On the Dark Side of Light:

Genesis and Prevention of Two Spectral Types of Retinal Light Damage

Ontstaanswijze en het voorkomen van
twee spectraal verschillende typen lichtschade aan het netvlies
(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor aan de Universiteit Utrecht
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College voor Promoties in het openbaar te verdedigen op dinsdag 8 juni 1999
des middags te 12.45 uur door Eelco-Marcel Busch geboren op 6 januari 1966 te Amsterdam.

Promotor: Prof.dr. D. van Norren
Co-promotor: Dr. T.G.M.F. Gorgels

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Chapter 1

General introduction

Brief overview of older literature

The records for damage to the eye by light dates back to Plato's Phaedo in which he describes Socrates words on blindness due to staring into the sun. The first written description of acute photic injury was provided by Botenus in the 17th century (Duke-Elder, 1972). The first (currently known) experimental work on retinal light damage was performed by Czerny in 1867, who focussed sunlight into animals' eyes. In 1912 Birch-Hirschfeld was the first to postulate that the visible portion of the solar spectrum was responsible for the retinal changes due to eclipse blinding. From the second half of the 19th century many studies were done on ocular light damage (reviewed in 1916 by Verhoeff and Bell, and by Walker). Until that time ocular light damage research was merely focussed of on the effects of brief, intense exposures.

From 1920 onwards the focus of the research gradually changed to human victims of chronic/prolonged exposures. During World War II Cordes (1944) found an increased number of retinal aberrations (fovemacular retinitis) in Navy personnel in Hawaii and the South Pacific. Smith (1944) found epidemiological evidence for actinic pigment degeneration (and a gradual decrease in visual acuity) due to light exposure. On the same tropical island they found actinic pigment degenerations in the maculae of outdoor personnel, but not amongst yeomen and pharmacy personnel. In prison camps in Southeast Asia "camp eyes" were described by Churchill (1945) and by Dekking (1947). The changes consisted of macular lesions similar to those due to sun gazing (Hope-Ross, Travers and Mooney; 1988, Kraushar, 1986). The condition was more prevalent amongst blue eyed prisoners (probably due to a less dense ocular pigmentation). Recent research suggests the condition described as camp eyes might be a multifactorial problem caused by malnutrition (lack of e.g. antioxidants vitamin E (Wiegand *et al.*, 1986) and vitamin C (Noell *et al.*, 1987)), in combination with daily exposure to bright sunlight. These studies indicate that (prolonged exposures to) ambient light levels under certain circumstances can cause damage to the human retina. There is still an ongoing debate whether cumulative exposure during life is a factor in retinal degeneration of old age ((Taylor *et al.*, 1990, Taylor *et al.*, 1992) vs (Bressler and Bressler, 1995, O'Shea, 1996, Darzins, Mitchell and Heller, 1997). We refrain from a more extensive discussion of this issue, because the emphasis of this thesis is on the effects of relative brief (up to several hours) exposure to optical radiation.

The focus of this thesis however, is on the effects of brief exposures to intense light sources. This condition occurs e.g. during the previously mentioned sun-gazing, but may also occur during ophthalmic practice.

Retinal light damage due to non-thermal mechanisms

Until the 1960's the nature of retinal degeneration due to acute exposures to intense light sources was thought to be a thermal process. In 1962 Vos calculated that eclipse blinding could not be a thermal process by itself, because the rise of temperature was not over the level of an ordinary fever. The notion of photochemical damage emerged only a few years later with a study by Noell and co-workers. A main breakthrough in the research on retinal light damage was probably caused by an accident. The story goes that during Christmas holidays in the lab of Werner K. Noell the lights in the animal facilities were accidentally left on. In the new year they observed a severe reduction in the amplitude of the electro retinogram (ERG) of the irradiated rats. Since illumination levels were relatively low, retinal burns due to temperature

rise could never have caused this damage.

Two action spectra for retinal light damage

Noell *et al.*(1966) determined by ERG measurements an action spectrum in rat for retinal light damage, and found the highest retinal sensitivity around 500 nm. Because the peak absorption of rhodopsin is around this wavelength, they proposed rhodopsin as the chromophore. When this study was published in the mid-1960's a new era in the research on retinal light damage started.

In 1976, Ham *et al.* investigated the threshold for retinal light damage in monkey as a function of the wavelength. They analyzed the retina by funduscopy shortly after irradiation by brief intense light. The minimum dose to cause visible aberrations was noted as threshold level. Monkey retina was the most sensitive for the shorter wavelengths in the visible light. After lens extraction, the sensitivity further increased into the UV (Ham, 1982).

Different histology for the two action spectra

Noell and co-workers also examined the damaged retinas histologically (Noell *et al.,* 1966). Shortly after irradiation they described “the outer nuclei to be in an early stage of pyknosis”...”the pigment epithelial cells are swollen”....“the pigment epithelial cytoplasm is in part replaced by large vacuoles” ...“the final outcome of these changes is the complete disappearance of the visual cell layer and the pigment epithelium”. In 1979 they considered damage to the RPE secondary to the damage in the photoreceptors: “this type of damage (and its sub types) is associated with the disappearance of the pigment epithelium unless the damage is near the threshold.”

Ham and co-workers observed in a single monkey experiment that UV radiation damage was mainly localized in the photoreceptors (Ham *et al.,* 1989). This manifestation differed from results of previous experiments, in which irradiations at 441 nm (blue light) had caused changes in the RPE and the photoreceptors (Ham *et al.,* 1978). Although a similar difference between near-UV and short wavelength visible radiation damage was found in squirrel retina (Collier, 1989), the existence of these two spectral damage types was controversial until recently. For example the RPE was the site of primary injury by repeated near UV irradiations in aphakic and pseudophakic monkeys (Li *et al.,* 1990). However, 1-14 days after exposure to broad band irradiation, in (albino) rats essentially identical histologic manifestations (Rapp, and Smith, 1992) were found for damage by irradiations centered respectively around 360 nm (UVA) and 500 nm (green light). Lawwill described yet another manifestation of damage to visible wavelengths: mitochondria in all retinal layers showed defects. Gorgels and van Norren (1995) recently solved this controversy. In rat retina they convincingly proved the existence of the action spectrum as described by Ham. Two spectral types of retinal light damage were found: At threshold radiation of 320-440 nm damaged only the photoreceptors, and radiation of 470- 550 nm mainly the RPE and to a lesser extend the photoreceptors. Little was known about the development of these damage types. Ham and co-workers’ (1989) results were based on a single monkey experiment, in which they started examining the UVA irradiated retina from two days after exposure onwards. Collier and Zigman (1989) started examining UVA and blue light induced lesions in squirrel retina from 24 hrs after irradiation. Gorgels and van

Norren examined rat retinas only at three days after irradiation.

Retinal light damage depending on irradiance conditions

The data in the 1966 study by Noell *et al.* were obtained under quite specific conditions. The rats were dark adaped for at least 24 hrs before exposure. The exposures lasted up to six hours, during which the animals had a body temperature of about 39.4^{o} C (103^{o} F). In order to obtain detectable damage at normal body temperature, exposures for up to days were required. Broad-band radiation was obtained from fluorescent tubes filtered by plexiglass (no 2092) "green in color and transmitted through the range from 490 to 580 mμ".

Ham's action spectrum was obtained under different circumstances. Monochromatic radiation was applied to small patches of retina in (light adapted), anaesthetized animals, with normal body temperatures. The irradiations lasted from a second up to minutes (Ham *et al.*, 1979).

The difference between action spectra determined by on the one hand Noell c.s. and on the other hand Ham and co-workers (or Gorgels and van Norren, 1995), might depend on irradiance conditions. In order to explain the discrepancy between the action spectra, Kremers and van Norren (1989) discerned two classes of retinal light damage depending on irradiation intensity. The first class of damage occurs under conditions of low retinal irradiance, when the rhodopsin is not fully bleached. Thus rhodopsin, or bleach products of rhodopsin, can be the intermediate in this type of irradiation. Both have been proposed as chromophore (Noell *et al.*, 1966; Williams and Howell, 1983). The second class of light damage occurs under higher retinal irradiances, when the rhodopsin is fully bleached. Under these conditions the action spectrum is different, indicating that the damage is mediated by a different chromophore. Because the rhodopsin is fully bleached, it is unlikely that rhodopsin is the chromophore under these conditions.

For retinal light damage under high irradiance conditions several compounds have been proposed as the possible chromophore. Recently, melanin could be excluded, because retinal light damage appeared similar in albino and pigmented animals (Gorgels and van Norren, 1998). Bleach products of rhodopsin are among the compounds that fulfill the requirements of having an absorption spectrum with increasing sensitivity for shorter wavelengths. Lipofuscin has also been proposed as chromophore. The amount of lipofuscin in the retina increases with age (Yin, 1996), as well as the sensitivity for light damage (O'Steen, Anderson and Shear, 1974; Organisciak *et al.*, 1998). In addition, lipofuscin has been demonstrated to generate oxygen radicals when illuminated with visible light. Because the cytochrome C oxidase complex has its maximum absorbance around 418 nm, this complex is a good candidate for the chromophore in blue light damage. Thus several potential chromophores are available.

The mechanism of acute photochemical light damage

Photochemical light damage results from photochemical reactions in which single photons have sufficient quantum energy to convert individual absorber molecules into one or more different molecules (free radicals) which may be toxic to the cell. In more detail the process is assumed to be as follows: Within the tissue there is a chromophore. The chromophore absorbs the energy of the photon and assumes an excited electrical state: called the singlet state S_1. There are three ways in which the singlet state can loose its energy (Valenzeno, 1993):

1 It can directly interact with its surrounding solvent (usually water).
2 It can emit a lower energy photon, a process known as fluorescence.
3 It can undergo a radiationless transition into a metastable or triplet state S_3.

The triplet state is more stable and longer lived than the S_1 state, and therefore has more time to react with other molecules. This state is thought to be the initial step in the pathway of most photochemical procedures. Further reactions are divided into two major types. In type I reactions the S_3 interacts with the surrounding medium to produce radicals. Type II reactions are those in which the S_3 interacts with molecular oxygen to produce singlet oxygen or superoxide radicals. These are both free radicals and can attack other molecules like for example polyunsaturated fatty acids in membranes (lipid peroxidation) (Borg, 1993), proteins or the DNA (Fridovich, 1993).

The role of oxygen in retinal light damage

In 1966 Noell and co-workers proposed three possible explanations for the damage they had observed: (1) photosensitized oxidation, (2) damage to a metabolic pathway, which was supported by their observations of an increase of the damage by elevated temperatures (3) the formation of a toxic photoproduct, of which the formation of Vitamin A in the photoreceptors is discussed extensively. In 1980 Noell reduced the hypotheses to the following: ”The sequence of events could be the following: Light is absorbed by rhodopsin and intermediates of the bleaching process accumulate. One of these intermediates is a photodynamic sensitizer and has an absorption spectrum within the spectral region of rhodopsin absorption or extending into this region.”

Over thirty years after the postulation of Noell’s hypotheses on the mechanism of retinal light damage, the genesis of retinal light damage has not been resolved. Oxidative processes appear to be involved in damage of the first class, because antioxidants (e.g. DMTU (Lam, Tso and Gurne, 1990; Organisciak *et al.*, 1992), Ascorbate (Organisciak *et al.*, 1985), vitamin E (Stone *et al.*, 1979)) protect against retinal light damage in the prolonged exposure model. But other factors also play a role in the genesis of the damage. “Rats reared under a high environmental light level are more resistant to retinal light damage than animals reared under low intensity light. Some adaptive events associated with visual transduction may also influence the extent of light damage in rats. Alterations in the levels of rhodopsin, alpha transducin and retinal S-antigen (arrestin), and their corresponding mRNAs have been found in rats changed from one rearing environment to another” (Organisciak and Winkler, 1994). Apparently, adaptive and oxidative events in retinal light damage are not separate events, but linked and interdependent processes, both contributing to this type of retinal damage.

For damage of the second class Ruffolo *et al.* (1984) demonstrated that with increased oxygen pressure the threshold for retinal light damage decreased. This finding indicated that

oxygen is also involved in this type of photochemical light damage. Other authors found the involvement of specific reactive oxygen intermediates in the process of retinal light damage (Yamashita, 1992, Zhang, 1994). Oxygen mediated damage is often caused by oxygen free radicals. Free radicals are defined as molecules with an unpaired electron. Molecular oxygen contains two unpaired electrons, which is a relatively stable configuration. When one electron is shifted into a higher energy orbit, this will increase the reactivity. When oxygen accepts an additional electron an oxygen radical is formed. (A much more detailed discussion can be found in "Oxygen radicals in tissue damage" (eds. Tarr and Samson, 1993).

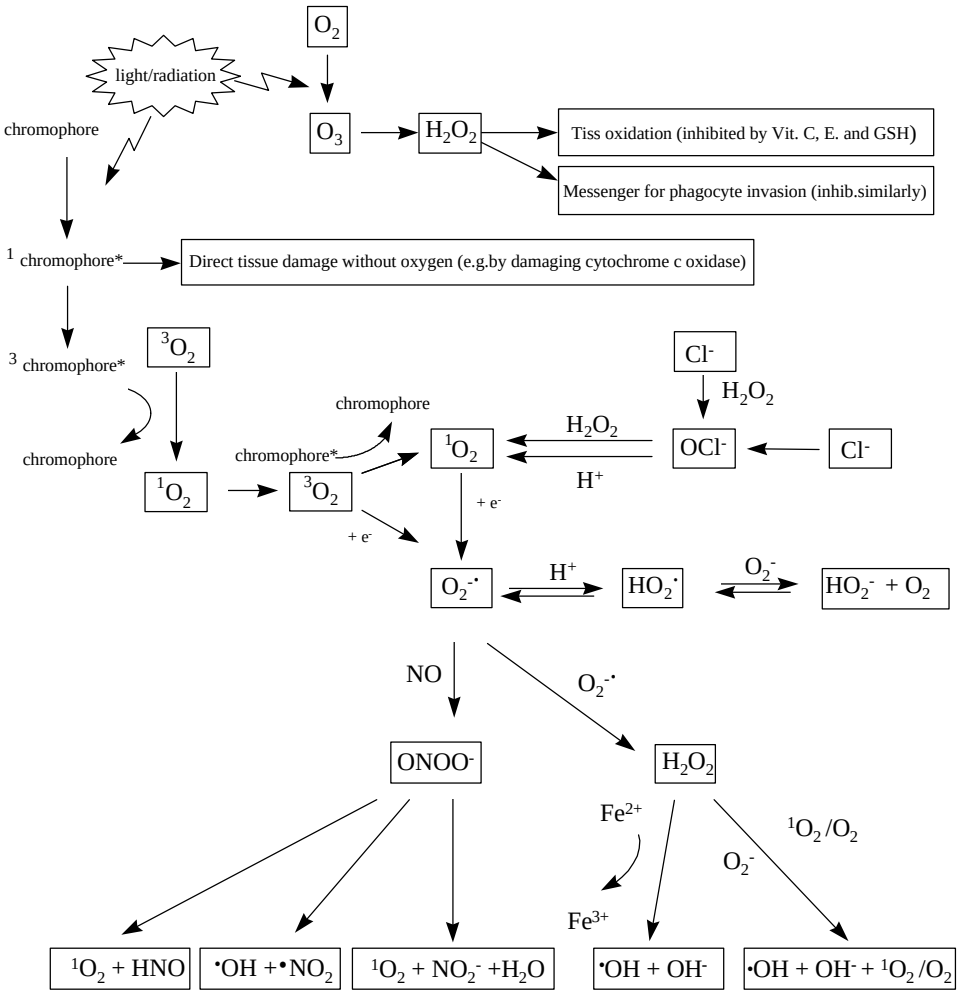
Reduction of oxygen

The reduction of oxygen to water is normally accomplished in four steps. The first step is acceptance of one electron by O_2, resulting in the formation of superoxide anion (1). In the second step superoxide gains another electron, reacts with two hydrogen ions and becomes hydrogen peroxide (2). In the next step hydrogen peroxide accepts one electron and splits into one hydroxyl radical and one hydroxide ion (3). In the final step the hydroxyl radical accepts one electron, reacts with two hydrogen ions and water is formed (4). When the reaction is complete, oxygen is thus reduced into water.

(1) $O_2 + e^-$	➔	$O_2\text{-}\cdot$	(superoxide)
(2) $O_2\text{-}\cdot + e^- + 2\ H^+$	➔	H_2O_2	(hydrogen peroxide)
(3) $H_2O_2 + e^-$	➔	$\cdot OH + OH^-$	(hydroxylradical + hydroxide)
(4) $\cdot OH + e^- + 2\ H^+$	➔	H_2O	(water)

Some of the intermediates in this process are very reactive, and can escape by reacting with other surrounding molecules. Hydrogen peroxide, one of the intermediates in the reduction of oxygen to water, has no unpaired electrons, but is relatively reactive. The group of oxygen radicals, combined with H_2O_2 are called reactive oxygen species (ROX). More on the toxicity of reactive oxygen species can be read in "Oxygen free radicals in tissue damage." In diagram 1 (adapted from Khan and Wilson, 1995) some possibilities for generation of ROX in retinal light damage are drawn.

The possibilities for causing photochemical damage to the retina in this (hypothetical) scheme are: (1) Light could interact with oxygen as the chromophore, and thus generate ozone. Ozone is degraded into hydrogen peroxide which can cause tissue oxidation and phagocyte invasion. (2) Light could interact with a chromophore without involvement of ROX. This would be direct tissue damage, or tissue damage by a chromophore radical. A candidate for such direct tissue damage by short wavelength light is the cytochrome C oxidase complex, which has an absorption peak around 420 nm. (3) Light could interact with a chromophore, which in activated condition reacts with oxygen (transfer of the unpaired electron), and superoxide is formed. Through the cascade described above, hydrogen peroxide, hydroxyl radical, and hydroxide ions can be formed. These can interact with tissue (tissue oxidation), or attract phagocytes. The hydrogen peroxide could interact
with chlorous ions to form reactive chlorous species. Additionally, superoxide could also react with nitrogen to form reactive nitrogen species.



Protection against ROX

In the eye several endogenous systems quench ROX (Castorina *et al.*, 1992, De La Paz, Zhang and Fridovich, 1996): β-Carotene (Handelman *et al.*, 1988), vitamin E (Friedrichson *et al.*, 1995), vitamin C (Organisciak *et al.*, 1990), glutathione (Naash and Anderson, 1989), superoxide dismutase (Behndig *et al.*, 1998) and catalase (Zhang, Agardh and Agardh, 1995; Ohta *et al.*, 1996). Each system has specific quenching properties. For example β-Carotene and vitamin E quench singlet oxygen, and vitamin C quenches superoxide.

Enhancing protection against rox in retinal light damage

Influencing the role of reactive oxygen species in retinal light damage would theoretically be possible by enhancing the quenching of these oxygen species. Three major intracellular antioxidant systems are enzymes. Enzymes generally are large molecules; e.g. MnSOD weights 23 kD per subunit and it can consist of multiple subunits (Fridovich, 1986). Such large molecules do not enter cells freely. Thus enhancing the function of the intracellular enzyme antioxidant systems cannot be accomplished by simply administering the enzyme. The concentration can only be influenced indirectly.

First, the intracellular synthesis of antioxidants can be enhanced. The intracellular concentration of the tripeptide glutathione is determined by its synthesis. The rate limiting factor in the synthesis is the intracellular concentration of the amino-acid L-cysteine (Banks and Stipanuk, 1994). Increasing the intracellular concentration of L-cysteine thus can increase the synthesis of glutathione. But due to the toxicity of cysteine the maximum dose is limited (Pedersen and Karlsen, 1980, Olney *et al.*, 1990, Roberts *et al.*, 1995). N-acetyl-cysteine is a compound containing cysteine. It is able to replenish GSH (Gross *et al.*, 1993), and it is less toxic than cysteine. Due to this decreased toxicity a higher dosage is possible. Additionally, thiols like N-acetyl-cysteine scavenge reactive oxygen intermediates (e.g. peroxide radicals and NO) by themselves (Gillissen *et al.*, 1997).

A second possibility is influencing the function of the enzyme complex SOD. The active sites of the SOD enzymes are the metal ions. To overcome the problem of the large size of the SOD enzymes, low molecular compounds have been synthesized to mimic the function of SOD, in a much smaller molecule (Day *et al.*, 1995). The use of porphyrin ring-structures is a common method to carry metal ions into cells. Such a porphyrin based mimic could be administered to enhance SOD-like activity.

A third possibility would be to increase the catalase activity. Catalase has the same size disadvantage as SOD. In a cell-free-system catalase protects retinal components against UV irradiation (Mattock, Lot and Parker, 1977), but in vivo intravenous injections with the enzyme catalase do not protect against retinal light damage (Zhang *et al.*, 1994).

Other possibilities are administering non-enzymatic inhibitors of specific processes which are caused by oxygen radicals. Vitamin E inhibits lipid peroxidation, and vitamin C scavenges ROX in an aqueous environment. Enhancing the functions of Vitamin C or Vitamin E could thus interfere with ROX mediated processes. For Vitamin C (Organisciak *et al.*, 1990) a protective effect has been described in the low retinal irradiance model. Vitamin E suppletion (Katz and Eldred, 1989) did not protect against retinal light damage. In the eye melanin can also act as a quencher of ROX (Bustamante *et al.*, 1993; Dunford *et al.*, 1995; Korytowski, 1995), and (Rozanowska *et al.*, 1997).

Clinical importance of retinal light damage

For the evaluation of the risk of acute exposure to high intensity light sources, animal models have been developed. Ham *et al.* (1976) determined the threshold for 441 nm radiation by a He-Cd laser for a 100 second exposure 0.20 W/cm^2. The solar irradiance at 440 nm for a 20 nm spectral band, at midday for an eye gazing directly at the sun at sea level, with a pupil diameter of 2 mm, is approximately 0.20 W/cm^2. Transferring these results on monkey retina to humans, results in the conclusion that 100 second of sun gazing at midday can produce a threshold lesion. Based on Ham's studies, guidelines for illumination levels and exposure duration were made, and the use of light in the ophthalmologic practice was adjusted. However, light damage during intra ocular surgery is still a very relevant topic (Minckler, 1995, Mainster *et al.*, 1997). Retinal light damage has been described during a variety of ophthalmologic procedures (Ross, 1984; Kingham, 1984; Lucke and Reme, 1984; Brod *et al.*, 1986; Kuhn, Morris and Massey, 1991). In 1992 Byrnes *et al.* described in a prospective study that during uncomplicated extra-capsular cataract surgery in 28 % of the patients evidence of light damage was found five days after surgery. The shortest procedure described during which photic injury was inflicted, took only 37 minutes, and 100% of the eyes with a total operating time of 100 minutes had photoretinal injuries. These data illustrate that retinal light damage is far from theoretical in ocular surgery. A complicated procedure, or complications occurring during a standard procedure, will increase operating time and thus the risk of iatrogen retinal light damage. A continued search for protective measures therefore seems meaningful.

Outline of this thesis

This thesis is focussed on retinal light damage by relatively brief and intense exposures. It has two major goals. (1) To document the natural development of two spectrally defined types of retinal light damage, (2) to obtain more information about the mechanism(s) of retinal light damage, and to evaluate possible protectors against retinal light damage.

In the first part of the thesis (Chapter 2) we describe the development of the two spectral types of light damage, from shortly after irradiation up to three months after irradiation. Chapter 3 is focussed on the size of the lesion, and the question whether repair occurs. In this Chapter lesions of different sizes are monitored for up to six months after irradiation.

