supplementation for preventing and slowing the progression of age-related cataract. Cochrane Database of Systematic Reviews. 3, 2004 .
63. Hegde K, Varma,SD. Protective effect of ascorbate against oxidative stress in the mouse lens. *Biochim Biophys Acta* 2004;5(1670):12-8.
64. Giblin F. Glutathione: a vital lens antioxidant.. *J Ocul Pharmacol Ther* 2000;16(2):121-35.
65. Shang,F, LU,M, Dudek E, Reddan,J, Taylor A. Vitamin C and vitamin E restore the resistance of GSH-depleted lens cells to H2O2. *Free Radic Biol Med* 2003;34(5):521-30.
66. Garland D. Ascorbic acid and the eye. *Am J Clin Nutr* 1991;54(6 (SUPPL)):1198-202.
67. Seth R, Kharb S. Protective function of alpha-tocopherol against the process of cataractogenesis in humans. *Ann Nutr Metab* 1999;43(5):286-9.
68. Masaki H, Okano Y, Ochiai Y, et al. Alpha-tocopherol increases the intracellular glutathione level in HaCaT keratinocytes.. *Free Radic Res* 2002;36(6):705-9.
69. Black HS, deGruijl FR, Forbes PD, Cleaver JE, Ananthaswamy HN, deFabo EC et al. Photocarcinogenesis:an overview. *Photochem Photobiol* 1997;40(1):29-47.
70. Schindl A, Rosado-Schlosser B, Trautinger F. Reciprocity regulation in photobiology. An overview. *Hautarzt* 2001;52(9):779-85.
71. Willis I, Menter JM. Effect of varying dose of UV radiation on mammalian skin: simulation of decreasing stratospheric ozone. *Invest Dermatol* 1983;80(5):416-9.
72. Takamura Y, Kubo E, Tsuzuki S, Akagi Y. Apoptotic cell death in the lens epithelium of rat sugar cataract. *Exp Eye Res* 2003;77(1):51-7.
73. Schwartz JL, Antoniades DZ, Zhao S. Molecualr and biochemical reprogramming of oncogenesis through the activity of prooxidants and antioxidants. *Ann N Y Acad Sci* 1993;28(686):262-78.
74. Greenblatt M, Bennett,WP, Hollstein,M, Harris C. Mutations in the p53 tumor supressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994;15(54):4855-78.
75. Schwartz J, Shklar G, Trickler D. p53 in the anticancer mechanism of vitamin E. *Eur J Cancer B Oral Oncol* 1993;29B(4):313-8.
76. Matsumura Y, Ananthaswamy HN. Toxic effects of ultraviolet radiation on the skin. *Toxicol Appl Pharmacol* 2004;195(3):298-308.
77. Cohen D, DeLeo VA. Ultraviolet radiation-induced phospholipase A2 activation occurs in mammalian cell membrane preparations. *Photochem Photobiol* 1993;57:383-90.
78. Peak JG, Peak MJ. Comparison of initial yields of DNA-to-protein crosslinks and single-strand breaks induced in cultured human cells by far- and near-ultraviolet light, blue light and X-rays. *Mutat Res* 1991;246:187-91.
79. Matsui MS, DeLeo VA. Longwave ultraviolet radiation and promotion of skin cancer. Review. *Cancer Cells* 1991;3:8-12.
80. Shindo Y, Hashimoto T. Time course of changes in antioxidant enzymes in human skin fibroblasts after UVA irradiation. *J Dermatol Sci* 1997;14:225-32.
81. Fryer MJ. Evidence for the photoprotective effects of vitamin E. [Review]. *Photochem Photobiol* 1993;58:304-12.
82. Krol E, Kramer K, Liebler D. Photoprotective actions of topically applied vitamin E. *Drug Metab Rev* 2000;32(3-4):413-20.
83. Dreher F, Maibach H. Protective effects of topical antioxidants in humans. *Curr Probl Dermatol* 2001;29:157-64.

84. Burke KE, Clive J, Combs G, Nakamura R. Effects of topical L-selenomethionine with topical and oral vitamin E on pigmentation and skin cancer induced by ultraviolet radiation in Skh:2 hairless mice. *J Am Acad Dermatol* 2003;49(3):458-72.
85. Stahl W, Heinrich U, Jungmann H, Sies,H, Tronnier H. Carotenoids and carotenoids plus vitamin E protect against ultraviolet light-induced erythema in humans. *Am J Clin Nutr* 2000;71(3):795-8.
86. McArdle F, Rhodes LE, Parslew RA, Close GL, Jack CI, Friedmann PS et al. Effects of oral vitamin E and beta-carotene supplementation on ultraviolet radiation-induced oxidative stress in human skin. *Am J Clin Nutr* 2004;80(5):1270-5.
87. Iwasaki K, Izawa M, Mihara M. UV-induced apoptosis in rat skin. *J Dermatol Sci* 1996;12:31-5.
88. Baba T, Hanada K, Hashimoto I. The study of ultraviolet B-induced apoptosis in cultured mouse keratinocytes and in mouse skin. *J Dermatol Sci* 1996;12:18-23.
89. Wester U. Personal information. Stockholm: Swedish Radiation Protection Institute; 1996.
90. Isaksson B, Andresson C, Asplund K, et al. Preventing disease with antioxidants. SBU reports; 1997.
91. West S. Cataract at the Cross Roads: increasing blindness and challenges for research. *Ophthalmic Research, Suppl* 2004;36(S1):S1.

10. APPENDICES