The second part of the thesis has a different focus. In Chapter 4 we tested whether two stereoisomers of N-Acetyl-Cysteine protect against retinal light damage. This was done to obtain more information on the mechanisms of the two types of retinal light damage, and to evaluate whether NAC might be able to protect against retinal light damage during intra ocular surgery. In the last study (Chapter 5) we further focussed on the mechanism of the type of light damage by short wavelengths. We investigated whether Aeol 11201 (a strong inhibitor of lipid peroxidation) influenced retinal light damage by 380 nm radiation. A brief summary and a general discussion of the results obtained are presented in Chapter 6.

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Chapter 2

Temporal sequence of changes in rat retina after UV-A and blue light exposure

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Abstract

Two spectral types of retinal light damage were induced in pigmented rats by irradiating small retinal patches at either 380 or 470 nm. The temporal sequence of changes in the retina was followed for up to 2 months by funduscopy and histology. For both damage types, fundus changes were best visible after 3 days. Histology showed that 380 nm specifically damaged photoreceptor cells, particularly the rods. All cell compartments of the rods, including the nucleus were affected already after 3 hrs. In the next days, damaged rods degenerated. At high doses (2.5 x the funduscopic threshold dose) all rods in the irradiated area were lost, resulting in a local photoreceptor lesion, which was still present at 2 months after the irradiation. At 470 nm, damage occurred both in the photoreceptor layer and in the pigment epithelium. Acute changes, at one hour after irradiation, consisted mainly of damaged mitochondria in these layers. Next, the pigment epithelium showed swelling, an altered melanin distribution and, at high doses (2.5 x threshold), interruptions of the monolayer. Degeneration of photoreceptor cells was initially limited to a few scattered cells, but 3 days after high doses focal areas of massive degeneration were seen. At late stages, the cells of the pigment epithelium recovered and the photoreceptor layer showed a loss of cells. The results show that the spectral damage types are distinct in the early phases, indicating that different mechanisms are involved. Yet, the end effect of both damage types after exposure at doses up to 2.5 times the funduscopic threshold is remarkably similar and consists of local photoreceptor lesions.

Introduction

The retinal toxicity of light at levels below the threshold for thermal damage was first described by Noell *et al.* (1966). They found damage in the retina of rats after exposing them for several hours to light at relatively low intensity. Later studies on retinal light damage can be divided into two categories with regard to the exposure conditions employed: (1) Long exposures of free running laboratory animals to relatively low levels of light. (2) Exposure of small retinal fields of anesthetized animals to short, intense irradiations. The latter setup is used as a model for clinical examples of phototoxicity such as solar retinitis and photic maculopathy during eye surgery (Tso, 1989; Byrnes *et al.*, 1992). The present study adheres to the second type of exposure conditions. Animal studies have indicated that this type of exposure also results in photochemical, rather than thermal damage (Friedman and Kuwabara, 1968; Tso *et al.*, 1972; Ham *et al.*, 1979). The wavelength dependency of light damage in these exposure conditions was investigated by Ham and colleagues. They showed that damage sensitivity increases for shorter wavelengths, with a maximum in the ultraviolet (Ham *et al.*, 1979, 1982). Subsequent histological studies from Ham's group suggested that two spectrally defined damage types add up to this sensitivity curve: Exposure at 350 nm damaged, in aphakic monkeys, predominantly the photoreceptors (rods and cones) (Ham *et al.*, 1982); at 441 nm, damage was found to be most prominent in the RPE (Ham *et al.*, 1978). These data were limited, however, to a very small number of monkeys. Specific photoreceptor damage was also found after 325 nm laser irradiations (Schmidt & Zuclich, 1980). In contrary, the RPE was the site of primary injury by repeated near UV irradiations in aphakic and pseudophakic monkeys (Li *et al.*, 1990). After exposure to visible wavelengths, damage was generally found in the RPE and photoreceptors (Friedman and Kuwabara, 1968; Tso *et al.*, 1972; Tso and Woodford, 1983). Lawwill (1982) described damage to mitochondria in all retinal layers after monochromatic irradiations at various wavelengths in the range of 457.9 to 514.5 nm. Spectral differences in retinal light damage after short, intense irradiations were also found in other species. In the squirrel (Collier and Zigman, 1989) 441 nm damaged both the RPE and the photoreceptors; exposure to 366 nm affected at threshold only the photoreceptors, and rods appeared more severely affected than cones. In the rat, two different pathologies were found at three days after monochromatic irradiations at various wavelengths: Photoreceptor damage was found at wavelengths in the range of 320 - 440 nm; wavelengths of 470 - 550 nm damaged most prominently the RPE (Gorgels and van Norren, 1995). However, another study on rats comparing broad-band green and UV-A exposures found no morphologic differences (Rapp and Smith, 1992).

From the above it is apparent that there are strong indications for the existence of at least two spectral damage types. Yet, several important questions remain unresolved. First, the distinction between the types is not clear, because the reports vary in the relative susceptibility of the various retinal elements, in particular that of RPE, cones, and rods. Species differences may account for some of the described variation. Also, with increasing dose progressively more retinal elements become affected (Gorgels and van Norren, 1995) and in time secondary damage may spread to other layers. Following this reasoning, a study employing different doses and focusing on the acute effects may clarify the distinction between the types. Second, only limited data are available on the (sub)cellular localization of the first manifestations of damage, as most studies start analysis at one or more days after the exposure. The acute effects are important in order to pinpoint the site of damage initiation. Third, the long term effects of light damage of small retinal patches are uncertain: Ham *et al.* reported in the

monkey a remarkable capacity for repair of mild photochemical lesions of both damage types:

The RPE damage at 441 nm recovered in time (Ham *et al.*, 1978) and the limited data on the UV-A induced lesions in the photoreceptor layers suggested that small lesions can be closed in by neighboring photoreceptors (Ham *et al.*, 1982). However, in the squirrel retina no evidence was found for filling in of photoreceptor lesions (Collier *et al.*, 1989). In the present study we wanted to address these questions by comparing the pathologies caused by separate wavelengths at various doses and at various time points after irradiation. Based on our previous study (Gorgels and van Norren, 1995), we chose to irradiate at 380 nm for the damage type with emphasis on the photoreceptors, and at 470 nm for the other damage type. The ocular media of the rat readily transmit 380 nm (Gorgels and van Norren, 1992). Damage was assessed by funduscopy and light microscopy at various time points ranging from 1 hour to 2 months after irradiation. In addition, electron microscopy was performed at the acute stage in order to examine the early radiation effects at the ultrastructural level.

Methods

Animals

Male Long Evans rats were obtained from Harlan CPB, Zeist, the Netherlands, at 30 days of age. They were kept in a 12-12 hours light-dark cycle, at 10-60 lux illumination by white fluorescent light. The animals used in the experiments were 60 to 130 days old.

Optics and irradiation

Irradiation of a 450 W Xenon arc was projected onto a rectangular patch of the retina (16 by 18^{o}) with optics basically consisting of three lenses. The first, condenser, lens at focal distance of the Xenon arc provided a parallel bundle. In the parallel bundle, interference filters were placed to select wavelength. We used filters with bandwidth 10 nm centered around 380 and 470 nm. A filter with peak transmission at 517 nm was used for aiming of the beam. In addition, the parallel beam passed a heat filter (Spindler and Hoyer, KG 3, 3 mm) and a water bath. Neutral density filters could be inserted to reduce irradiance. The second lens (f = 120 mm) focused the beam. The focus was conjugated with the pupil plane and a diaphragm was placed here to reduced the size of the entrance pupil. The third lens (f = 53 mm) focused the beam in the pupil plane of the rat eye, ensuring Maxwellian view conditions. In a plane conjugate to the retina, a rectangular diaphragm was placed with neutral density filters separated by black bars. The beam was thus divided into four bundles with intensities of about 100, 45, 25, and 10%. Thus, four retinal patches of 6 x 7^{o} were irradiated at different intensities in one exposure. The irradiance provided by this configuration was measured and retinal irradiance was calculated as described previously (Gorgels and van Norren, 1995). Retinal irradiance ranged from 0.5 to 8 mW/cm^2 at 380 nm and from 25 to 250 mW/cm^2 at 470 nm. Exposure time was 6-8 min at 380 nm and 60-90 min at 470 nm. Within these ranges of exposure duration and retinal irradiance, the dose, i.e. irradiance times exposure duration, determines damage morphology and threshold (Gorgels and van Norren, 1995). The small fraction of light reflected from the fundus, was deflected by a mirror at a pupil plane and was used to observe and monitor the retinal position. If the position changed during irradiation, the

experiment was discarded.

For irradiation the rat was sedated with ether, anesthetized with pentobarbital (50 mg/kg) by intraperitoneal injection, and kept anesthetized during the irradiation by intravenous infusion with saline (1 ml/hr) containing pentobarbital (15 mg per kg per hr). Atropine sulfate (0.1 ml of a solution of 0.5 mg/ml) was injected subcutaneously. Pupils were dilated with drops of phenylephrine 5% and cyclopentolate HCl 1%. Body temperature was measured by a rectal thermometer and kept between 37.5 to 38.5°C by an electrical blanket. The rat was placed in a holder and the head was fixed at three points: both acoustic ducts and the upper front teeth. The cornea was moistened during the experiment by a continuous flow of saline (4 ml/hr). The rat was positioned in the bundle using green light (517 nm) of low retinal irradiance (5 - 10 mW/cm^2). Both irradiations, at 380 and at 470 nm, were performed in the same eye. They were placed close to each other ($20\text{-}25^0$ distance) in the central, slightly superior part of the retina. The 380 nm irradiation was done first, followed by the 470 nm irradiation at a slightly more temporal location. Control experiments (n=3) with the 380 nm irradiation as the second irradiation at the more temporal position revealed no influence of this procedure on damage sensitivity and morphology (analyzed at 3 days after irradiation). During the preparation of the animal for the irradiation the lighting conditions were dim white fluorescent light of 50 lux, which was turned off as the irradiation started. After irradiation the animals recovered in the dark for 2-3 hrs and were placed back in the cage in the cyclic environment of dim white light.

Analysis

Data were obtained during the first hours and at 1, 3, 7, 21, and 63 days after irradiation. The effect of the irradiation was analyzed by funduscopy and histology. The rats were anesthetized and the pupils were dilated as described above. Indirect funduscopy was performed at a low level of retinal irradiance (< 50 mW/cm^2). A filter was used to block wavelengths shorter than 500 nm. Next, the animal was either allowed to recover or was fixed for histology. The fixation procedure consisted of perfusion through the heart with phosphate buffered saline, quickly followed by the fixative of 2% paraformaldehyde and 2% glutar-aldehyde in sodium cacodylate buffer, pH 7.4. The eye was removed and immersed in the same fixative overnight at 4°C. After opening the posterior chamber of the eye, the funduscopically visible damage sites could be seen using a stereo microscope. The position of the patches without visible changes were determined using the retinal vasculature as landmarks. The irradiated parts with adjacent control retina were resected and postfixed in 1% OsO_4 in buffer for 1 hr. The tissue segments were dehydrated in ethanol and embedded in Epon. Semithin sections (1 µm) were collected, stained with toluidine blue and examined light microscopically. Ultrathin section were counterstained with uranylacetate and leadcitrate and examined with a Jeol electron microscope. The effects of the irradiation were detected by comparing irradiated area with adjacent control tissue within the same section.

Results

Data were obtained of in total 33 animals. For each time point of analysis at least three to four irradiated eyes were evaluated with histology. The animals used for the longer survival experiments were usually also examined funduscopically at earlier time points.

Funduscopy

Irradiations at 380 nm were performed at doses of 0.25 up to 3 J/cm^2. At 470 nm much higher doses are required for funduscopic lesions (Gorgels and van Norren, 1995) and the employed dose range was 250 - 1350 J/cm^2. At both wavelengths fundus changes took time to develop (Fig. 1, Table 1): There were no changes in fundo immediately after exposure. A maximal brightness was reached at 3 days after irradiation and thereafter the visibility declined. In Fig. 2 the average dose for a just visible change in fundo is plotted as a function of the time after irradiation at 380 nm (Fig. 2A) and at 470 nm (Fig. 2B). The plot illustrates that for both wavelengths the lowest threshold dose is reached at 3 days after irradiation.

Histology

The histologic manifestations of both damage types were studied at various doses and time points. In the presentation of the results we focussed on the development of the damage over time. The differences between the spectral types were emphasized in a previous paper (Gorgels and van Norren, 1995). Yet, to also facilitate a comparison of the damage types in the present material the following precautions were made: (1) Doses were expressed as multiples of the funduscopic threshold dose (T). T was 0.61 J/cm^2 for 380 nm and 513 J/cm^2 for 470 nm. (2) The morphologic features of both damage types are summarized in Table 2. (3) In two figures the morphology of the two damage types at equal relative doses are presented side by side.

Acute effects at 380 nm.

Histologic material was collected at 3 hrs after the irradiation. With light microscopy, changes were seen in the photoreceptor cells. The number of cells affected depended on the dose. No changes were seen at T (0.5-0.7 J/cm^2). At doses of 1.5 x T, some scattered photoreceptor cells (5-15%) were damaged (Fig. 3A,B, Table 2). At doses of 4 x T practically all photoreceptors were damaged (Fig. 7A). Damaged photoreceptors had darkly stained nuclei, inner segments (IS) and outer segments (OS). Increased staining was also noticed in the outer plexiform layer (OPL). Interestingly, in severely damaged retina with all rod nuclei affected, there were still normal appearing cone nuclei present in the ONL, approximately in the same numbers as in control retina (i.e.< 2%). Cone nuclei were distinguished from rod nuclei by their larger size, ovoid shape, and the multiple small clumps of heterochromatin (LaVail, 1976). In addition, the RPE showed a conspicuous increase in number of phagosomes(Fig. 7A). Other retinal layyers appeard normal.

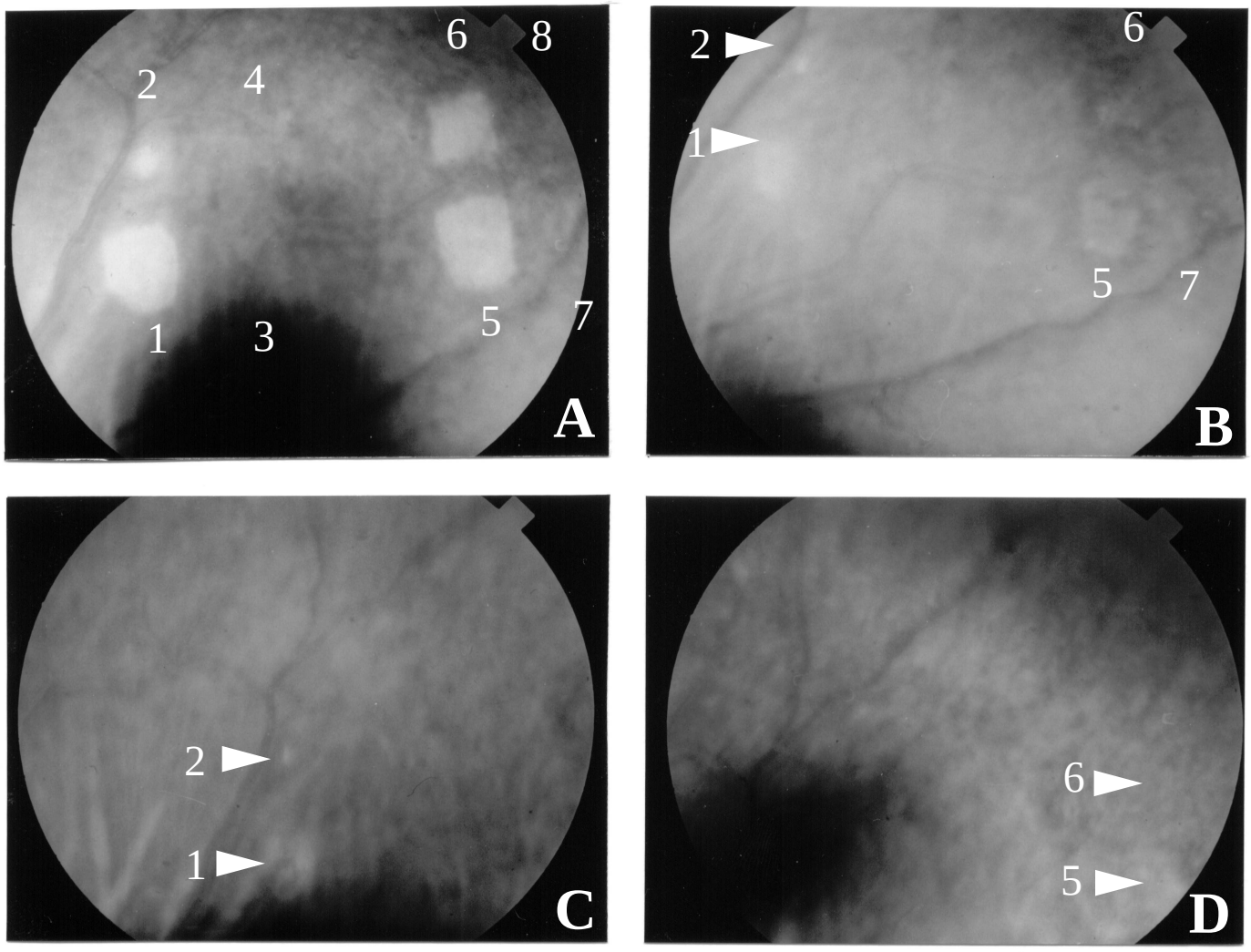

Fig. 1. Fundus photographs of the same eye taken 1 (A), 7 (B) and 21 (C,D) days after irradiation at 470 and 380 nm. During an irradiation four rectangular retinal patches were exposed at different doses. At 470 nm, doses of 1327 (1) and 858 (2) J/cm2 caused bright spots at day 1, which became smaller and less bright at day 7, and were reduced to white speckles at day 21. Lower doses of 448 (3) and 264 (4) J/cm2 caused no fundus changes. At 380 nm doses of 2 (5), 1.3 (6), and 0.74 (7) J/cm2 were visible as grey spots at day one. They became less bright at day 7. At day 21, a faint, mottled appearance remained. A dose of 0.46 (8) J/cm2 caused no visible changes.

Ultrastructural analysis was performed on retina exposed to 1.5 x T (1 J/cm2; n=2) and to 5 x T (3 J/cm2; n=3). The irradiated area was compared with adjacent control tissue. In agreement with the light microscopic observations, the irradiation had caused damage in rods. At 1.5 x T a small fraction of the rods was damaged (Fig. 3B), whereas at 5 x T all were affected (Fig. 3C). Changes consisted of condensation of nuclear chromatin and cytosol. The electron-dense appearance of the cytosol was noticed in the spherules, around the nucleus and in IS. Rod spherules contained besides synaptic vesicles also several larger electron-lucent vesicles (Fig 3E). Mitochondria often had an electron-lucent ground substance and broken cristae. Damaged IS were thin and contained many free ribosomes. The OS surface often was

undulating. At the base of the OS the disks were separated by large spaces. The epithelial cells had a heavy load of phagosomes but appeared otherwise normal (Fig. 3D). Between the damaged rods in the tissue irradiated at 5 x T, normal appearing cones were seen with normal nuclei, cytosol and axon terminals (Fig. 3E).

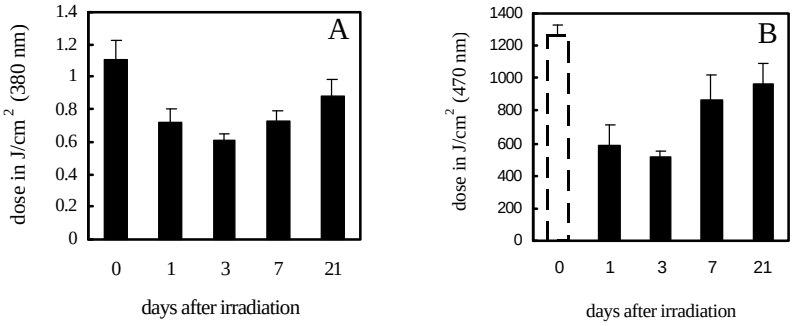

Fig. 2. Damage threshold doses (± SEM) as assessed by funduscopy at different time points after irradiation at 380 nm (A) and at 470 nm (B). Only the data of the experiments with survival of at least 21 days were used in the histogram (n=7). Analysis at day 0 was performed at 2-3 hrs after irradiation. Threshold dose for 470 nm at day 0 is the average of only two experiments (dotted bar); the other five experiments showed no fundus changes at day 0.

Progression of damage at 380 nm.

The acute changes in the rods, appeared to indicate irreversible cell damage: In the next days light microscopy showed progressive degeneration and loss of rods. In contrast, the increase in phagosomes in the RPE was a transient feature. After one day the irradiated RPE did not deviate from the control. The progression of the damage was mainly studied at three doses: Low (T), moderate (1.5 x T) and high (2.5 x T).

Low dose (T)

At the funduscopic threshold dose (0.5-0.7 J/cm^2), damage was manifested as a few scattered photoreceptors (< 1%) with dark staining of the nucleus and OS at day 1 - 7 after irradiation. At subthreshold doses of 0.3-0.5 J/cm^2, occasionally a dark staining photoreceptor cell was also observed.

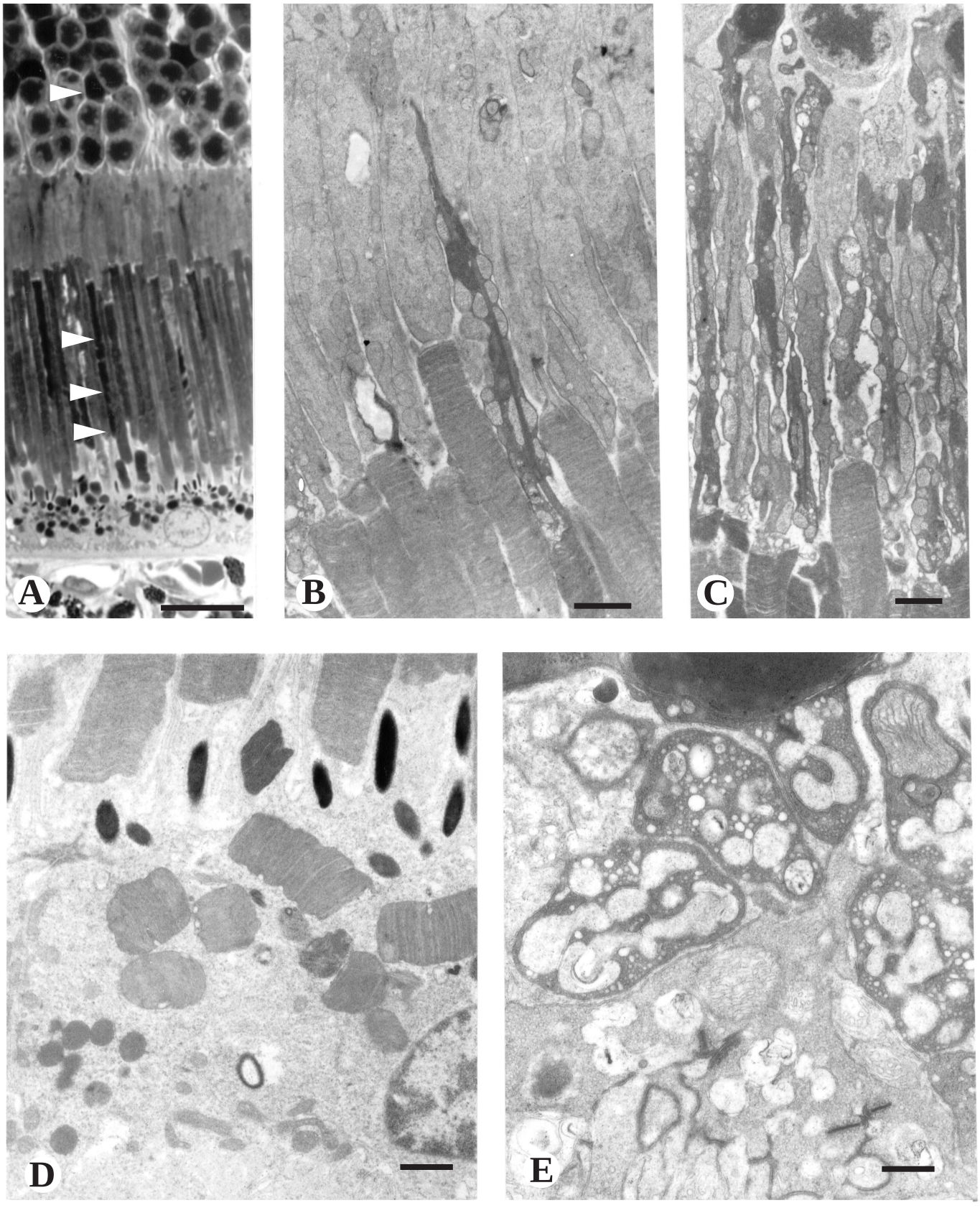

Fig. 3. Acute effects of irradiation at 380 nm. Material was collected at 3 hrs after exposure to 1.5 x the threshold dose (**A,B**) and at 5 x threshold (**B,C,D,E**). A: light micrograph showing dark staining (arrowheads) of a few nuclei, inner and outer segments of photoreceptor cells. **B**: Electron micrograph of the inner segment layer. A few inner segments (arrows) are darkly stained, thin and have swollen mitochondria. **C**: Practically all photoreceptor elements are damaged. **D**: Retinal pigment epithelium is loaded with phagosomes, but appears undamaged. **E**: Rod spherules have an electron-dense cytosol. Besides synaptic vesicles, several larger clear vesicles are present. Cone pedicles (asterisk) have a normal appearance. Bars: 20 µm (**A**), 2 µm (**B,C**), 1 µm (**D,E**).

Moderate dose (1.5 T)

At doses of 0.8-1.0 J/cm2, the percentage of damaged photoreceptors rose from 5 - 15% at 3 hrs (Fig. 3A,B) to 10- 30% of the irradiated photoreceptor cells after 3 days. At day seven, a loss of photoreceptor cells was apparent. At 21 days and 2 months after irradiation, the irradiated area had lost 30 - 40 % of the photoreceptors when compared to the surrounding control retina (Fig. 4B).

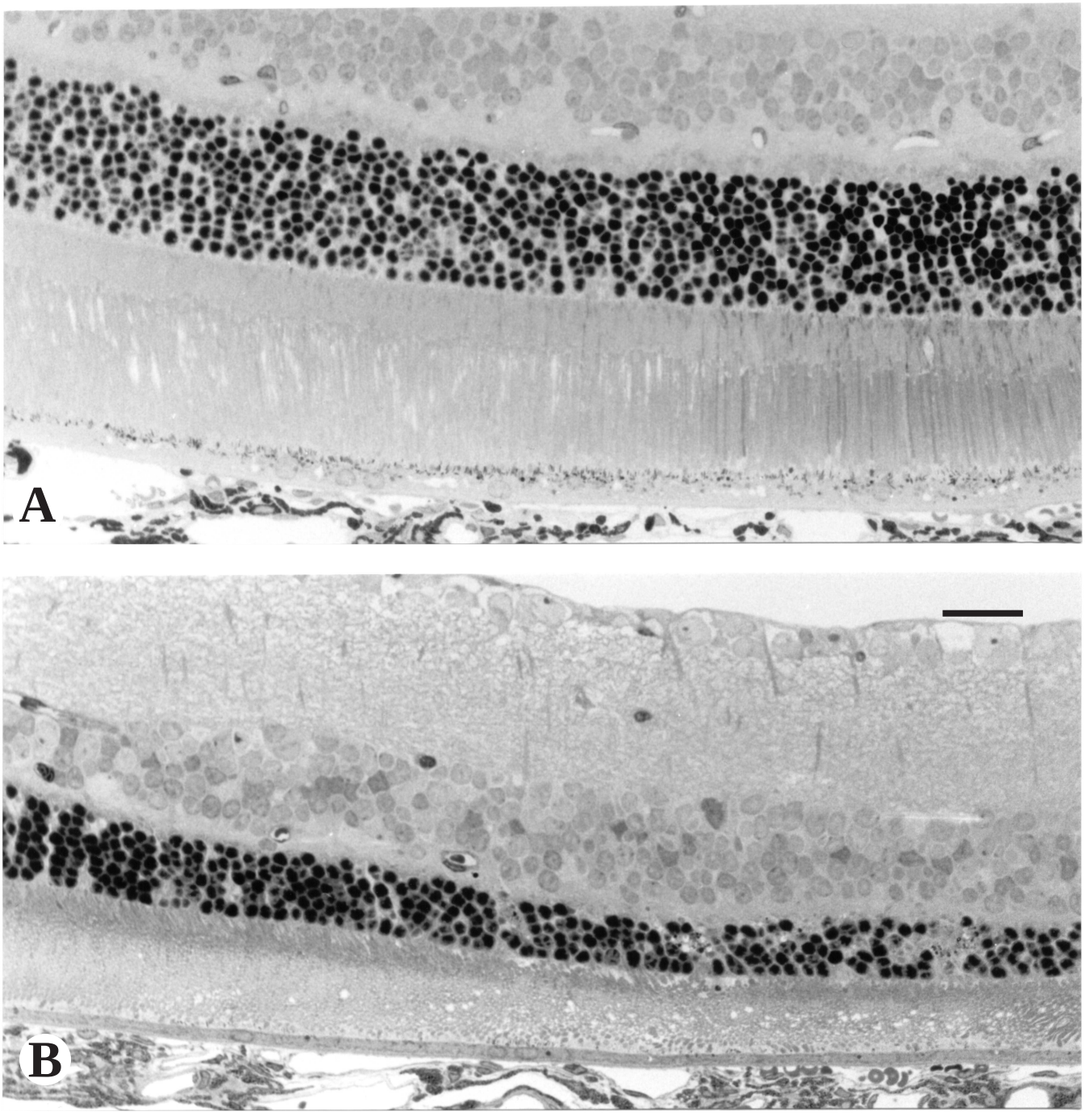

Fig. 4. Light micrographs of retina after exposure to 380 nm at 1.5 x threshold. The irradiated area is on the right; control retina is on the left. **A**: Three hrs after irradiation. Several photoreceptors are darkly stained. **B**: Two months after irradiation: The irradiated area shows aloss of photoreceptors. Bar: 25 µm.

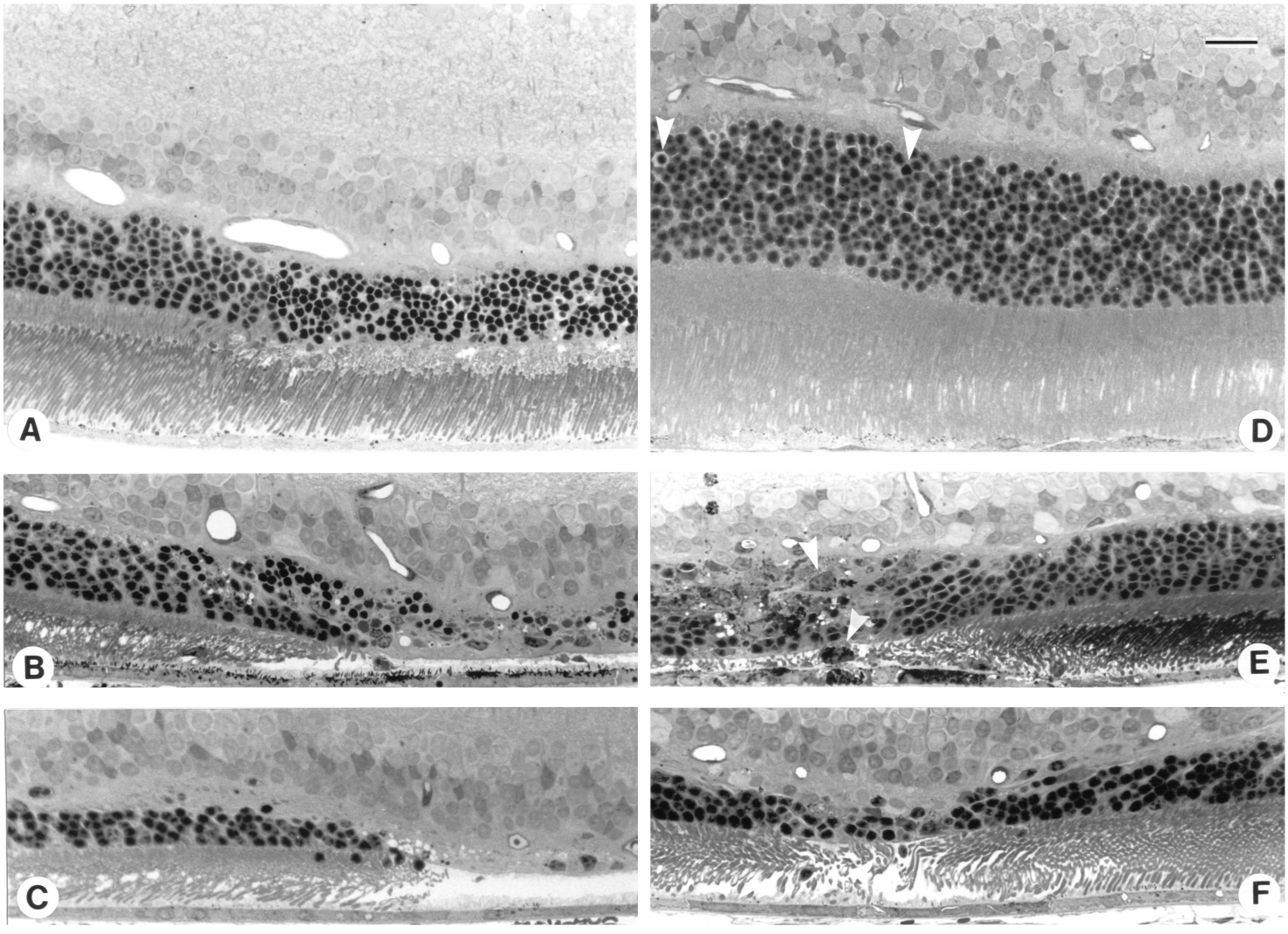
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Fig.5. (previous page) Comparison of damage by 380 (**A**,**B**,**C**) and 470 nm (**D**,**E**,**F**) at doses of 2.5 x threshold. Micrographs show damage at one day (**A**,**D**), 7 days (**B**,**E**), 3 weeks (**C**) and 2 months (**F**) after exposure. Irradiated retina is on the central part of the figure; Control retina is on the outside. After one day: Exposure to 380 nm (**A**) caused dark staining of practically all photoreceptors and the photoreceptor layer is thinner than in control. The RPE is normal. Exposure to 470 nm (**D**) resulted in a few pyknotic nuclei (arrowheads) in the ONL. The OS layer is thicker than control. The RPE cells are swollen and lightly stained. After one week: Exposure to 380 nm (**B**) caused cell loss in the outer part of ONL and dark staining of nuclei in the inner part of ONL. On the sides the outer rows of neighboring ONL nuclei have shifted into the lesion. The area irradiated at 470 nm (**E**) shows cell loss and an oblique orientation of columns of ONL nuclei on the sides. Debris and cigar-shaped melanin granules are found in cells in the photoreceptor layer and inner retina (arrowheads). The RPE is swollen and contains dark inclusions. Three weeks after irradiation at 380 nm (**C**) only a few ONL nuclei, possibly of cones, are left. **F:** Two months after irradiation at 470 nm, the photoreceptor layer shows cell loss in the center and oblique orientation of the columns of ONL nuclei on the sides. RPE cells stain darker than control. Bar: 25 µm.

High dose (2.5 T)

One day after doses of 1.3 - 1.7 J/cm^2 most ONL nuclei had a homogeneously dark staining and the IS appeared crumbly (Fig. 5A). Phagocytes had appeared in the ONL. After 3 days, all photoreceptors except for a few cones were damaged (Fig. 6C). Debris and phagocytes were numerous in the photoreceptor layer. The RPE appeared normal or slightly swollen. After 7 days, the OS and IS had virtually all disappeared (Fig. 5B). In the ONL, the inner part still contained photoreceptor nuclei which were pyknotic, whereas in the outer part most nuclei had been removed. The columns of nuclei of the adjacent unexposed photoreceptors had obtained an oblique orientation with the outer rows of nuclei pointing into the lesion, suggesting that these nuclei had shifted into the lesion area. Twenty-one days after irradiation (Fig. 5C) nearly all photoreceptors had been removed. Two months after irradiation, a lesion specific for the photoreceptors remained. The lesion area measured approximately 175 µm in diameter, whereas the irradiated area had been approximately 350 µm in diameter (Fig. 7). A few photoreceptors with cone morphology and a few degenerating photoreceptor nuclei were still present in the lesion area. On the sides the columns of neighboring photoreceptor nuclei had an oblique orientation. No damage was observed in other retinal layers.

Acute effects at 470 nm.

Material was collected at 1 hour after the irradiation. Light microscopy revealed changes in both the RPE and the photoreceptor cells in retina irradiated at 2.5x T (1200-1350 J/cm^2; Fig. 8A): Most RPE cells and IS contained small light vesicles. A few photoreceptor nuclei showed dark staining of their chromatin. Some of these had the morphology of cone nuclei. Ultrastructural analysis of retina irradiated at 1.5 x T (900 J/cm^2; n=2) and at 2.5 x T (1300 J/cm^2; n=2) showed mitochondrial changes in RPE and photoreceptor cells as the most conspicious effects. The number of cells affected depended on the dose. At 1300 J/cm^2, practically all epithelial and photoreceptor cells were affected (Fig.8B-D). Mitochondria were swollen and had broken cristae. In addition, the RPE contained vacuoles and the cytosol was slightly more condensed than in the control RPE. In the photoreceptor cells, damaged mitochondria were noticed in the IS, ONL and in the OPL, both in rod spherules and in cone pedicles. In other aspects, the cones and rods generally

appeared normal. Rarely, a swollen, empty IS was seen and a few nuclei in the ONL had dense chromatin.

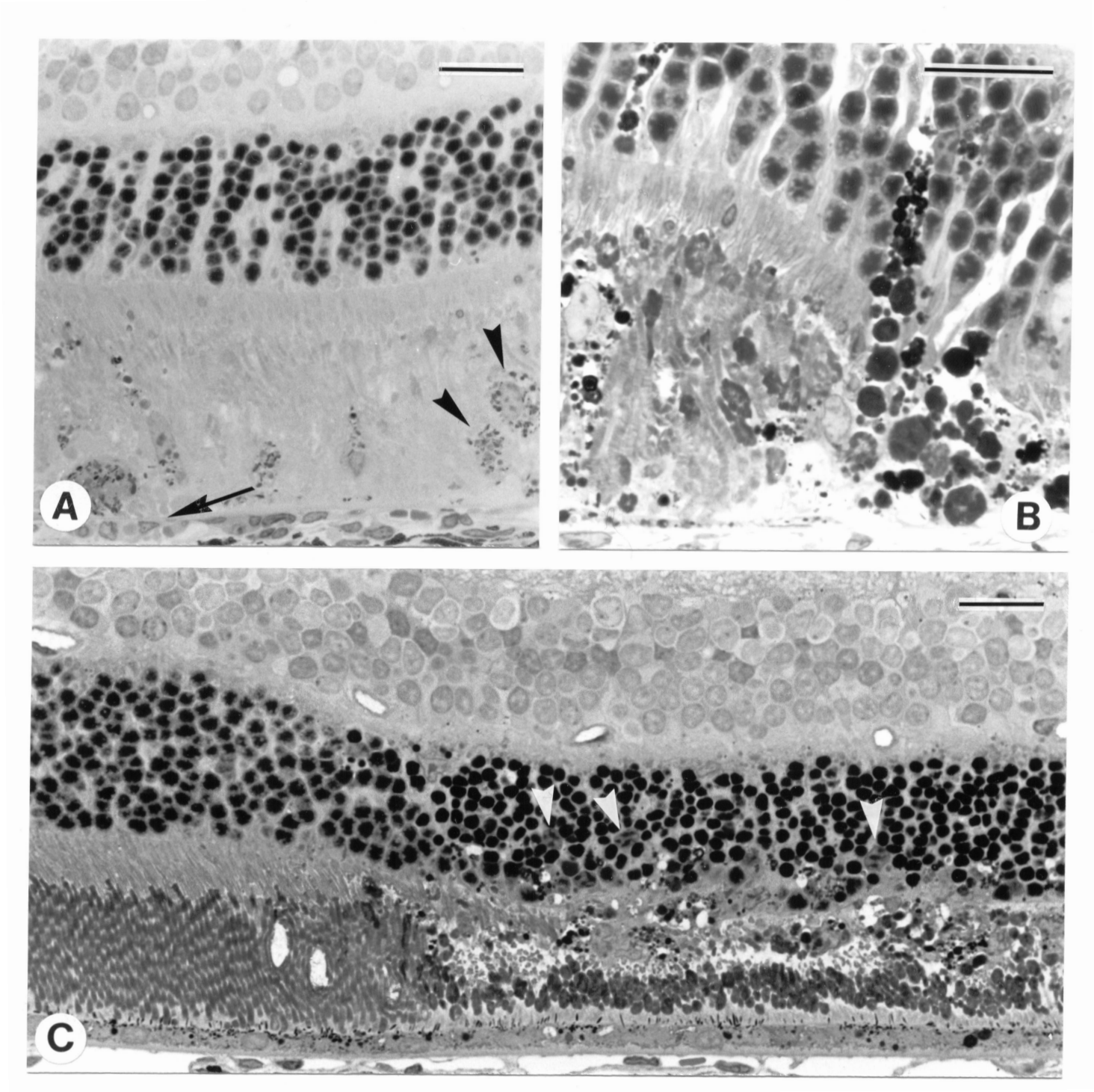

Fig. 6. Comparison of damage by 470 (**A,B**) and 380 nm (**C**) at 3 days after irradiation at a dose of 2.5 x threshold. **A**: Phagocytes with cigar-shaped melanin granules (arrowheads) are present between the OS. Leukocytes are located at Bruch's membrane (arrow). **B**: Columns of debris are present in the ONL and the subretinal space. On the right RPE cells are absent. **C**: In the area irradiated at 380 nm (on the right) all ONL nuclei show dark staining except for cone nuclei (arrowheads). The OS are shortened. The RPE appears unaffected or slightly swollen. Bars: 25 µm.

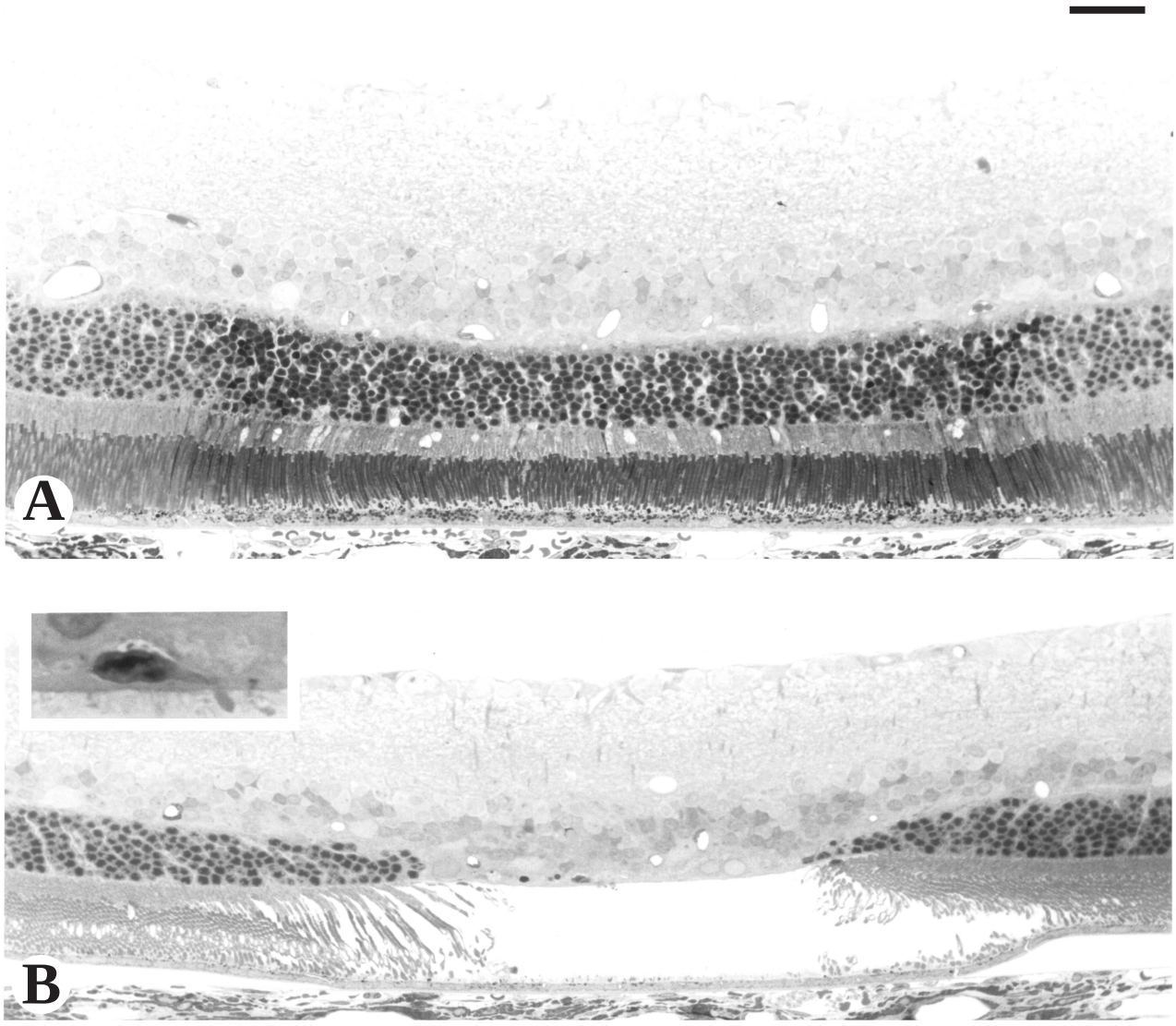

Fig. 7. Micrographs of irradiation at 380 nm, illustrating the size of the damaged area after 3 hours (**A**) and 2 months (**B**): The photoreceptor lesion after 2 months is smaller than the damaged area after 3 hours. Inset: Surviving ONL nucleus in the lesion shows morphology of cone nucleus. The large space between the OLM and the RPE is a preparation artifact. Dose: 4 x threshold (2.5 J/cm2). Bar: 25 µm.

Progression of damage at 470 nm.

Low dose (T)

At the funduscopic threshold dose of 400 - 600 J/cm2, damage was found in the RPE and in the photoreceptor layer. The RPE showed an altered distribution of the melanin granules after one day. The granules were not seen in villi, but had accumulated at the apical side of the cells. At day 3, RPE cells were swollen and their melanin granules were spread out in the cytoplasm. Some RPE cells contained many dark inclusions. At later time points the RPE

deviate from the control. Photoreceptor damage consisted of a few (< 1%) dark staining nuclei and OS at 1 and 3 days after irradiation.

Moderate dose (1.5 T)

Doses of 650 to 850 J/cm^2 caused similar, but more severe, changes in the RPE cells and involved more photoreceptors. The RPE cells often contained small light vesicles after one day (Fig. 9A) and numerous dark inclusions after 3 days (Fig. 9B). In the following weeks the appearance of RPE cells returned to normal (Fig. 9C,D). Photoreceptor damage consisted of a few (<1%), scattered, dark staining photoreceptors at day 1 and 3. Phagocytic cells containing debris and typically also melanin granules were present in the OS layer. After one week, pyknotic cells and debris became scarce. After 2 months the photoreceptor layer in the center of the exposed area was slightly thinner than in the surrounding control retina, corresponding to a loss of up to 5% of photoreceptors.

High dose (2.5 T)

One day after exposure at 1200 - 1350 J/cm^2, some RPE cells had lost their cuboidal shape (Fig. 5D). After 3 days, the RPE monolayer was interrupted (Fig. 6B). Mitotic figures were occasionally observed in RPE cells. In the choroid, there were leucocytes at Bruch's membrane in the irradiated area (Fig. 6A). After 7 days, RPE cells were still swollen but a continuous monolayer was present (Fig. 5E). At late stages, the RPE cells had regained a normal shape, but the cytoplasm stained darker than control and some cells were hypopigmented (Fig. 5F).

The number of degenerating, dark staining photoreceptor cells was still modest (1-3%) at one day after irradiation. The thickness of the IS and OS layers was increased (Fig. 5D). After 3 days, there were still scattered dark staining photoreceptors, but in addition, we often observed columns of debris, suggestive of degeneration of entire columns of photoreceptor nuclei (Fig. 6B). This was observed where the underlying RPE cells were absent. Phagocytes with debris and melanin were prominent in the photoreceptor layer (Fig. 6A). Occasionally, mitotic figures were seen in the phagocytes. After one week, cell loss was evident, especially in the center. On the sides the columns of photoreceptor nuclei had an almost tangential orientation, suggesting that the outer rows of nuclei had moved towards the center of the irradiation (Fig 5E). After 3 weeks, most of the pyknotic nuclei had been removed. In the center of the irradiated area, the ONL contained none or just a few photoreceptor nuclei. Towards the sides the number of photoreceptors increased as the columns of nuclei did not stand upright but were bent towards the center of the lesion. Probably these nuclei had occupied the area vacated by degenerated photoreceptors. This configuration made counts of the photoreceptor cell loss at this stage unreliable. In the inner retina, no damage was seen. Cells containing melanin granules were observed in the OS, ONL and INL. Two months after irradiation, a similar picture was seen, with a further decline in phagocytes (Fig. 5F).

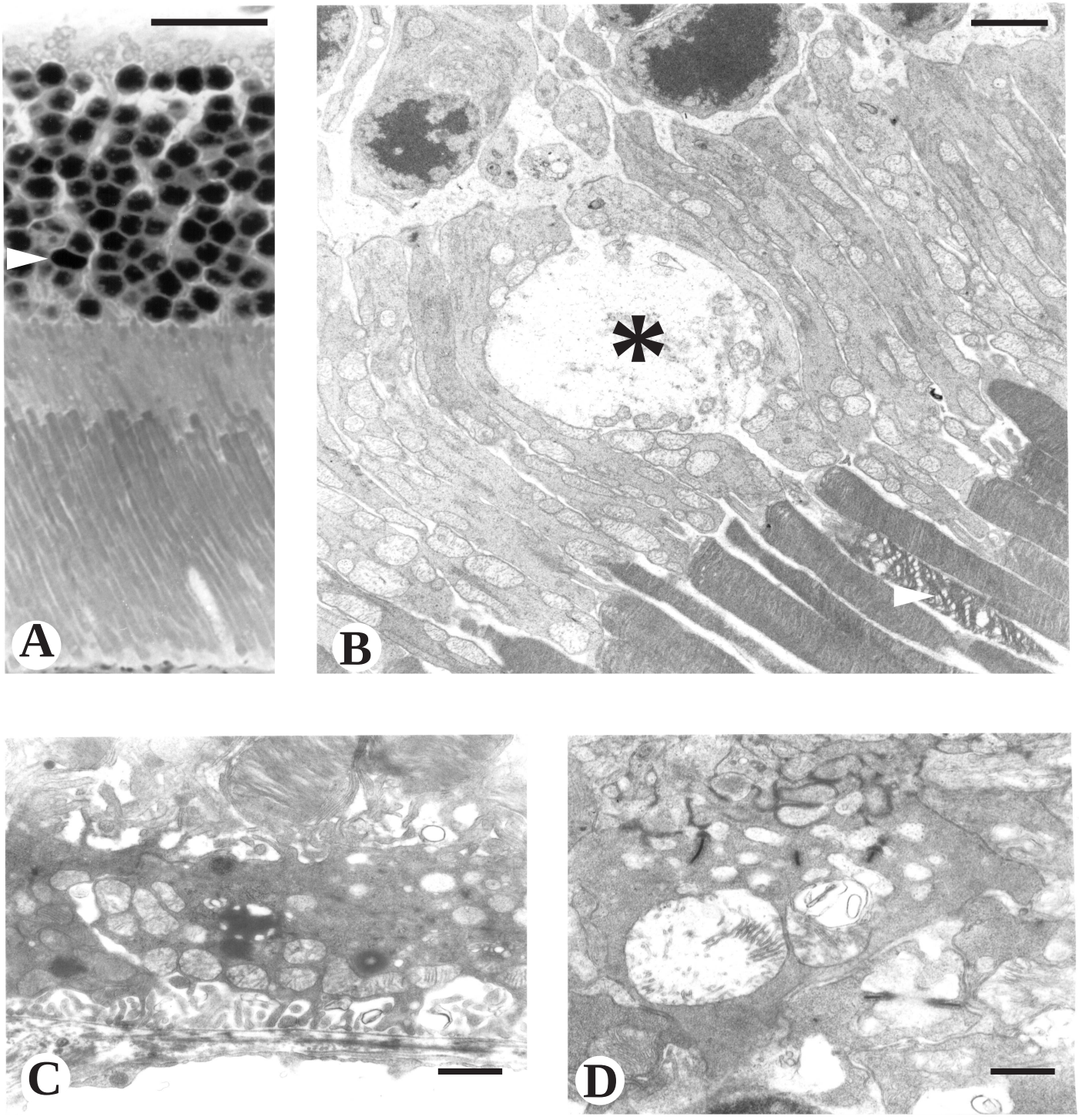

Fig. 8. Acute effects of exposure to 470 nm. Material was collected at one hour after irradiation at a dose of 2.5 x threshold (1300 J/cm2). **A**: Light micrograph showing a darkly stained photoreceptor nucleus (arrow) and light vesicles in inner segments and pigment epithelium. **B,C,D**: Electron micrographs showing swelling and disruption of internal structure in mitochondria in inner segments (**B**), pigment epithelium (**C**) and outer plexiform layer (**D**). In addition, a large, swollen inner segment (*) and a vesiculated outer segment (arrow) are present in B.
Bars represent 20 µm (**A**), 2 µm (**B**), 0.5 µm (**C**), 1 µm (**D**).

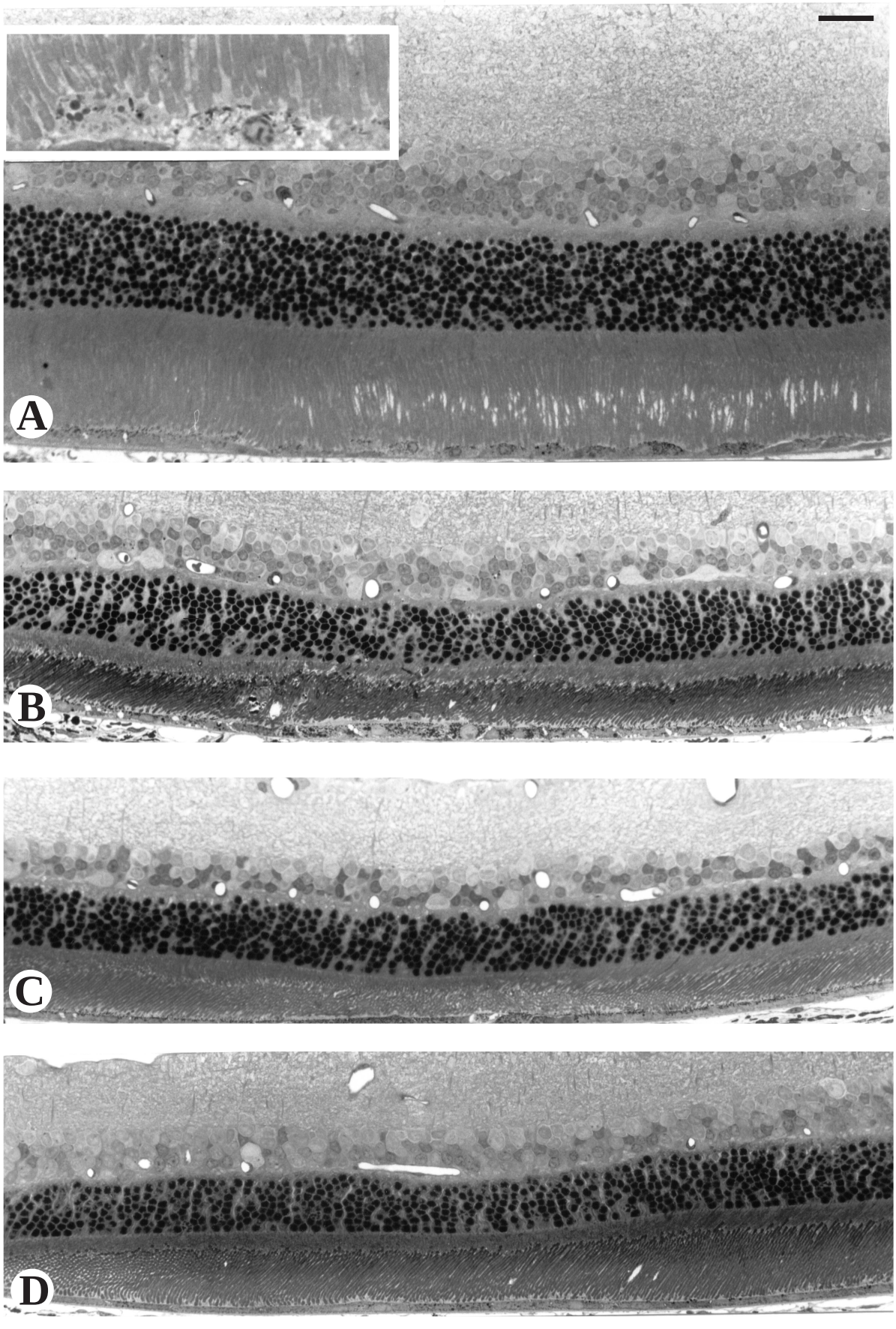
A
B
C
D

Fig. 9. (previous page) Development of damage by 470 nm at a dose of 1.5 x threshold (700-900 J/cm^2). The irradiated area is in the center with control retina on the sides. **A**, after one day: The apical RPE surface is undulating. The cells show no melanin in villi and often contain many light vesicles (see inset). **B**, after 3 days: the RPE is swollen and contains many dark inclusions; phagocytes have appeared between the OS and a few photoreceptors are darkly stained. **C**, after one week: The swelling in the RPE is reduced; OS layer is thinner than control. **D**, after 2 months: The RPE shows marginal swelling. The thickness of the photoreceptor layers is slightly decreased. Bars: 25 µm.

Discussion

In photochemical processes wavelength is an important parameter. The action spectrum depends on the absorption spectrum of light energy by the chromophore. Different chromophores with different absorption spectra will cause spectrally distinct damage types. In the present study on retinal light damage we compared the effects of irradiations at 380 nm and at 470 nm. Marked differences were found in the early course of the development of damage, while at late stages the damage types become remarkably similar in appearance. As will be discussed later in detail, both damage types resemble damage types previously reported in monkey, thus allowing for an important generalization between rat and monkey.

Damage at 380 nm

At 3 hrs after the irradiation, the first time point of analysis, rods showed signs of damage with no damage at the other cell types in the retina. At later timepoints we witnessed the complete loss of the local rod population without distinct changes in the other retinal layers. A remarkable finding was that the small population of cones in the rat retina (< 2% (Szel and Rohlich, 1992)) was far less susceptible to intense radiation.

Other studies in rat and squirrel also described that one day after threshold doses of UV-A the damage was confined to the photoreceptor cells (Collier and Zigman, 1989; Rapp and Smith, 1992). For RPE damage higher doses were required (Collier *et al.*, 1989; Rapp and Smith, 1992; Gorgels and van Norren, 1995). A clear specificity for rods has not reported before, but a study on the squirrel mentioned that at the border of the lesion, only rods were affected at day one (Collier and Zigman, 1989). In the monkey, Ham *et al.* (1982) found that irradiation at 350 nm caused degeneration of rods and cones accompanied by mild damage to the RPE. Most likely their dose was higher, relative to threshold, than our highest dose, since a funduscopic lesion was visible immediately after exposure. It leaves room for speculation that at lower doses a different susceptibility of rods, cones and RPE cells will also show in the monkey. A relative sparing of cones was also reported in light damage studies using a much different setup, i.e. long exposures of free running rats to low levels of white fluorescent light (Cicerone, 1976; LaVail, 1976). These conditions may well induce yet another type damage, but it is important to note that fluorescent light contains UV-A, which in rats readily passes the ocular media to reach the retina (Gorgels and van Norren, 1992).

With electron microscopy the damage was localized at the subcellular level. At 3 hrs after irradiation all cell compartments were affected and clearly the nucleus was already involved, showing condensation of the chromatin. Other studies on acute UV-A effects are scarce.

Schmidt and Zuclich (1980) examined monkey retinas 1 hr after high doses of 325 nm. Their basic observation of changes in the nucleus, IS and OS of photoreceptor cells agrees well with our findings.

Our findings are clearly at odds with reports (Rapp *et al.,* 1994) showing that mild UV-A exposures cause mainly changes in outer segments: Damaged OS appeared pale and showed increased spacing between the disks. The damaged photoreceptors apparently can recover as the damaged disks are gradually replaced by normal appearing disks. In our material, damage was found in all cell compartments including OS. In areas with just a few scattered dark photoreceptors, the others appeared normal. None of the dark staining rods appeared to survive. Possibly, the origin of this discrepancy lies in a difference in exposure conditions, affecting the concentration of visual pigment. The conditions that led to specific OS changes consisted of dark adaptation before exposure and, compared to our study, a low retinal irradiance and a long exposure duration. In these conditions retinal light damage probably is mediated by rhodopsin (Noell *et al.,* 1966; Organisciak and Winkler, 1994). Alternatively, the different findings on OS morphology may also originate from differences in fixation of the tissue. A recent study showed that the appearance of OS vesiculations in light damaged retina depends on the fixation method (Szczesny *et al.,* 1996).

When the damaged photoreceptors have been removed, the irradiated area shows a thinning of the photoreceptor layer or, at high doses, a photoreceptor lesion with the other retinal layers intact. Ham *et al.* (1982) suggested, on the basis of their observations in the monkey, that small lesions (500 µm) might be closed in by photoreceptors from surrounding retina within a month. In the squirrel, much larger lesions of 29^{o} were not covered by photoreceptors at 30 days after irradiation (Collier *et al.,* 1989). In our study using a spot size of 350 µm, clearly a photoreceptor lesion persisted after two months. Yet, there were indications that to some extent, filling in from the sides had occurred. After two months, the lesion appeared smaller than the originally irradiated retinal area. In addition, there were morphological indications that as soon as damaged photoreceptors were removed, neighboring photoreceptor cells shifted into the lesion. In an ongoing study, we are currently investigating this possible capacity for redistribution of photoreceptor cells in more detail.

Damage at 470 nm

The most important finding at 470 nm was that damage involved both RPE and photoreceptors, even at threshold levels. In the photoreceptor layer, acute radiation effects (after 1 hr) consisted of extensive damage in a few scattered photoreceptors, both in rods and cones. Less extensive damage, limited to the mitochondria, was observed in many of the photoreceptors cells in the irradiated area. In the RPE, similarly mitochondrial changes were prominent. These results agree well with observations of Lawwill (1982) in monkey retinas after irradiations at various visible wavelengths. He concluded that mitochondria are the structure most sensitive to anatomic change.

At low and moderate doses, the RPE cells are probably not irreversibly damaged, because in a few weeks the RPE recovers completely. At high doses, the RPE monolayer was interrupted, suggesting that some RPE cells had degenerated. Alternatively, the gaps in the RPE monolayer could also have developed by detachment of RPE cells to become phagocytic cells in the outer retina. Melanin granules were often observed in the phagocytic cells and the occasional mitoses in phagocytic cells suggest that some of them are actually epithelial cells. At late stages a

continuous monolayer was observed, which may indicate that small gaps in the monolayer can be closed in by RPE cells. Yet, the RPE monolayer still showed subtle differences from control RPE: The cells were hypopigmented and had a dark staining cytoplasm.

Degeneration of photoreceptor cells by 470 nm was initially limited to a few cells scattered in the irradiated area, but 3 days after high doses, there was focally massive photoreceptor loss: an entire column of nuclei in the ONL could be degenerating with neighboring columns unaffected. We propose that there are two kinds of photoreceptor damage at 470 nm: First, there is acute damage to a few photoreceptors scattered in the irradiated area, resulting from a direct radiation effect. Second, there is local massive photoreceptor degeneration, occurring later when RPE cells are missing in the monolayer.

Whereas during the first days after the irradiation, the RPE changes were the most prominent features of damage, during the later stages the loss of photoreceptors becomes the most important feature: The RPE recovers, while a lesion or a thinning of the photoreceptor layer remains. Much similar to our findings at 380 nm, we observed that neighboring photoreceptors tend to occupy the space vacated by the degenerated photoreceptors. But again, as with 380 nm lesions, the retina showed only a limited capacity to redistribute the photoreceptors.

This damage type with prominent RPE changes has not been reported before in rat, apart from our previous study with analysis at three days after irradiation (Gorgels and van Norren, 1995). In other species a similar pathology has been described: In monkeys (Ham *et al.,* 1978) irradiation at 441 nm caused RPE damage followed by recovery, and also a few damaged cones and rods were encountered at early stages. These findings resemble our findings at low and moderate doses. Many features of the damage we found at high doses were also induced by white light in monkeys and rabbits (Friedman and Kuwabara, 1968; Tso *et al.,* 1972; Tso and Woodford, 1983; Hoppeler *et al.,* 1988). Apparently, white light causes in these species predominantly this type of damage, rather than the damage without RPE involvement found at 380 nm. The low transmittance of the ocular media at short wavelengths, probably protects the retina from the latter damage type (Gorgels and van Norren, 1995).

Mechanism of damage

The early manifestations of damage at the different wavelengths were clearly distinct, indicating that different mechanisms are involved. The acute changes in the rods after 380 nm suggest that rods are directly targeted by the irradiation and that the chromophore may well be a rod specific substance. This might be rhodopsin (Noell *et al.,* 1966) or, rather one of its photoproducts. Using data on the relation between light intensity and bleaching in the rat (Cone, 1963; Perlman, 1978), we calculated that rhodopsin was already fully bleached before the start of the irradiation by the green light that we used for aiming the beam. Electron microscopy at 3 hours after irradiation, revealed no subcellular localization of the source of damage, as signs of damage found were found throughout the cell. The further degeneration and removal of the damaged rods follows in the next days without distinct changes in the other retinal layers. The RPE remained intact and no indications of invasion of leucocytes were noticed. The phagocytes involved may well be resident macrophages since they first appear in the ONL.

At 470 nm, both the RPE and the photoreceptors (rods and cones) were affected. While not excluding other possibilities (Noell *et al.,* 1966; Ham and Mueller, 1989; Rozanowska *et al.,*

1995), the acute changes in mitochondria lend support to previous suggestions that a mitochondrial enzyme, such as cytochrome c oxidase is the chromophore for this type of damage (Lawwill, 1982; Pautler *et al.*, 1990). After a few days, we observed a distortion of RPE shape, the monolayer was interrupted at high doses and probably an influx of leucocytes occurred. The massive degeneration of photoreceptor cells is somewhat delayed as compared with 380 nm and it occurs where the RPE is severely damaged. The disturbance of RPE may thus be one of the factors which cause this degeneration of photoreceptor cells. At late stages, as the RPE recovers and the photoreceptor death takes its effect, the morphology of 470 nm damage becomes remarkable similar to that of 380 nm, i.e. a local loss of photoreceptor cells.

Differences in details, such as presence of melanin granules in phagocytic cells and hypopigmentation and dark staining cytoplasm of RPE cells, become apparent only at close examination. In albino retina these details probably will not show at all (Rapp and Smith, 1992).

The present findings again emphasize the sensitivity of funduscopy as a means of detecting retinal damage (Gorgels and van Norren, 1995). The threshold dose for funduscopic changes was the same as or only slightly higher than that for light microscopic changes in all stages of damage development. Thus, funduscopy is a useful tool in studies of retinal light damage for instance when evaluating the protective efficacy of pharmaca. It might save animals, effort and time. It should be noted, however, that discrimination between the damage types is not possible by funduscopy alone, since the fundus appearance of the two damage types is very similar. In addition, fundus lesions are best visible during the first days. Structural damage and misalignment of photoreceptors seem to contribute substantially to the brightness of the fundus appearance. During the first days after irradiation at 380 nm, a bright fundus lesion corresponds to specific damage to the photoreceptor cells. At 470 nm, with additional RPE changes and edema, the fundus appearance is chalk-white. In late stages, when damaged photoreceptors have been removed and none or just a few are left, fundus changes are barely visible and could easily be overlooked.

Acknowledgements

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Chapter 3

Filling-in after focal loss of photoreceptors in rat retina

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Abstract

We investigated the fate of isolated photoreceptor lesions in rat retina over a time span of six months. With a carefully selected dose of UV-A (380 nm) we caused complete loss of photoreceptors in sharply demarcated areas of 200, 400 or 800 µm wide, without visible damage to other retinal layers. One day after irradiation all rods were pyknotic. Three weeks later practically all damaged photoreceptors were removed. The size of the lesion had decreased as the surrounding photoreceptors had migrated into the lesion. The outer segment tips had moved inwards up to 200 µm, but the innermost nuclei in the outer nuclear layer had moved inwards substantially less. The distance over which the photoreceptors migrated increased with lesion size, but only 200 µm defects were filled-in completely on the level of the outer segments. Between three weeks and six months after irradiation no further decrease in lesion size occurred. We conclude that after local loss of photoreceptor cells the bordering photoreceptors rapidly shift into the lesion area, but complete filling-in is limited to very small lesions.

Key words: retina, light damage, photoreceptor, repair, experimental, rat, UVA

Introduction

Local injuries to the retina, such as vascular accidents or photocoagulations, often result in permanent visual field defects. This is caused by the fact that neurons in the adult CNS are postmitotic. In humans, lesions of photocoagulations have been monitored funduscopically over long periods. It was found that the lesion persists and may even increase in size (Brancato *et al.*, 1990, Morgan and Schatz 1991, Dastgheib, Bressel and Green, 1993).
Damage to the retina generally involves multiple layers. For example photocoagulation causes damage to choroid, retinal pigment epithelium (RPE) and generally also to the neuroretina. Yet, not all insults to the retina cause damage throughout several layers. Ham *et al.* (1982) exposed small patches (500 µm, approx. 2^o of visual angle) of retina in aphakic monkeys to monochromatic irradiations at 350 nm. A dose of 5.5 J/cm^2 locally destroyed photoreceptors without notable damage to other retinal layers. After 5 days the damaged PRs had been removed and there was a lesion strictly limited to the PR layer. When the authors examined the retinal patches one month after irradiation, no lesion could be identified. The authors speculated that the lesion had been filled-in by surrounding healthy PRs. In squirrels and rats, UV-A radiation can also cause specific damage to the photoreceptors. Collier, Waldron and Zigman (1989) examined the retina of the squirrel in relation to much larger UV-A induced PR defects (29^o). In contrast to Ham's findings, they observed a persisting PR lesion without spread of damage to other retinal layers at one month after irradiation, but also without filling-in. In Chapter 2 studied in rat the development of PR lesions of 5^o wide (350 µm). After two months a lesion was still present in the PR layer. Yet, the lesion area appeared to be smaller than the irradiated area, suggesting that to some extent neighboring PRs had moved into the damaged area.

Considering these conflicting findings, the fate of local PR lesions (with all other retinal layers intact) remains unclear. Possible repair processes occurring in such lesions are of great interest in terms of visual capacity. When healthy PRs move into damaged areas, it means that blind spots are removed or at least reduced in size. In the present study in rat retina the fate of lesions of 200-800 µm (2.8-11.4^o) wide, limited to the photoreceptors, was followed up to six months.

Materials and methods

Animals

Twelve male pigmented Long Evans rats (24 eyes) were obtained from Harlan CPB, Zeist, the Netherlands, when they were 30 days of age. They were kept in a 12-12 hrs light-dark cycle, at 15-25 lux illumination by white fluorescent light. Room temperature was 22-24^o C. The animals were 60 to 120 days old at the time of irradiation. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Optics

The optical configuration is similar to that used in a previous study (Gorgels and van Norren, 1995). Briefly, a 450 Watt Xenon arc and a monochromator (MM12, Carl Zeiss, Oberkochen, Germany) provided the radiation. The beam was directed into the rat's eye under Maxwellian view conditions. In a plane conjugate to the retina a rectangular diaphragm was placed which determined the size of the irradiated retinal area. Diaphragms of three different sizes were used with retinal dimensions of 200, 400 or 800 µm wide. All were 1000 µm high. Retinal irradiance was calculated from measured irradiance as described previously (Gorgels *et al.*, 1995).

Anaesthesia

The rats were sedated with ether and anaesthetized with an initial intraperitoneal dose of pentobarbital 50 mg per kg. Anaesthesia was maintained by intravenous infusion with 15 mg pentobarbital per kg per hr in saline (1 ml/hr). Pupils were dilatated with phenylephrine 5% and cyclopentolate HCl 1%. Atropine sulfate 0.15 mg was injected s.c. The cornea was moistened during the experiment by a continuous flow of saline (4 ml/hr). Body temperature was measured by a rectal thermometer and kept between 37.2^{o} and 38.0^{o}C by an electrical blanket. After the irradiation the animals recovered in the dark until they could maintain physiological body temperature (approximately 2 hours). Thereafter they were returned to the cyclic light environment**.**

Irradiation

The anaesthetized rat was fixed in a restrainer; the eyelids were kept open with tape. Using 570 nm light of low intensity (retinal irradiance < 0.5 mW/cm^2), the beam was aimed at a position slightly superior in the nasal part of the retina. The size limiting diaphragm was placed in the beam. The retina was irradiated at 380 nm (bandwidth 10 nm) for 9 to 12 minutes to achieve a dose of 3 J/cm^2. For the second and third irradiation the rat was rotated, respectively, 20^{o} and 40^{o} in the horizontal plane (Fig. 1), in temporal direction. In each eye two 800 µm areas and one of either 200 µm or 400 µm were irradiated; the positions of the different sizes were randomized. The rationale of inflicting two 800 µm lesions was that we anticipated that these large lesions would not fully recover, thereby providing retinal markers for identifying the position of the smaller lesion. During irradiation the field position was regularly checked for movement of the eye by observing the autofluorescence of the 380 nm radiation. After the irradiation the position was again checked with green light of low retinal irradiance.

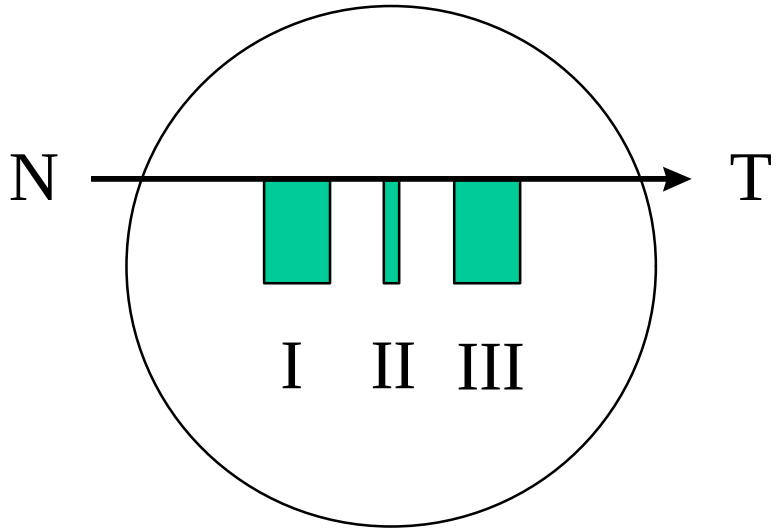


Fig. 1. Schematic drawing of the irradiation order in the retina of the right eye. N: nasal direction, T: temporal direction, I: first irradiation, most nasal location, in this case 800 µm wide; II: second irradiation 20° more temporally located, in this case 200 µm wide; III third irradiation, 800 µm wide.

Funduscopy.

One day after irradiation funduscopy was performed in all 12 animals; four animals were prepared for histology. For funduscopy the following aspects of the lesion were noted: size, appearance, color, demarcation. The remaining animals were analyzed at day 21, and again four were used for histology. The remaining four had funduscopy at day 183, and were used for histologic analysis. For funduscopy the rats were sedated with ether and anaesthetized with pentobarbital 50 mg per kg by intra-peritoneal injection. Pupils were dilatated with phenylephrine 5% and cyclopentolate HCl 1%. Fundus photos were made using a Topcon fundus camera, field 50°. After fundus-photography indirect funduscopy was performed. The animal either recovered to be analyzed on a later time point, or was perfusion fixed for histology.

Histology

The rats were fixed by an intra-cardiac infusion with saline followed by paraformaldehyde 2% and glutaraldehyde 2% in sodium cacodylate buffer, pH 7.4. The eye was removed and immersed in the same fixative overnight. The next day, the eyecup was inspected using a stereo-microscope. A tissue segment was excised containing the three irradiated rectangles together with adjacent control retina. The tissue segments were rinsed in buffer and postfixed for 1 hr in OsO_4 1% fixative. Next, they were dehydrated in ethanol and embedded in Epon. The sections were stained with toluidine blue and examined by light microscopy.

Measurement of lesion size

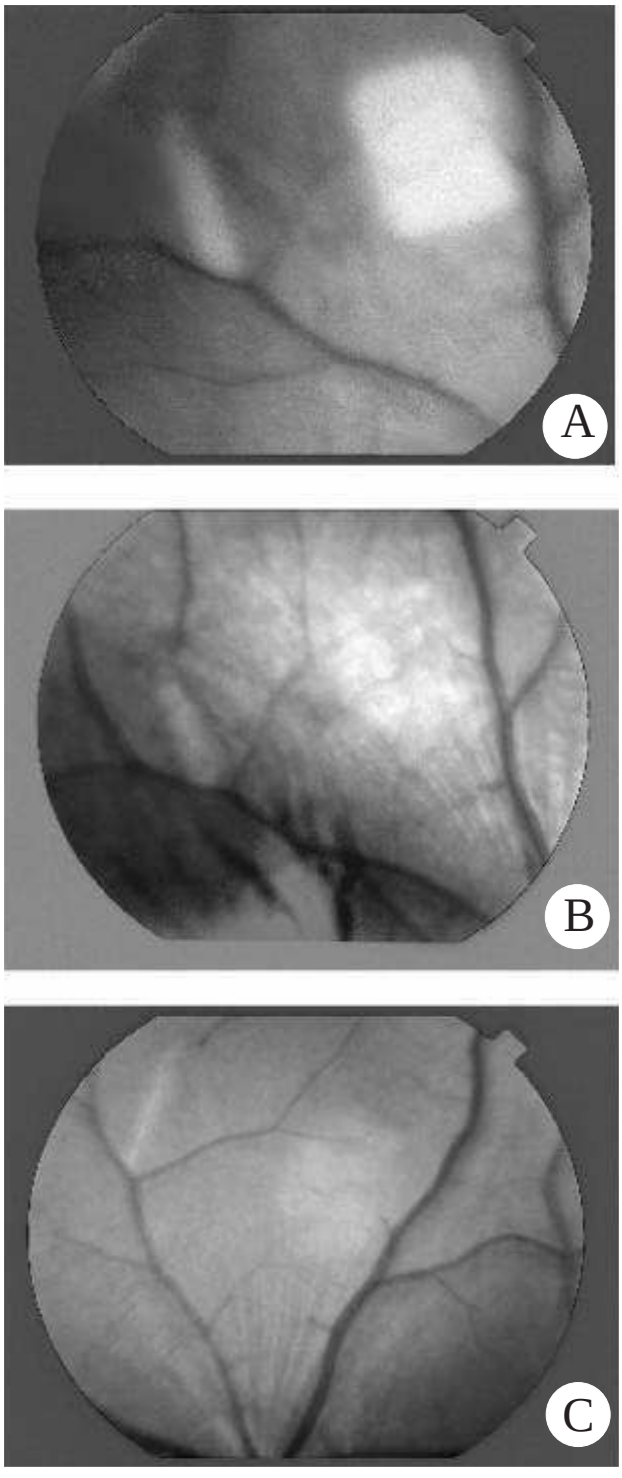
The diameter of the affected area was measured from photo's using a digital image

analyzer. Lesion size was measured as the distance between the two flanking (normal appearing) PR columns. Measurements were performed at four levels: the nuclei on the inside of the outer nuclear layer (ONL), the nuclei on the outside of the ONL, the inner segments (IS), and the distal endings of the outer segments (OS). In order to obtain reliable measurements of lesion size several precautions were taken. All lesion diameters were measured twice without knowledge of original lesion diameter and time point after irradiation. The difference between the two measurements was less than 5 %. All lesions were inflicted with the same three (fixed) diaphragms. Experiments during which eye movements occurred were excluded. Retinal areas with large blood vessels that may filter the incident light, were excluded from analysis. At least four different irradiated areas were examined for each time-point and lesion size. The sections were cut at right angles to the irradiated rectangles, in order to obtain reliable measurements of the width of the lesion. Most lesions were cut over the full length of the lesion, in order to evaluate whether the lesion size remains constant throughout the lesion. Lesion sizes were measured after observing a constant lesion size over at least 100 µm into the lesion.

Results

Funduscopy

One day after irradiation the fundus showed homogeneously bright white rectangles that matched the irradiated areas (Fig. 2A). After three weeks the brightness of the irradiated areas had decreased, and the patches often were mottled and appeared smaller than at one day after irradiation (Fig. 2B). After six months the irradiated area appeared only slightly lighter than control retina, but the 200 µm lesion as well as the 800 µm lesion remained visible (Fig. 2C). In addition, close to the irradiated area the fundus was slightly darker than the more remote fundus.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B
C

Fig. 2. (previous page) Fundus photographs of the same two photoreceptor lesions at three time points after irradiation. The left lesion was originally 200 µm wide, the right lesion was 800 µm wide. Please note that the photos have a somewhat different orientation. The position of the lesions should be appreciated relative to the course of the surrounding retinal vessels. A: One day after irradiation both lesions appear bright, white and sharply demarcated. B: Three weeks after irradiation both lesions appear less bright and less demarcated. C: Six months after irradiation both lesions are still visible, but the 200 µm lesion in particular appears much smaller than one day after irradiation. Both lesions are only slightly brighter than control retina.

Light microscopy.

After cutting the small side of the rectangle the lesion diameters remained constant over several hundreds of μm. One day after irradiation nearly all photoreceptors in the irradiated area were damaged, showing dark staining and nuclear condensation. No cell loss was apparent. An example of a 200 µm lesion is presented in Fig. 3A; a lesion of 800 µm is shown in Fig. 4A. The RPE appeared normal, the inner retina edematous. The border between the affected and the unaffected photoreceptors was not wider than two ONL columns (approx. 10 µm), indicating a sharply demarcated irradiated area. The size of the lesion was roughly similar throughout the depth of the PR layer (Fig.5B). The width of the affected area was measured as the distance between the unaffected PR columns on both sides of the lesion. This was done at four depths in the PR-layer. Measurements of the lesion size are presented in Fig. 5 and summarized in Table I.

Table 1 Lesion diameter in µm (measured as the distance between the flanking, unaffected photoreceptor columns on both sides of the lesion), at three time points after irradiation. Measurements were taken at four levels: iONL= the innermost Outer Nuclear Layer nuclei, oONL= the outermost Outer Nuclear Layer nuclei, IS= Inner Segments, OS/RPE= tips of the outer segments. SD= standard deviation.
ANOVA had the following results: the lesion size decreased (from day 1 to day 21, at all levels and for all irradiation sizes ($p<0.05$); no further decrease occurred between day 21 and day 183. Asterisks (*) indicate that the size of the lesion is smaller at the level of the iONL than at the other levels ($p<0.05$).

Irradiation width	Day	Layer			
		iONL (SD)	oONL (SD)	IS (SD)	OS/RPE (SD)
200 µm	1	149 (± 32)	159 (± 29)	166 (± 31)	172 (± 34)
	21	91 (± 23)*	51 (± 17)	39 (± 11)	12 (± 1)
	183	92 (± 22)*	48 (± 25)	38 (± 21)	9 (± 9)
400 µm	1	358 (± 16)	370 (± 22)	377 (± 24)	388 (± 30)
	21	141 (± 24)*	77 (± 19)	62 (± 13)	41 (± 17)
	183	181 (± 47)	135 (± 76)	116 (± 72)	70 (± 64)
800 µm	1	745 (± 38)	769 (± 46)	782 (± 52)	797 (± 41)
	21	444 (± 57)	347 (± 85)	322 (± 93)	295 (± 99)
	183	536 (± 29)*	419 (± 32)	394 (± 20)	348 (± 28)

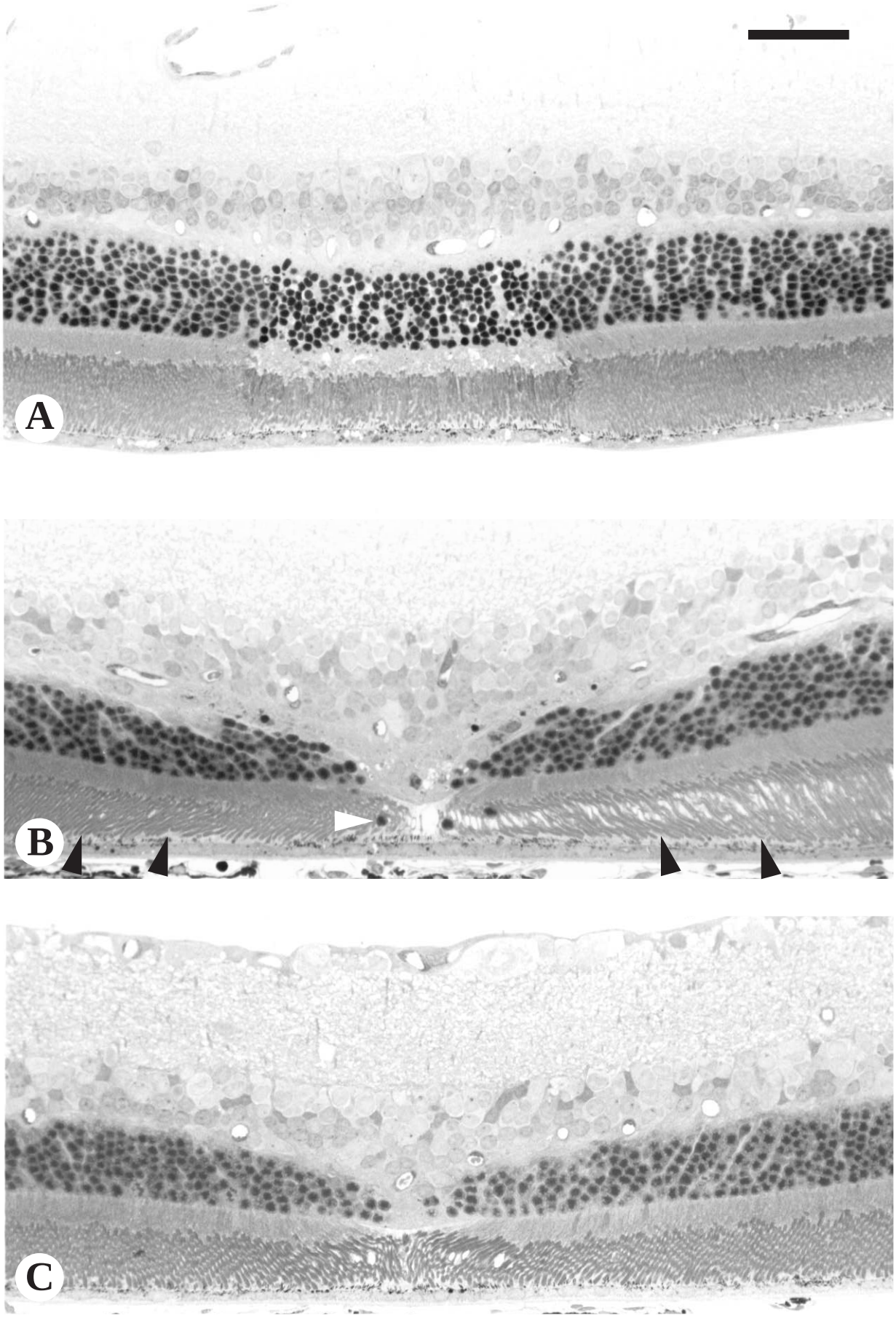
A
B
C

Fig.3. (previous page) Light micrographs of rat retina after exposure of an area of 200 µm wide to 380 nm at a dose of 3 J/cm2. **A**: One day after irradiation: Homogeneously dark staining of ONL, IS and OS is seen in a sharply demarcated area, surrounded by control retina. **B**: Three weeks: All affected photoreceptors have been removed. The lesioned area is filled by inclined photoreceptors; INL thickness is increased. Subretinal space is increased (black arrowheads). Some photoreceptor nuclei are located between the inner and outer segments (white arrowheads). **C**: Six months: At the level of the outer segments the size of the lesion has slightly decreased further (not statistically significant) by migration of surrounding photoreceptors, the lesion is closed. Bar: 50 µm.

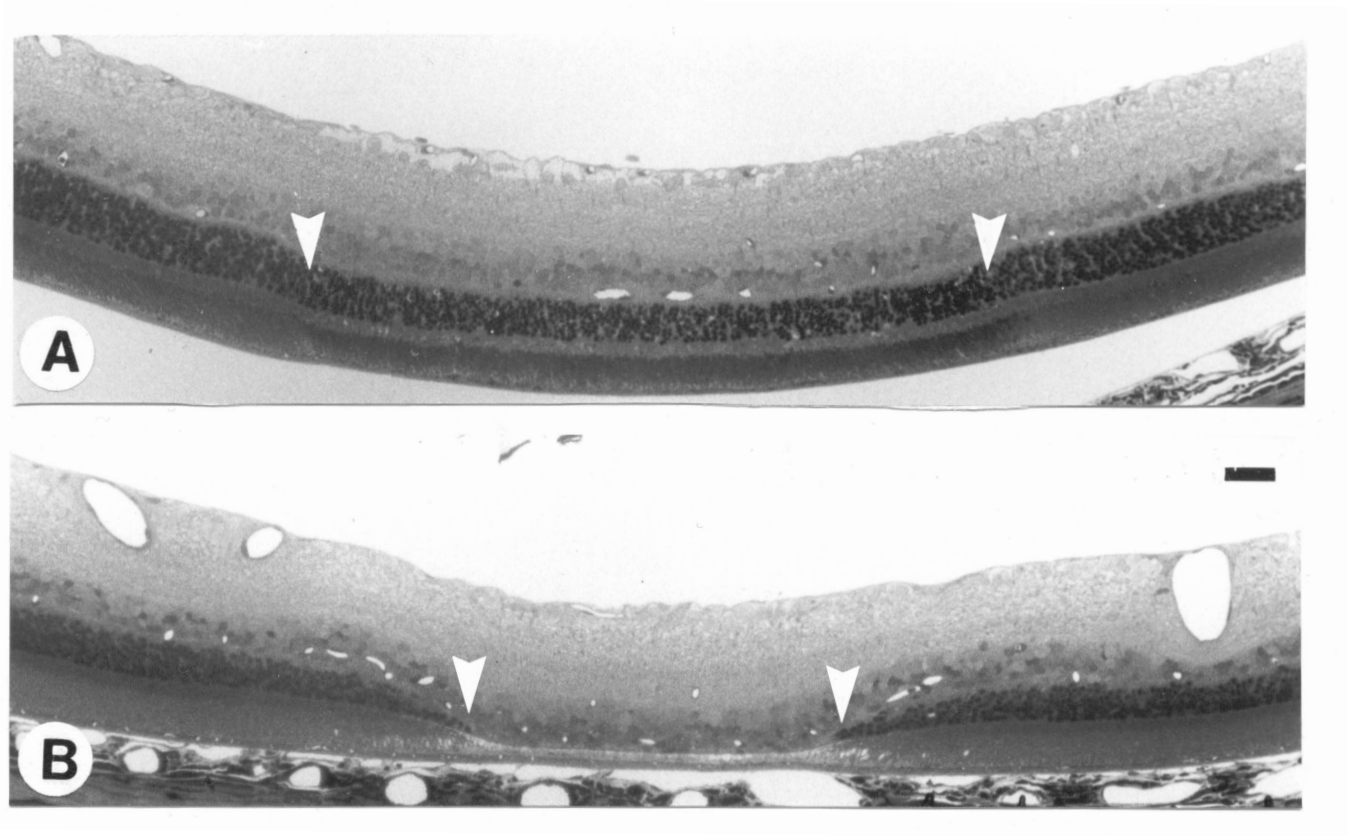

Fig. 4. Light micrographs of rat retina after exposure of an area of 800 µm wide to 380 nm at 3 J/cm2; The borders of the lesion are indicated by arrowheads. **A**: One day after irradiation: Homogeneously dark staining and decreased thickness of ONL, IS and OS in a sharply demarcated area, flanked by control retina. **B**: Six months: The size of the lesion has decreased. The OLM has come to rest upon the RPE. Bar: 80 µm.

Three weeks after irradiation, practically all irradiated photoreceptors had been removed (Fig. 3B). Occasionally, a few degenerating PR nuclei and a few apparently unaffected cone nuclei were seen in the lesion. In addition, vacuoles and macrophages were present. The inner retina appeared normal, apart from some dark inclusions in the inner nuclear layer (INL) and ganglion cell layer (GCL). In the center of the lesion the INL thickness had increased at the expense of the ONL. In the 400 µm and 800 µm lesions the outer limiting membrane (OLM) came to rest upon the RPE (not shown). The RPE in the irradiated area had returned to normal. Quantitative analysis of the lesion size at three weeks showed a substantial decrease of the lesion size as compared to one day after irradiation. This decrease depended on the retinal depth. The least decrease was observed at the level of the innermost ONL nuclei (iONL), the largest occurred at the level of the tips of the OS (Fig. 5). For example, at the latter level, the lesion size of the irradiations of 800 µm had decreased to 350 µm, whereas at the iONL level it decreased to 540 µm. For the smallest irradiation the OS had, in most cases, filled the lesion, but at the iONL level there was still a gap of 90 µm.

Because of the difference in migration between the various PR levels, the orientation of the photoreceptors at the flanks of the lesion was strongly deranged. ONL columns and IS were strongly inclined. This effect of distorted PR orientation extended beyond the borders of the irradiated area. The orientation gradually reached normal at a distance of approx. 100 µm from the border of the original lesion (for all sizes). Sometimes, in the area adjacent to the defect, a few photoreceptor nuclei were located external to the OLM.

Six months after irradiation the lesions consisted of a local PR-defect without apparent damage to other retinal layers (Fig. 3C, 4B). The ONL could hardly be discerned; it contained some sparse cone nuclei. The lesion size had not decreased further (Fig. 5). In the larger lesions, there was even a slight, but not statistically significant, increase in lesion diameter. The PR columns on both sides of the defect had an oblique orientation, gradually changing to normal in the area surrounding the lesion. The subretinal space in the retina surrounding the lesion was less enlarged than three weeks after irradiation.

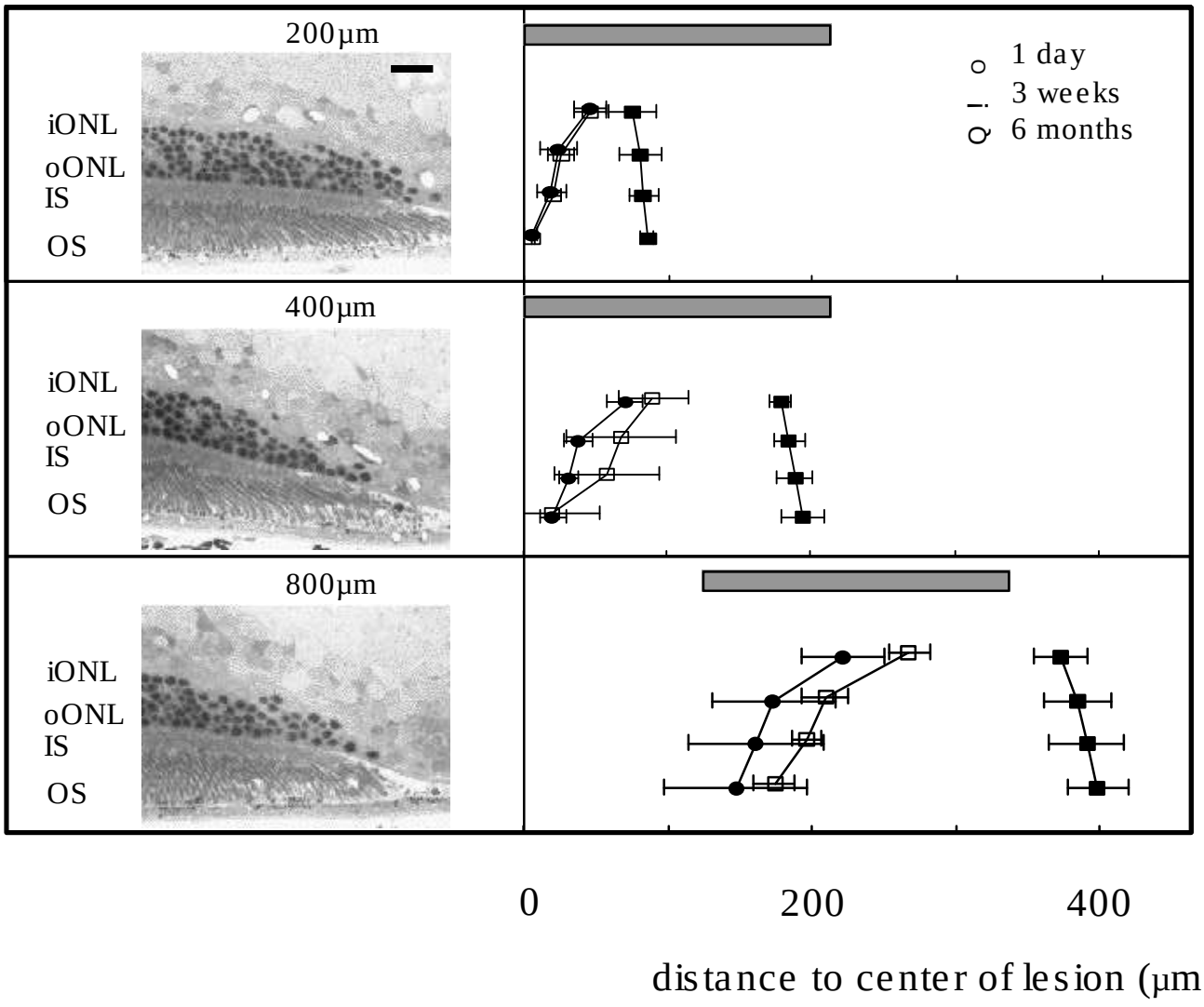

Fig 5. Left: Micrographs illustrating the oblique orientation of the photoreceptors flanking the lesion at 6 months after irradiation. Bar: 50 µm. The grey bar on the right half of the figure indicates the area from where the light micrograph is taken.
Right: Graph depicting the distance of the border of the lesion to the center of the lesion (± SD) at 1 day, 3 weeks and 6 months after irradiation for 3 lesion sizes. The distance was measured at four different levels, i.e. the outermost ONL nucleus (oONL), innermost ONL nucleus (iONL), inner segment (IS) and outer segment tips (OS). The tips of the outer segments of the flanking photoreceptors move inwards the most.

Discussion

We investigated the fate of lesions in the photoreceptor layer produced by sharply focused UV-A irradiations. After the loss of the PRs in the irradiated area, the bordering PRs shifted into the lesion, thereby reducing the size of the lesion. For lesions of 200 µm wide the outer segments of photoreceptors of both flanks met, filling in the lesion completely within three weeks after irradiation. Larger lesions of 400 and 800 µm also substantially decreased in size, but were not filled in completely. Apparently there is rapid migration of photoreceptors into the lesion. This process is completed in a few weeks, since no progression in the process of filling in was found between three weeks and six months after irradiation.

Previous reports on this subject yielded conflicting results. Ham *et al.* (1982) exposed monkey eyes to UV-A irradiations with a retinal spot size of about 500 µm. In histologic material taken at 5 days after irradiation they noticed that a PR lesion had developed, but one month after irradiation no lesion could be identified. In contrast to these findings, we could still identify the lesion at later time points. Even the smallest lesion (200 µm) was not filled-in completely at the level of the ONL. To explain this discrepancy apart from species differences, it is important to note that Ham's observation was based on a few lesions in a single eye. Moreover, the size of the lesions, as observed after five days was much smaller than the irradiated spot size of 500 µm. Possibly, the original lesion (the one that could not be identified) had also been smaller than 500 µm. In our case, early lesions always matched the irradiated area.

No filling-in was found in a study on lesions in the photoreceptor layer of the squirrel retina (Collier *et al.*, 1989a). The main difference with our study probably is that we took several precautions for a reliable measurement of the lesion size. In Collier and Zigman's study (1989) relatively large lesions (29º) were examined and there appeared to be no precise measurement of the lesion size. In such conditions a relatively small reduction in size might go unnoticed. In addition, it is uncertain whether the irradiation was sharply demarcated. Rather it appeared that there was a border zone, with partial damage (Collier *et al.*, 1989b). This will also obscure an inward migration of neighboring photoreceptors.

The current study shows that lesions in the PR layer can be closed in by PR cells surrounding the lesion, but at the same time it shows the limitations of this process. Reduction of the lesion size by cell division of the PRs in adult retina is unlikely, but cannot be excluded. Redistribution of the PRS over the retinal surface would seem to be a more plausible explanation. The decrease in size of the lesion is remarkable when compared with photocoagulation lesions. The size of these lesions remains constant, or even increases due to pigment atrophy (Morgan *et al.*, 1989, Schatz *et al.*, 1991). Multiple factors will influence a movement of PRs into the lesion. Photocoagulates generally show the formation of scar tissue which will block a filling-in of the lesion by PRs. However, a distinct glial scar was not observed in our UVA induced lesions. On the other hand, it has been demonstrated that Muller cells show a clear reaction in response to light damage (Burns and Robles, 1990). One may speculate that this may lead to contraction of the lesion pulling the neighboring PRs inwards. Another important difference is that UV-A induced lesions have an intact RPE monolayer, while the RPE is destroyed in photocoagulates. An intact RPE may be essential for PR movement**.** The connection between the PRs and the RPE appears to be flexible to some extent

(Roider *et al.*, 1992), and a lack of RPE apparently blocks migration of PRs (Eysel, Gonzales-Aguilar, and Mayer, 1980). In the present study we noticed that the tips of the OS always moved inwards further than the rest of the PR. This suggested that the RPE may provide a suitable substrate for PR movement. On the other hand the restrictions for further movement into the lesion are most likely to be found on the proximal side of the PR. The innermost ONL nuclei move inwards less than the outermost ONL nuclei suggesting that the synaptic contacts in the OPL may impose limits on the movement. In our model the inner retinal layers of the irradiated area show no degeneration. Therefore a continuation of the inward movement of the PR's would depend on a displacement of the synapse itself, on the synaptic remodelling or axonal elongation. These processes occur to some extent between day 1 and day 21 after irradiation since the distance between the flanking innermost ONL nuclei declined by 300 µm in the 800 µm lesions, which is more than their distance to the OPL. In this period the INL in the irradiated area increased in thickness which may indicate that the irradiated area is compressed by the surrounding retinal areas moving inwards. Yet these processes have only limited capacity as no further reduction was noticed between 21 days and six months after irradiation.

The altered orientation of the PRs which have entered the lesion could explain the apparent discrepancy between the histological and funduscopical findings. Funduscopically, even the smallest lesions remained visible at six months after loss of the photoreceptors (Fig. 2), while the histological analysis reveals a closed lesion on the level of the OS tips (Fig. 3C). In the smallest lesions this increased reflectance observed in the irradiated area could well be caused by the disturbed alignment of the PRs (Fig. 5). Due to the inclined orientation, the PRs are no longer directed towards the pupil entrance. Thus, the light guidance/light capturing capacity of the PRs will be decreased (Enoch, 1979). The light not captured, is partially scattered and reflected, causing a brighter (than control) funduscopic appearance of the filled lesion (Fig. 2, left lesions). However, the altered orientation of the photoreceptors cannot explain the persistence of lesions of 400 and 800 µm wide, because the center of these lesions does not contain any photoreceptors. The glial tissue replacing the photoreceptors (Fig 4B) could induce this increased reflection (Fig. 2 B,C) .

The redistribution of PRs which we observed in our animal model may have relevance for several pathologic conditions involving local PR loss (e.g. by vascular accidents, light damage or hereditary PR loss (Flannery *et al.*,1990, Huang *et al.*,1993). Similar to our histological findings, Bedell *et al.* (1981) measured psychophysically a disturbance of PR orientation in the region bordering a region of choroidal atrophy. This disorientation could be caused by migration of PRs into the lesioned area. In the current study we measured that the capacity for redistribution is limited: The limit lies at about 200 µm which is 3°. In addition, it should be noted that the orientation of the PRs is very disturbed. The orientation of the OS influences the photoreceptor function, in particular that of cones (Enoch and Lakshminarayanan, 1991). Rods are less direction-sensitive (Tobey and Enoch, 1973). Since in rat retina 99% of the photoreceptors are rods, the filling-in may in fact effectively remove blind spots, at least at the level of the retina. In the current study the disorientation of PR columns spreads about 100 µm around the originally irradiated area (for all three sizes of lesion). This finding might have clinical correlates. Recently, Ezra *et al.* (1997) demonstrated that the visual acuity after a single macular hole operation is worse when the removed preretinal tissue (operculum)

contained retinal elements of the deeper layers. In patients with such ‘true opercula’ a defect possibly remains at the level of the PRs after surgery. A filling-in process similar to our findings could cause disorientation of PRs in the surrounding retina, explaining the loss of visual acuity. Additionally, when the defect is anatomically closed by a second operation, the photoreceptors probably become realigned, and the prognosis becomes similar to that of patients with false opercula. Realignment of photoreceptors in human retina might be a slow process, because the visual acuity has been described to improve for two years after macular hole surgery (Leonard *et al.* 1997).

Acknowledgements

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Chapter 4

The effects of two stereoisomers of N-acetyl-cysteine on photochemical damage by UVA and blue light in rat retina

Eelco M. Busch, Theo G.M.F. Gorgels, Joan E. Roberts, Dirk van Norren

(manuscript under second review)

Abstract

High doses of light can cause damage to the retina e.g. during intra ocular surgery. Previously, thiols have been demonstrated to protect against retinal damage in various damage models. Such protection is very promising for clinical practice. Retinal light damage can be caused by a relatively short exposure to high irradiance levels. These conditions occur during intra ocular surgery. In the current study we therefore investigated whether the thiol N-acetyl-cysteine protects against retinal light damage under high irradiance conditions in the rat retina. Two stereoisomers of this thiol were tested for protection against two spectrally defined types of retinal light damage. Shortly after administration N-acetyl-L-cysteine in doses of 270-1000 mg/kg IP protected against 380nm (UVA) light but not against 470nm (blue) light. Two hours after injection the protection had diminished. We observed no protection by the stereoisomer N-acetyl-D-cysteine. From this study we conclude that N-acetyl-L-cysteine protects stereospecifically against retinal damage in the UV, but not in the visible part of the spectrum. This limits the possible applications.

Key words: oxygen radicals, reactive oxygen species, reactive nitrogen species, N-acetyl-L-cysteine, N-acetyl-D-cysteine, glutathione, rat

Introduction

Exposure to high doses of light can cause damage to the retina. The classical example is eclipse blinding, of which the first known record dates back to Plato's Phaedo. Recently, in two editorials the hazard of full spectrum light from the operation microscope in the ophthalmic practice has been stressed (Minckler, 1995; Mainster *et al.*, 1997). During standard procedures such as extra capsular cataract surgery, photic maculopathy can develop within the average operating time (Byrnes *et al.*, 1995). Several risk-factors (e.g. duration of the operation, light intensity (Minckler, 1995) and spectral composition) have been identified, but the exact mechanism of retinal light damage is still unknown. Probably the damage results from toxic photochemical reactions, initiated by absorption of light in the retina. Investigating whether a compound with known molecular properties protects against retinal light damage thus serves two purposes. (1) This may shed light on the (protection) mechanism. (2) It may identify a compound that can clinically protect the retina against damaging light exposure experienced during operations. The current study describes the effects of two such compounds.

Kremers and van Norren discerned two classes of retinal light damage, which can be obtained under different circumstances. The first class occurs with long exposures under incomplete bleach conditions, as for instance described in the classical study by Noell *et al.* in 1966. The second class of retinal light damage occurs with short light exposures (generally less than one hour), under fully bleached conditions. The latter condition is typical for clinical practice, and was therefore chosen in the present study. Ham and Mueller (1989) described two spectrally defined damage types in the monkey retina after short irradiations to high doses of light. Near UV light (350nm) caused specific loss of photoreceptors, while exposures to 441nm (blue) light caused a highly inflammated RPE. Gorgels and van Norren (1995) gave a more extensive description of these types in the rat. Irradiation at 320-440nm caused damage throughout photoreceptors, while after exposure to 470-550nm at threshold levels, changes were noted mainly in the RPE. In the present study we chose two wavelengths for irradiation, 380nm and 470nm, each representative for a spectral type of damage.

Blue light damage in the macaque retina has been shown to increase when O_2 pressure in the blood increases (Ruffolo *et al.*, 1984). This result suggests the involvement of reactive oxygen species (ROS). One intermediate in retinal light damage in both mice (damage of the first class) and rats (Yamashita *et al.*, 1995) (damage of the second class) has been shown to be the reactive oxygen species H_2O_2. Thus it is plausible that retinal light damage might be alleviated by administering ROS quenchers.

There are at least four endogenous ROS quenchers in the eye: β-Carotene, vitamin E (α-tocopherol), vitamin C (ascorbic acid) and glutathione. Each has specific and unique quenching properties. For instance β-Carotene and vitamin E quench singlet oxygen, and vitamin C quenches superoxide. Glutathione does *not* efficiently quench either singlet oxygen or superoxide, but it does quench other reactive species which are released during an inflammatory response: peroxide, nitric oxide, hydroperoxide, and hypochlorous acid.

Glutathione, a tripeptide, is not efficiently transported into cells (Davidson *et al.*, 1994), and thus cannot be directly administered as an exogenous drug. Other thiols can be given that will either enhance the endogenous synthesis of glutathione (NAC) and/or directly scavenge

inflammatory ROS. It has been demonstrated that exogenous thiols can cross blood ocular barriers and protect the lens and the retina against light damage (Lam *et al.*, 1991, Zhang *et al.*, 1994, Wong *et al.*, 1994). For example Lam *et al* (1991) and Wong *et al*. (1994) described protection of the thiol DMTU against retinal light damage. In their experiments retinas of albino rats were exposed over a prolonged period to light of low intensity, resulting in damage classified by Kremers and van Norren (1989) as damage of the first class. Roberts *et al*. (1991) found that the thiol WR-77913 provided photo-protection of the lens, while Remé *et al*. (1991) described protective properties against retinal light damage in dark adapted albino rats for the same thiol. As explained before, the conditions in these experiments were quite different from the conditions under which light damage occurs in humans. We sought to determine whether thiol compounds would also protect against retinal light damage by short irradiation at high exposure levels (damage of the second class).

For our experiments we chose the thiol NAC because it is widely used in humans, e.g. against chronic obstructive pulmonary disease, and against acetaminophen intoxication. We inflicted light damage of the second class by irradiating at high exposure levels at two distinct wavelengths (380nm and 470nm) and assessed the protection of the non-toxic thiol NAC against damage from either or both wavelengths of light.

Two different mechanisms of protective action have been proposed for NAC: direct s cavenging of ROS by NAC (Aruoma *et al.*, 1989; Gillissen *et al.*, 1997), or indirect scavenging by enhancing glutathione synthesis (Butterworth *et al.*, 1993; Gross *et al.*,1993). In order to differentiate between these two mechanisms, we also evaluated protection by the stereoisomer N-Acetyl-D-Cysteine (D-NAC), which can act as a direct scavenger (Sarnstrand *et al.*, 1995), but cannot be incorporated into glutathione (Sjodin *et al.*, 1989).

To carry out these experiments we irradiated small patches of rat retina at 380nm or at 470nm in non-treated control animals, and in animals treated with L-NAC or D-NAC. We used funduscopy and light microscopy to assess damage.

Materials and methods

Animals

Male pigmented Long Evans rats, were obtained from Harlan CPB, Zeist, the Netherlands at the age of 30 days. They were kept in a 12-12 hr light-dark cycle, at 10-90 lux illumination by white fluorescent light. Room temperature was 22-24ºC. The animals used in the experiments were 60 to 130 days old. Animals were treated to conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Optics

Optics were similar to those described in Gorgels and van Norren and in Chapter 2. Briefly, under Maxwellian view conditions, a rectangle of light measuring 12 by 12.6^{o} was projected on the retina. Radiation was obtained from a 450 Watt Xenon arc. Wavelengths were selected by a monochromator (MM12, Carl Zeiss, Oberkochen, Germany) or by interference filters. We used a bandwidth of 10nm centered around 380nm or 470nm. In a plane conjugate to the retina a set of neutral density filters divided the radiation into four bundles with intensities of approximately 100, 50, 25, and 12 %; these bundles were separated by black bars of 1^{o}. The output of all bundles was measured with a radiometer, and retinal irradiance was calculated from measured irradiance as described previously[7]. Light reflected from the retina was branched off by a mirror and focused by two lenses into the observer's eye.

Anaesthesia

The rats were sedated with ether, anaesthetized with pentobarbital (50 mg/kg) by intra peritoneal injection. The animals were kept anaesthetized during irradiation by intravenous infusion with pentobarbital 15 mg/kg/hr. Body temperature was measured by a rectal thermometer and kept between 37.2^{o}C and 38.0^{o}C by an electric blanket. Atropine sulphate (0.1 mg) was injected subcutaneously. Pupils were dilatated with phenylephrine 5% and cyclopentolate HCl 1%. Following irradiation the animals recovered in the dark until they could maintain physiological body temperature (approximately 3 hr). Thereafter they were returned to the cage**.**

Irradiation

Anaesthetized rats were fixed in restrainers, and eyelids were kept open with tape. The cornea was moistened during the experiment by a continuous flow of saline (4 ml/hr). The rat was positioned using weak light of 570nm (Eret <50 mW/cm^{2}). A position in the central retina was found, slightly superior. After irradiation, the position was checked and the rat was repositioned approximately 20^{o} more temporally, where the second and, another 20^{o} more temporally, the third irradiation were performed. By observing the retinal blood vessels in the irradiated area, the position could be monitored. When eye movements of more than 1^{o} occurred the results were discarded. Body temperature was checked every ten minutes.

Anti-oxidants

Solutions of N-Acetyl-(L)-Cysteine (Sigma, the Netherlands) and of N-Acetyl-(D)-Cysteine (kindly provided by Dr B. Särnstrand ASTRA-Drako, Sweden) were made in saline. The pH was set to 7.4 by adding NaOH. Control rats received 10 ml/kg doses saline. All injections were administered in anaesthetized animals, intra-peritoneally and in a double blind protocol.

The irradiation scheme is drawn in Fig. 1

In the first series of experiments the rats received either 1000 mg/kg, 500 mg/kg or 270 mg/kg L-NAC dissolved in saline (10 ml/kg). The injection-irradiation intervals were planned such that three irradiations could be done in a single experiment. The factor of 10^3 difference in damage sensitivity for 380nm radiation and sensitivity for 470nm radiation (Chapter 2) was overcome by increasing the duration from 8 to up to 90 minutes and increasing the retinal irradiance from 5 mW/cm^2 to 300 mW/cm^2 for the 470nm irradiation.

All irradiations were performed by an experimenter who was unaware of the contenets of the injection. The first irradiation with 380nm was administered 15 minutes after injection. The 470nm irradiation started 30 minutes after the injection, and the final, second 380nm irradiation 120 minutes after injection. In the second series of experiments, injections with L-NAC (500 mg/kg) or saline were administered. After 15 minutes animals were subjected to radiation of 470nm for 45 minutes, and 60 minutes after the injection the irradiation with 380nm was performed. In the third series rats received either D-NAC (500 mg/kg) or saline. After 15 minutes the 380nm irradiation was administered and 30 minutes after injection the 470nm irradiation was performed. The setup of these experiments was similar to the setup of the experiments in series 1. The total (anaesthesia) duration of these experiments was slightly (<10%) shorter than in the experiments in series 1, due to the lack of the second 380 nm irradiation.

Analysis

Data was obtained three days after irradiation. The effects were examined in two ways by an observer unaware of the substance injected (NAC or control). (1) Indirect funduscopy with orange light was performed at a low level of retinal irradiance (< 50 mW/cm^2). The rats were sedated with ether, and anaesthetized with pentobarbital by intra-peritoneal injection. Pupils were dilated as described above. After funduscopy, material was prepared for histological analysis. In each series of experiments NAC injected animals were compared to saline injected controls. The funduscopic threshold dose was defined as the minimal dose required to cause a visible decoloration of the irradiated area. In order to compensate for possible differences in regional sensitivity, and for differences between experiments, the threshold doses were normalized. The threshold for funduscopically visible damage was set to 1 for the control group, thus the value for the experimental group was relative to the threshold of the control group.

(2)With histology we analyzed a representative selection of the specimens which were irradiated at 380nm 15 minutes after injection with L-NAC (500 mg/kg) or saline, the group in which we funduscopically observed the best protection. The rats were perfusion fixed by intracardiac infusion with phosphate buffered saline followed by fixative of paraformaldehyde 2% and glutaraldehyde 2% in 0.1 M sodium cacodylate buffer (pH 7.4). The eye was removed and immersion fixed in the same fixative overnight. The irradiated parts were resected with adjacent control retina and postfixed for 1 hr in OsO_4 1% fixative. The tissue was dehydrated in ethanol and embedded in Epon. From each damage area 200 µm was cut and every 50 µm some semi-thin sections (1 µm) were collected, stained with toluidine blue and examined.

These lesions were analyzed by light microscopy, and the photoreceptors (PRS) were counted in an area of 160 µm wide. The area irradiated at 0.6-0.8 J/cm^2 was counted over the full diameter of the damaged area.

Results

Data were obtained for a total of 59 animals. Every dose presented is the mean (± SEM) of three to seven experiments. The differences were analyzed by a separate variance *t*-test for difference between two sample means (ANOVA).

Funduscopy

In the first series of experiments, we tested 3 doses of L-NAC. When NAC was administered 15 minutes before the start of the irradiation, the threshold dose for damage by 380nm radiation increased significantly for all three doses of NAC (up to 70% for a dose of 500 mg/kg; Table 1, Fig. 1). The effect did not differ significantly between the three doses. Two hours after injection, NAC did not protect against irradiation with 380nm (Fig. 1; only the data for the 500 mg/kg experiments are presented, Table 1). None of the doses protected against 470nm radiation one hour after injection (Fig. 1, Table 1).

In the second series of experiments we tested whether the lack of protection against 470nm radiation in the first experiment was caused by the interval between injection and irradiation. Therefore, we started irradiating rats with 470nm at fifteen minutes after injection of NAC 500 mg/kg. The NAC caused no significant protection. In the same experiment 60 minutes after injection, the threshold for 380nm radiation was still increased in the NAC treated animals (60%, $p<0.01$). These data are also presented in Fig. 1 and Table 1.

In order to investigate whether protection by NAC is glutathione mediated, we also tested whether D-NAC 500 mg/kg (which cannot be converted into glutathione) protects against light damage. We observed neither a significant difference between D-NAC and saline injected animals in threshold for damage by 380nm (15 minutes after injection), nor an effect on the threshold for damage by 470nm (30-120 minutes after injection, Fig.1, Table 1).

Table 1. Threshold doses for funduscopic damage by irradiation at 470 and at 380nm. The normalized average threshold doses (± SEM) are presented for control, saline injected, animals (=1) and for animals treated with L-NAC (3 doses) and D-NAC. Irradiation at 380 nm was performed at 15-25, 60-70 and 125-135 minutes after injection. Irradiation at 470 nm was performed at 15-60 and 30-120 minutes after injection.
n = number of experiments, (*) = significant difference between NAC treated and control animals ($p<0.05$).

Series	Dose	380nm 15-25 min.		470 nm 15-60 min		470nm 30-120 min.		380 nm 60-70 min		380nm 125-135 min.	
		n	Thresh. (SEM)	*n*	Thresh. (SEM)	*n*	Thresh. (SEM)	*n*	Thresh. (SEM)	*n*	Thresh. (SEM)
1: L-NAC	control	6	1 (0.006)			6	1 (0.077)			6	1 (0.016)
	L-NAC 270 mg/kg	6	1.44 (0.004)*								
	L-NAC 500 mg/kg	8	1.59 (0.031)*			8	1.10 (0.035)			8	1.22 (0.043)*
	L-NAC 1000 mg/kg	4	1.47 (0.033)*								
2:L-NAC	control			5	1 (0.053)			8	1 (0.125)		
	L-NAC 500 mg/kg			5	1.18 (0.066)			8	1.84 (0.23)		
3: D-NAC	control	5	1 (0.018)			3	1 (0)				
	D-NAC 500 mg/kg	5	1.16 (0.047)			6	1.13 (0.06)				

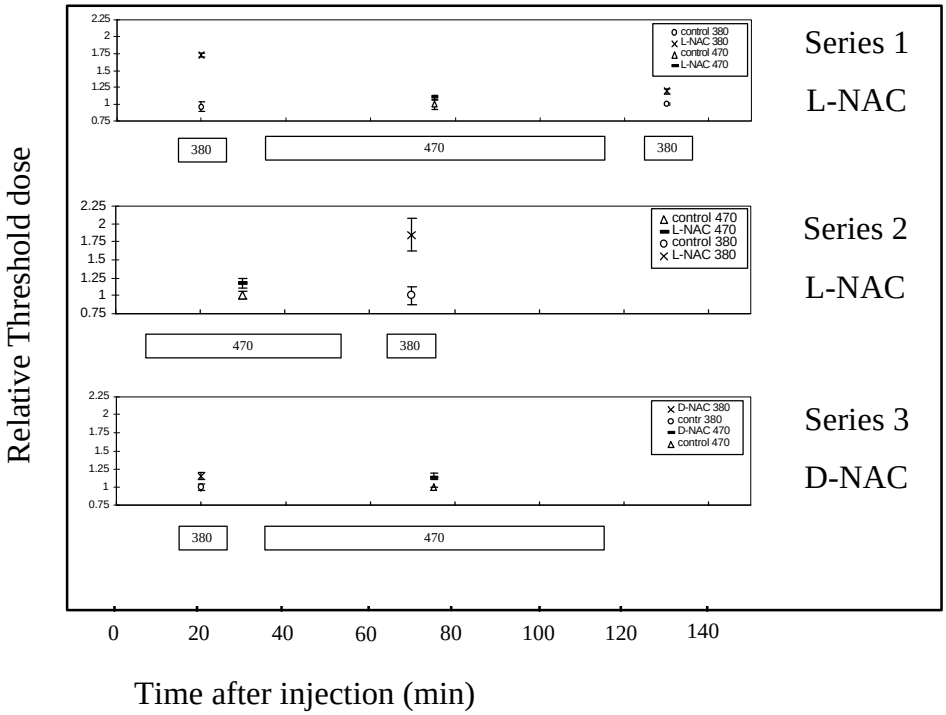

Fig.1. Irradiation scheme and thresholds for funduscopically visible damage for three series of experiments. The bars under x-axis indicate wavelenght and duration of the irradiation. The horizontal axis is time after intra peritoneal injection (T=0). The vertical axis is the normalized threshold dose (control = 1). Data are mean ± SEM of 3 to 8 experiments. Series 1: L-NAC ameliorates significantly ($P<0.05$) the effects of irradiation at 380 nm presented 15-25 minutes after injection. No protection was observed againts 380 nm 125-135 minutes after injection, or against 470 nm radiation30-120 minutes after injection. Series 2: L-NAC ameliorates significantly ($P<0.05$) the effects of irradiation presented 65-75 minutes after injection. No protection was observed against 470 nm radiation presented 15-55 minutes after injection. Series 3: D-NAC ameliorates neither the effects of irradiation at 380 nm presented 15-25 minutes after injection, nor against 470 nm radiation 30-120 minutes after injection.

Light microscopy

For histological evaluation of the effect of L-NAC (500 mg/kg) on light damage, we examined areas which were irradiated at 380nm 15 minutes after injection, and compared them with adjacent non-irradiated areas. In the non-irradiated samples of the retina, we observed no difference between L-NAC treated animals and control animals. In control animals doses of 0.6-0.8 J/cm^2 affected 2-4 % of the photoreceptors. The affected nuclei had a homogeneously dark staining. The corresponding IS had a crumbly appearance and the OS were darkly stained. These results resemble earlier findings in similar conditions (Chapter 2). In the L-NAC treated group, the number of pyknotic PRS was between 1 and 2% of the photoreceptor cells, a significant decrease as compared to the control group ($P<0.05$, Table 2). The morphology of these affected PRS did not differ from the saline injected group. The results of the quantitative analysis are summarized in Table 2.

Table 2. Number of pyknotic and normal appearing photoreceptor nuclei in control and L-NAC (500 mg/kg) treated animals after irradiation at three doses of 380nm. Nuclei were counted in an area of 160 µm wide of cross sections of irradiated retina, for the lowest dose of 0.6-0.8 J/cm^2 the full width of cross sectional area of the irradiated retina was examined. (*) significant difference ($p<0.05$) between control animals and NAC treated animals.

Dose (J/cm^2)	control		L-NAC	
	normal nuclei	pyknotic nuclei	normal nuclei	pyknotic nuclei
0.6-0.8		6.3 (± 0.4)		1.6 (± 0.2)*
1.2-1.4	45 (± 11)	115 (± 10)	149 (± 15)*	65 (± 13)*
1.9-2.1	22 (± 4)	151 (± 4)	60 (± 15)	138 (± 15)

After irradiation with intermediate doses (1.2-1.4 J/cm^2), in the control group 40-80% of the PRS were pyknotic. The IS and OS layers were slightly reduced in thickness. In the ONL, some cell loss had occurred. In the NAC treated group, the percentage of affected nuclei was 20-40%; a reduction of 50% as compared to the control group ($P<0.05$; Table 2). In addition, the number of non-affected nuclei showed a significant increase ($P<0.05$) as compared to control.

After irradiation with the highest doses (1.9-2.1 J/cm^2), we observed no significant effect of NAC. In both groups, most of the irradiated photoreceptors were damaged. The IS and OS layers were reduced in thickness. In the outer part of the ONL, cell loss had occurred. Debris and phagocytes were numerous in the photoreceptor layers. The RPE appeared normal or slightly swollen. In the inner retina, small dark spots were occasionally seen in the OPL and inner nuclear layer (INL).

Discussion

During eye surgery retinal damage may inadvertedly occur due to the light of the operation microscope. Animal studies designed to study this iatrogenic effect showed that in the monkey as well as in the rat two spectral types of retinal damage occur. In the present study we explored whether NAC could attenuate or block these damage types. We found that L-NAC offered protection for damage by 380nm; the dose for funduscopic threshold damage was raised by nearly a factor of two. However, the other damage type, which we investigated by irradiation at 470nm, was not significantly influenced by L-NAC administration.

An important second goal of our study was to gain insight in the mechanism of retinal light damage. The observed protection against radiation of 380nm suggests that this type of damage is mediated by non-specific oxidative processes.

In response to stress (e.g. inflammation, oxidation or heat), retinal tissues produce hypochlorous acid, hydrogen peroxide (Roberts *et al.*, 1991), peroxylradical, superoxide and nitric oxide (NO) (Issels *et al.*, 1988; Cotgreave *et al.*, 1991; Gross et al.; 1993; Rathburn *et al.*, 1996). Although NO is not by itself a strong oxidizing agent, when superoxide is present, NO reacts with it to form the highly oxidizing species peroxinitrite, which can extensively

damage retinal tissue. However, peroxinitrite is not formed in the presence of thiols, including NAC, because the thiols remove NO by reacting with it to form thiol esters (S-nitroso thiols), thus protecting the retina.

In addition NAC and other thiols are excellent reducing agents. They efficiently reduce peroxyl radicals to hydroperoxides, which are then further reduced by gluatathione peroxidase. Since it is peroxyl radicals that initiate lipid peroxidation and destroy membrane layers of the photoreceptor cells, removal of peroxyl radicals by NAC prevents this damage.

Besides acting an antioxidant, NAC can exert protective effects by increasing the production of the endogenous antioxidant glutathione (GSH) (Issels *et al.*, 1988; Cotgreave *et al.*, 1991; Butterworth *et al.*, 1993; Gross *et al.*, 1993; Rathburn *et al.*, 1996). This tripeptide contains cysteine and NAC can act as a cysteine donor for GSH synthesis. Recently it was suggested that extracellular NAC may increase cellular uptake of cysteine (Ferrari, Yan and Greene, 1995). The experiments with D-NAC (the stereoisomer of L-NAC) indicate that the L-configuration is required for protection. D-NAC has similar direct scavenging properties as L-NAC. The main difference between the stereoisomers is that D-NAC is not efficiently deacetylated (Sjodin *et al.*, 1989). Since the main portion of NAC is deacetylated prior to transport into the cell (Banks and Stipanuk, 1994), and little of the cysteine from NAC is found as free cysteine in the cell (Banks and Stipanuk, 1994), and the intracellular cysteine concentration is considered the limiting factor in the synthesis of glutathione (Banks and Stipanuk, 1994), the lack of protection by D-NAC suggests that the protection comes about by increased GSH synthesis rather than by direct scavenging. Remarkably, in an in vitro model (Ferrari *et al.*, 1995), GSH was also increased after prolonged exposure (48 hr) to D-NAC. Apparently, this does not occur in the in vivo conditions of the present experiment. The slightly different setup of the D-NAC series as compared to Series 1, most likely did not affect the threshold dose. The (raw) threshold doses in the control experiments in the D-NAC series were similar to those of the controls in Series 1. Additionally, the threshold dose for 380 nm radiation is similar whether or not irradiations are preceeded or followed by another irradiation (Chapter 2).

Damage by 380nm mainly consists of PR loss (Gorgels and van Norren, 1995). The increase of funduscopic threshold dose corresponds to saving of PRS at low doses; at doses of 0.6-0.8 J/cm^2 we observed a reduction of loss of PRS of 66%. Increasing the amount of NAC administered up to 1000 mg/kg did not result in a better protection. This suggests that the rate of cellular uptake of NAC (Ishii, Sugita and Bannai, 1987) or the rate of GSH synthesis (Banks and Stipanuk, 1994) are imposing limits on the protective effect. At high concentration a pro-oxidant effect of NAC has been described (Sprong *et al.*, 1998). In our study the highest concentration appeared to be less effective (not significant), suggesting a paradoxal effect.

A remarkable finding is that NAC failed to protect against damage from 470nm light. In the post injection interval in which we observed protection against 380nm radiation, the damage by 470nm was not attenuated. Irradiation at 470nm damages mainly the RPE cells, in contrast to 380nm which damages specifically photoreceptor cells (Chapter 2). This lack of protection against 470nm radiation cannot be explained by failure of NAC to penetrate into the RPE cells: In cultured RPE cells intracellular GSH is resynthetized within 60 minutes after incubation with cysteine (Lam *et al.*, 1991), and GSH can equally be replenished by NAC and Cysteine (Banks and Stipanuk, 1994), in lymphocytes).

We must conclude that the damage mechanism from 470nm irradiation differs from that of 380nm. There are chromophores in the retina (melanin, retinal lipofuscin) that absorb light at 470nm. Upon visible light irradiation, melanin produces superoxide (Rezka and Jimbow, 1993; Burke *et al.*, 1998) and retinal and lipofuscin produce singlet oxygen; these two highly efficient reactive oxygen species are not quenched by thiols (Roberts *et al.*, 1991; Aruroma *et al.*, 1989). However, melanin alone is not the chromophore (Gorgels and van Norren, 1998).

Previous studies on retinal light damage have demonstrated protective properties of thiol compounds in various animal models (Lam, Tso and Gurne, 1990; Roberts *et al.*, 1991; Reme *et al.*, 1991; Organisciak *et al.*, 1992; Wong *et al.*, 1994; Li *et al.*, 1993), suggesting that thiols might be used during intraocular surgery to protect patients against light hazards. The experimental conditions of these studies, generally consisted of exposure of whole retinas of free running animals to relatively low illumination levels, after a prolonged period of dark adaptation. These conditions, in general, are quite different from the lighting conditions that patients are subjected to. The difference in conditions might lead to a difference in light damage (Ham and Mueller, 1989; Organisciak and Winkler, 1994). In fact, the limited data on light damage in humans show prominent RPE damage (Tso, 1989; Green and Robertson, 1991), whereas in general the pathology of the above mentioned animal studies has mainly PR damage. The current study was specifically designed for the risks in the ophthalmologic practice. The setup therefore employed short, focussed exposures to high illumination levels in order to cause damage of the second class (Kremers and van Norren, 1989). Under these conditions two spectral light damage types occur (Gorgels and van Norren, 1995). The damage by 380-440nm affects mainly the PRS. In this damage type we observed protection similar to the protection in the previously mentioned animal studies. The damage by wavelengths in the range of 470 - 550nm (blue light damage) resembles more closely the pathology in humans; the RPE is involved in the damage. Unfortunately, this damage was not prevented by injections of NAC.

We conclude that NAC will effectively protect the retina against damage induced by lights of shorter wavelenghts. To protect against damage of longer wavelengths, another kind of quencher may be more effective. These quenching studies have demonstrated that the mechanism of short and long wavelength damage are distinct. We are presently using the same methodology to explore the mechanism of blue light damage with the aim of finding an effective protector against this clinically important type of retinal light damage.

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Chapter 5

Amelioration of retinal light damage by a potent inhibitor of lipid peroxidation.

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Abstract

High doses of optical radiation in the wavelength range from 320-440 nm, can specifically damage photoreceptor cells. We evaluated whether the new metalloporphyrin Aeol 11201 offers protection against this type of retinal damage. Aeol 11201 is catalytic antioxidant which we demonstrated to be a potent inhibitor of lipid peroxidation in vitro. The compound was tested against retinal damage by 380 nm radiation in Long Evans rats. The irradiations started at 30 to 180 minutes after intravenous injection (50 mg/kg) of the compound. A group of four small rectangles (5×6^{o}) of rat retina was irradiated during 15 minutes at four different doses. At three days after irradiation the threshold for funduscopically visible alterations was determined. In irradiations which started at 30 to 60 minutes after injection of Aeol 11201, the retinal sensitivity for damage had decreased significantly by 60%, as compared to solvent injected controls. No protective effect was observed at later time points after injection. The results of these experiments suggest the involvement of lipid peroxidation in this type of light damage to photoreceptor cells.

Introduction

High doses of light may cause serious and irreversible damage to the retina, resulting in a permanent loss of, in particular, photoreceptors (PRs) and retinal pigment epithelium (RPE) (for reviews see Tso, 1989; Organisciak and Winkler, 1994; Rapp, 1995; Reme *et al.*, 1996). The exact mechanism of this type of damage to the retina is still unknown. The type of alterations largely depends on exposure conditions. For investigation of retinal light damage basically two damage models are used. In the first model free running animals, usually rats, are exposed over a prolonged period to relatively low illumination levels (Noell *et al.*, 1966). In this model the damage action spectrum resembles the rhodopsin absorption curve. In the second model small patches of retina in anaesthetized animals are exposed to high illumination levels over a relatively short period of time (Ham *et al.*, 1982). The latter model mimics the specific conditions of exposure to the sun and to irradiating devices such as used in ocular surgery. Under the latter conditions two spectral damage types occur. The shortest wavelengths (320-440 nm) are most harmful and specifically damage the photoreceptors (Gorgels and van Norren, 1995; Chapter 2). Longer wavelengths (470-550 nm) require much higher doses and then cause alterations of both photoreceptors and the retinal pigment epithelium (Gorgels and van Norren, 1995; Chapter 2).

In the search for the primary event(s) at molecular level in retinal light damage, Ruffolo *et al.* (1984) found an increased sensitivity when the oxygen pressure was raised. More recently, the formation of reactive oxygen intermediates (ROx) has been demonstrated in the prolonged exposure models (Yamashita *et al.*, 1992), as well as under high illumination conditions (Rozanowska *et al.*, 1997). In addition, increase of retinal light damage was obtained by inducing a dietary deficiency of antioxidant vitamin C (Woodford and Tso, 1994). Amelioration of retinal light damage was obtained by administering various anti-oxidants in both the low illumination model e.g. DMTU (Lam *et al.*, 1990; Organisciak *et al.*, 1992; Li *et al.*, 1993), WR-77913 (Reme *et al.*, 1991) and Vitamin C (Organisciak *et al.*, 1990), and the high illumination model e.g. B-carotene (Ham *et al.*, 1984), Vitamin E (Kozaki *et al.*, 1994) and L-NAC (Chapter 4).

Substances with protective properties against specific ROx can be used as a tool to obtain information about the molecular mechanism of retinal light damage. For example DMTU and Vitamin C both protected free running rodents to damage in the low exposure model. Because both are antioxidants with relatively specific properties, these findings indicated the involvement of their respective substrates of ROX. Hydroxyl radical involvement can be indicated by protection by DMTU (Fairshter *et al.*, 1984, Fox, 1985), and e.g. singlet oxygen or peroxyl radical involvement by protection by Vitamin C (Sies, Stahl and Sundquist, 1992). We recently found that N-acetyl-L-cysteine (L-NAC) protected against photoreceptor damage by high exposure levels of 380 nm radiation (Chapter 4). Thiols, including NAC, remove NO and reduce peroxyl radicals to hydroperoxides, which are then further reduced by glutathione peroxidase. L-NAC also enhances the synthesis of glutathione (Butterworth *et al.*, 1993; Banks and Stipanuk, 1994). Glutathione quenches mainly hydrogen peroxide and inhibits lipid peroxidation through glutathione peroxidase, but also (inefficiently) scavenges superoxide and the hydroxyl radical (Younes and Siegers, 1980; Rao *et al.*, 1988). Due to the diversity of

substrates, the protective effect of NAC does not supply detailed information about the molecular basis of retinal light damage. Evaluating protection of a more selective scavenger might provide additional information about the mechanism of retinal light damage under high retinal irradiance conditions.

Aeol 11201 is a mangano-porphyrin, having a methyl ester attached to each of the methionine bridge carbons of the porphyrin. Due to this structure, it is highly lipophilic. In this study we first demonstrated Aeol 11201 to be a powerful (and more selective) inhibitor of lipid peroxidation. Next, we tested whether Aeol 11201 can increase the threshold for retinal damage by 380 nm radiation.

Methods

Determination of antioxidant properties

Lipid peroxidation activity measurement

Preparation of rat brain homogenates.

Frozen adult Sprague-Dawley rat brains (Pel-Freez, Rogers, AR) were homogenized with a polytron (Turrax T25, Germany) in 5 volumes of ice cold 50 mM potassium phosphate at pH 7.4. Homogenate protein concentration was determined with the Coomassie Plus protein assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. The homogenate volume was adjusted with buffer to give a final protein concentration of 10 mg/ml and frozen as aliquots at -80ºC.

Oxidation of rat brain homogenates.

Rat brain homogenates (2 mg protein) were incubated with varying concentrations of antioxidant at 37ºC for 15 minutes. Brain homogenates with no antioxidant added were used as controls. Oxidation of the rat brain homogenate was initiated by the addition of 0.1 ml of a freshly prepared anaerobic stock solution containing iron(II) chloride (0.25 mM) and ascorbate (1 mM) as previously reported by Braughler *et al.* (1987). Samples (final volume 1 ml) were placed in a shaking water bath at 37 ºC for 30 minutes. The reactions were stopped by the addition of 0.1 ml of a stock butylated hydroxytoluene (60 mM) solution in ethanol.

Lipid peroxidation measurement.

The concentration of thiobarbituric acid reactive species (TBARS) in rat brain homogenates was used as a index of lipid peroxidation (Bernheim, Bernheim and Wilber, 1984; Witz *et al.*, 1986; Kikugawa *et al.*, 1992; Jentzsch *et al.*, 1996). Malondialdehyde standards were obtained by adding 8.2 µl of 1,1,3,3-tetramethoxypropane in 10 ml of 0.01 M HCl and mixing for 10 minutes at room temperature. This stock was further diluted in water to give standards that ranged from 0.25 to 25 µM. Samples or standards (200 µl) were acidified with 200 µl of 0.2 M phosphoric acid in 1.5 ml locking microfuge tubes. The color reaction was initiated by the

addition of 25 µl of a 0.11 M thiobarbituric acid solution and samples were placed in a 90° C heating block for 45 minutes. TBARS were extracted with 0.5 ml of n-butanol by vortexing samples for 3 minute and chilling on ice for 1 minute. The samples were then centrifuged at 12,000 x g for 3 minutes, 150 µl aliquots of the n-butanol phase were placed in each well of a 96-well plate and read at 535 nm in a Thermomax platereader (Molecular Devices, Sunnyvale, CA) at 25°C. Sample absorbencies were converted to MDA equivalencies (µM) by extrapolation from the MDA standard curve. None of the antioxidants at concentrations employed in these studies affected the reaction of MDA standards with thiobarbituric acid and reactions without TBA were used as subtraction blanks.

Statistical analyses.

Data were presented as their means $\pm$ SE. The inhibitory concentration of antioxidants that decreased the degree of lipid peroxidation by 50% (IC_{50}) and respective 95% confidence intervals (CI) were determined by fitting a sigmoidal curve with variable slope to the data (GraphPad Prizm, San Diego, CA).

Analysis of catalase activity.

Catalase-like activity was measured as previously described by Day, Fridovich and Crapo (1997). Briefly, a Clark-type electrode was used to monitor the release of oxygen as an indicator of catalase like activity. The reaction chamber volume was 1.6 ml and the electrode was calibrated with an air saturated 50 mM phosphate buffer at 25o C (0.24 mM of oxygen) and zeroed by consuming the dissolved oxygen by the addition of excess sodium dithionite. Reactions were carried out in a 50 mM phosphate buffer, pH 7.8, containing 0.1 mM EDTA. H_2O_2 solutions were prepared by diluting a 30% (v/v) stock solution with ultrapure water (Milli-Q PF, Millipore, Bedford, MA). H_2O_2 concentrations were verified spectrophotometrically using a molar extinction coefficient at 240 nm (E = 44 M^{-1} cm^{-1}) (Beers and Sizer, 1952).

Analysis of superoxide dismutase (SOD) activity.

The SOD like activity of Aeol 11201 was measured in an assay described by McCord and Fridovich (1969).

Light damage experiments

Animals

Male, 30 days old pigmented Long Evans rats were obtained from Harlan CPB, Zeist, the Netherlands. They were kept in a 12-12 hrs light-dark cycle, at 10-90 lux illumination by white fluorescent light. Room temperature was 22-24ºC. The animals used in the experiments were 60 to 130 days old. Animals were treated conform the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Optics

The optical configuration was similar to that used in a previous study (Gorgels and van Norren, 1995). Briefly, a 450 Watt Xenon arc and a monochromator (MM12, Carl Zeiss, Oberkochen, Germany) provided the radiation. The beam was directed into the rat's eye under Maxwellian view conditions. In a plane conjugate to the retina a set of neutral density filters divided the radiation into four beams with intensities of approximately 100, 50, 25, and 12 %. The width of a beam was 5 × 6º, separated by a black bar of 1º. Retinal irradiance was calculated from measured irradiance as described previously (Gorgels and van Norren, 1995).

Anaesthesia

The rats were sedated with ether and anaesthetized with an initial intraperitoneal dose of pentobarbital 50 mg per kg. Anaesthesia was maintained by intravenous infusion in a tail vein with 15 mg pentobarbital per kg per hr in saline (1 ml/hr). Pupils were dilatated with phenylephrine 5% and cyclopentolate HCl 1%. Atropine sulfate 0.15 mg was injected subcutaneously. The cornea was moistened during the experiment by a continuous flow of saline (4 ml/hr). Body temperature was measured by a rectal thermometer and kept between 37.2º and 38.0ºC by an electrical blanket. After the irradiation the animals recovered in the dark until they could maintain physiological body temperature (approximately 2 hours). Thereafter they were returned to the cyclic light environment.

Irradiation

The anaesthetized rat was fixed in a restrainer. The eyelids were kept open with tape. Using 570 nm light of low intensity (retinal irradiance < 0.5 mW/cm^2), the beam was aimed at a position slightly superior in the nasal part of the retina. The neutral density filters were placed in the beam. The animal was injected through the same intavenous catheter used for the anaesthesia with either Aeol 11201 or control (see later). The start of the first irradiation was

30 minutes after injection. The retina was irradiated at 380 nm

(bandwidth 10 nm) for 15 minutes, to achieve a highest dose of 2 J/cm^2. For the second irradiation, at 60 minutes after injection, the rat was rotated 25^o in the horizontal plane, in temporal direction. The third irradiation was performed in the contralateral eye at 110 minutes after injection. Similarly, in another series of experiments, irradiations were performed 60 and 180 minutes after injection in two eyes. During irradiation the field position was regularly checked for movement of the eye by observing the autofluorescence of the 380 nm radiation. After irradiation the position was again checked with green light of low retinal irradiance. When eye movements occurred during irradiations the measurements were discarded. In this way the exact dose on each position could be calculated.

Anti-oxidant

The Aeol 11201 (100 mg) (Fig. 1) was dissolved in dimethylacetamide (Sigma) / PEG-400 (Sigma) (1:1) 4 ml and diluted in dextrose 5% (12 ml). The solution was filter sterilized and stored in dark at room temperature until use. The animals were injected 50 mg/kg of either dissolved Aeol 11201 or a similar amount of the solvent through the same intravenous catheter used for the anaesthesia.

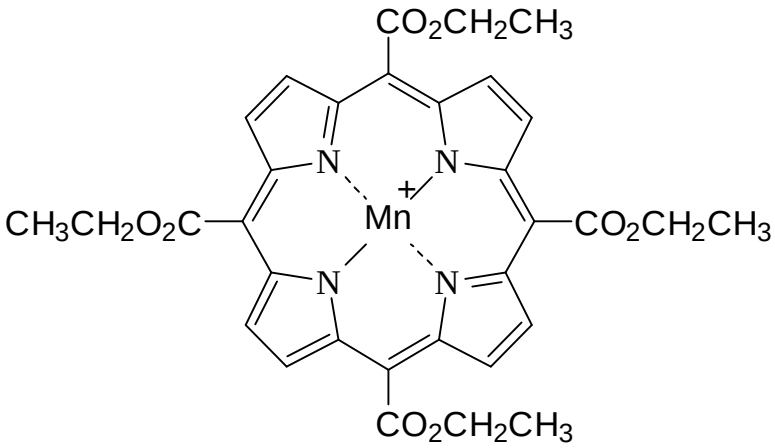

Fig. 1. Structure formula of Aeol 11201.

Funduscopy

Because funduscopic damage is best visible at three days after irradiation (Chapter 2), this time point was chosen for assessment. Rats were sedated with ether and anaesthetized with pentobarbital 50 mg per kg by intra-peritoneal injection. Pupils were dilatated with phenylephrine 5% and cyclopentolate HCl 1%. An observer unaware of the type of injection (compound or control) noted the following aspects of the lesion at each irradiated area: size, appearance, color and demarcation. An example of the funduscopy after irradiation can be found in Chapter 2. We refrained from basing the judgement on fundus photographs, because fundus photographs of a constant high quality were difficult to obtain due to the small pupil size. This held in particular for photographs of a more peripheral locations. The lowest dose causing visible aberrations was noted as threshold dose. After funduscopy some retinas were prepared for light microscopy as described previously (Chapter 2).

Results

Determination of antioxidant properties

Three in vitro assays were used to characterize the antioxidant properties of Aeol 11201. The lipid peroxidation inhibiting capacity was measured in a TBARS assay using oxidized brain homogenates. Figure 2 shows the reduction of lipid peroxidation as function of the Aeol 11201 concentration. An S-curve was fitted through the datapoints, and the concentration which inhibits the lipid peroxidation by 50% (IC_{50}) was calculated (Fig. 2).

The IC_{50} of Aeol 11201 in this assay was 3.4 µM.For comparison, the IC_{50} of a well-studied inhibitor of lipid peroxidation Trolox, a watersoluble analog of vitamin E was reported to be 200 μM in this lipid peroxidation model (Day, Batinic-Haberle and Crapo, 1998). In the catalase assay the ability to produce O_2 from H_2O_2 was measured. Purified catalase produces in this assay 35000 U/mg (Day et al., 1997), where one unit is defined as the amount of enzyme required to scavenge 1 mol H_2O_2 /min. Aeol 11201 activity was 0.25 U/mg, which is less than 0.1 % of the typical activity of catalase on weight base.

The SOD mimicking activity was measured according to McCord and Fridovich (1969). In this assay the oxidation of cytochrome C by superoxide is measured spectrophotometrically. They defined one unit as the amount of superoxide dismutase required to inhibit rate of reduction of cytochrome C by 50%. The activity of purified CuZnSOD was 3300 U/mg, while we measured an activity of 13.5 U/mg for Aeol 11201 in the same test (≈0.4% of CuZnSOD activity). In Table 1 the antioxidant capacities as measured in the three assays are summarized.

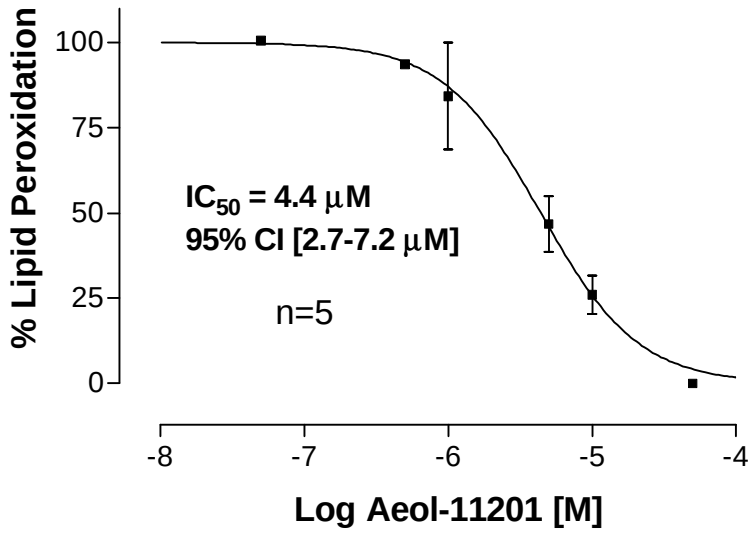

Fig. 2. Percentage of lipid peroxidation in TBARS assay presented as a function of Log [Aeol 11201] (M). Sigmoidal curve fitted with variable slope to the data, each datapoint represents 2-4 experiments. Errorbars represent SEM. The concentration of antioxidant that reduced lipid peroxidation by 50% (IC_{50}) is presented with 95% confidence interval.

Table 1. Antioxidant activities of Aeol-11201

Compound	*SOD (U/mg)*[a]	*Catalase (U/mg)*[b]	*Lipid Peroxidation IC_{50}, (μM; 95% CI)*[c]
Aeol-11201	13.5	0.25	3.4 (1.6-7.2)
CuZnSOD	3300[d]		
Catalase		35000[e]	
Trolox			200[f]

[a] Unit is defined as the amount of compound needed to inhibit cytochrome c reduction by one-half

[b] Unit is defined as the amount of compound that consumes 1 μmol H_2O_2/minute.

[c] The IC_{50} is the concentration of compound that inhibits 50% of iron-ascorbate initiated lipid peroxidation

[d] McCord, J.M. and Fridovich, I. (1969)

[e] Day *et al.*, (1997)

[f] Day *et al.*, (1998)

Light damage experiments

After a single intravenous injection of Aeol 11201 at a dose of 50 mg/kg, or an equivalent amount of the solvent, small pieces of rat retina were irradiated at four doses of 380 nm. Two series of experiments were done. In the first series irradiations were performed after 30, 60 and 110 minutes after injection. In the second the irradiations were done after 60 and 180 minutes. Each irradiation consisted of 4 different doses on juxtaposed retinal patches, which allowed us to determine the threshold dose as the lowest dose to cause visible damage at three days after irradiation. The threshold doses are presented in Fig. 3. Every dose presented is the mean (± SEM) of 4 to 12 experiments. The data from two series of experiments, were pooled and were normalized using the controls as a standard, in order to exclude local differences in retinal sensitivity, and possible differences between the two series. The threshold doses were compared by a separate variance *t*-test for difference between two sample means (ANOVA). Light microscopical analysis revealed no differences between the non-irradiated retina of Aeol 11201 treated animals and controls (not shown).

In the irradiations performed 30 minutes after injection we observed an increase in funduscopic threshold dose of 64 % ($p<0.05$), 60 minutes after injection the increase in threshold dose was 45% ($p<0.05$). At 110 as well as 180 minutes after injection we observed no significant difference between experimental and control animals.

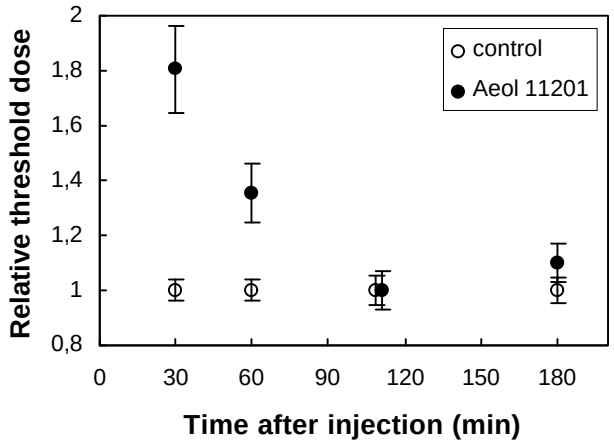

Fig. 3. Threshold dose for 380 nm funduscopical visible light damage after injections with Aeol 11201 (solid circles) or control (open circles). The bars represent average threshold dose for irradiations 30, 60, 110 and 180 minutes after intravenous injection. Thresholds presented are normalized averages for 4 to 12 experiments. Errorbars represent SEM.

Discussion

In this study we demonstrated that Aeol 11201 protects against retinal damage by 380 nm radiation. Aeol 11201 is a potent inhibitor of lipid peroxidation, virtually without catalase or superoxide dismutase activity. In the Aeol treated group the threshold for light damage was raised over 60 %. We observed protection up to 60 minutes after intravenous administration. Radiation of 380 nm (in the doses applied) specifically damages the photoreceptors (Gorgels and van Norren, 1995; Chapter 2). The protection against radiation of 380 nm by Aeol 11201 strongly suggests that lipid peroxidation is involved in this type of light damage to photoreceptors.

Three in vitro assays were used to determine the antioxidant properties of Aeol 11201. Using the TBARS concentration as an index of lipid peroxidation, we showed that it is a potent inhibitor of lipid peroxidation in rat brain homogenates, approximately 60 times as powerful as Trolox (Day *et al.*, 1998). On the other hand, Aeol 11201 is a poor mimic of catalase and superoxide dismutase: In the catalase assay, Aeol 11201 activity was less than 0.1 % of the typical activity of catalase on weight base. Aeol 11201's SOD-activity compared to CuZnSOD was similarly low; an activity of 13.5 U/mg, as compared to 3500 U/mg for purified CuZnSOD (McCord and Fridovich, 1969). Thus it appears that Aeol 11201 is *in vitro* a rather selective lipid peroxidation inhibitor.

The protection was measured using the unique features of the high dose/short irradiation model. During one irradiation period four doses were applied simultaneously on small areas, surrounded by control retina. Thus, the effects of four different doses of radiation were tested, at various time points after administration of a compound. By comparing the irradiated areas to surrounding control retina, in each animal the threshold can easily be determined (in a semiquantitative way). Previously we have demonstrated that in the current model the threshold for funduscopic damage by 380 nm highly correlates with the threshold for photoreceptor damage as assessed by light and electron microscopy (Gorgels and van Norren, 1995; Chapter 2).

The results of the current study add to our understanding of retinal light damage. In the photoreceptors GSH dependent enzymes are present in the OS (Naash and Anderson, 1989), protecting the membranes against hydrogen peroxide and lipid peroxidation. A previous study suggested that enhancement of glutathione synthesis protects against retinal light damage by 380 nm (Busch et al., Submitted for publication). While glutathione (amongst other scavenging properties) is an excellent quencher of lipid peroxidation, the current study underscores the importance of lipid peroxidation in the retina. Previously, other authors have also demonstrated lipid peroxidation in retinal light damage (Feeney and Berman, 1976; Kagan et al., 1981; Organisciak, Favreu and Wang, 1983; Wiegand et al., 1983). The essential difference with the current study is the model in which the retinal light damage is studied. These authors generally studied retinal light damage by exposing whole retinas, for a relatively long period of time to relatively low exposure levels. Retinas damaged by such exposures often demonstrate changes consisting of OS vesiculation, combined with damage to the RPE (Noell et al., 1966; Reme et al., 1991; Li et al., 1993;Organisciak et al., 1995; Wang et al., 1995). In the current model, retinas damaged by short wavelength radiation (320-440 nm) show damage to the photoreceptors, virtually without changes in the RPE (Gorgels and van Norren, 1995; Chapter

2). The different morphology of retinas damaged by short, intense irradiation (Ham and Mueller, 1989; Collier and Zigman, 1989; Rapp, Tolman and Dhindsa, 1990; Gorgels and van Norren, 1995; Busch et al., 1999), suggests that different mechanisms are involved. However, the results of the current study indicate that lipid peroxidation is also involved in retinal light damage after short intense irradiations. Due to the large proportion of polyunsaturated fatty acids in the outer segments (Flieser and Anderson, 1983; Organisciak et al., 1992), lipid peroxidation appears a plausible explanation for damage to the photoreceptors.

The model with high intensities and short exposure appears well suited for assessing the light hazards in the ophthalmic practice (Friedman and Kuwabara, 1968; Tso, 1989). In animal models of monkey and rat two spectral types of retinal light damage were found (Ham and Mueller, 1989, Gorgels and van Norren, 1995, Busch et al., 1999). In the rat, wavelengths in the range of 320-440 nm specifically damage the photoreceptor cells; wavelengths from 470-550 nm damage RPE and PRs. In the present study we irradiated at 380 nm and examined the spectral damage type of short wavelengths. This type of damage may, in principle, occur in human patients during eye surgery by short visible wavelengths (400-440 nm). The risk may even increase during vitreal and retinal surgery, when light sources are used that bypass the protective (UV) filtering of the human lens. The Aeol 11201 raised the threshold for funduscopic visible damage by 60%, which translated to the clinical practice could mean an equivalent extension of the safe period for retinal light damage of this type. Future studies may clarify whether the compound also has protective effects against the other type of light damage occurring at longer wavelengths.

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Chapter 6

Summary and discussion

This thesis is on light damage to the retina. Photochemical light damage to the retina can even occur under ambient light conditions as demonstrated by Noell *et al.* (1966) They found green light (500 nm) the most powerful in causing retinal damage. Under higher irradiance conditions the action spectrum changes: Short wavelengths are then the most harmful (Ham *et al.*, 1979; Ham, Mueller and Sliney, 1982; Gorgels and van Norren, 1995). With ever increasing irradiance the domain of thermal damage is entered. The highest retinal irradiance used in the experiments in this thesis is approximately 300 mW/ cm^2. This is a rather high level, but insufficient to increase the retinal temperature over 10^oC; the generally accepted safety limit (Ham *et al.*, 1979). Another argument against thermal damage, is the duration of the exposure. Thermal damage occurs in less then a second, or not at all (Ham *et al.*, 1979; van Norren, Keunen and Vos, 1998). In the experiments in this thesis longer exposures (from 8 to 100 minutes) were required to cause damage. Thus, we have always dealt with photochemical rather than thermal mechanisms in retinal light damage as investigated in this thesis.

The aims of the studies in this thesis were:

(1) To document the development of two spectrally determined types of photochemical light damage.
(2) To obtain more information about the mechanisms of these two spectral light damage types, and to evaluate possible protectors against retinal light damage.

In this chapter first a short summary will be presented of the research findings. Next the discussion will focus on the mechanism of retinal light damage and clinical implications of the results, and the chapter ends with recommendations for further research.

In *Chapter 2* a comparison is made between the natural development of two spectral types of retinal light damage. In all experiments sharply demarcated rectangles were projected on the retina, and compared to the surrounding control retina. For each irradiation the threshold dose was determined. The threshold was defined as the minimum dose to cause just visible aberrations.

The threshold level for funduscopically visible aberrations is a factor 1000 lower for 380 nm radiation then for 470 nm radiation. The funduscopic threshold dose correlates very well with the threshold for light microscopically visible damage; for both damage types the thresholds for funduscopically visible damage and histologically visible damage are approximately similar. This result can be used to reduce the amount of work and animals required to evaluate whether a compound protects against retinal light damage, because histology is no longer required for determination of the threshold dose. Histological analysis in those experiments was only necessary for verification whether the damage caused is of the same type in the treated and control groups, and for evaluation of the toxicity of compounds. We applied the method of using funduscopy in the studies on the effects of Aeol 11201 and NAC (Chapters 4 and 5).

When the morphology of the two damage types is compared at their respective threshold doses for funduscopic visible aberrations, there is a clear difference. At two times threshold level, damage by 380 nm radiation is limited to the rod photoreceptors; a relative sparing of the cones occurs. At one hour after irradiation at 470 nm, a comparable dose (two times threshold level) caused alterations in the mitochondria throughout the photoreceptors, as well as in the retinal pigment epithelium.

Three weeks after damage by irradiation at 470 nm, a lesion in the photoreceptors remains, and the underlying RPE has returned to a hypopigmented monolayer. Apart from the hypopigmentation, these lesions appear similar to the lesions three weeks after irradiations at 380 nm. For both spectral damage types we observed no clear difference between the morphology of the lesions at three weeks and two months after irradiation.

The fate of lesions limited to the photoreceptors was the subject of study in *Chapter 3* Radiation of 380 nm applied in limited doses can cause a rapid, specific and complete loss of photoreceptors, without major changes to the surrounding retinal layers. One day after irradiation all rods were pyknotic. Three weeks later practically all damaged photoreceptors were removed. After creating such a sharply demarcated photoreceptor lesion, the bordering photoreceptors shift inwards rapidly. The capacity for this redistribution of the photoreceptors is limited. Within three weeks the size of defects of 400 and 800 μm wide decreases, but only the defects up to 200 μm are filled in completely. No further decrease was observed between three weeks and six months after irradiation. The shift inwards of the bordering photoreceptors has an effect on the retina adjacent to the irradiated area. The outer segment tips had moved inwards up to 200 µm, but the innermost nuclei in the outer nuclear layer had moved inwards substantially less. Due to this difference in distance migrated, the photoreceptors have an oblique orientation. This altered orientation extends beyond the originally irradiated area.

Chapter 4 and 5 were focussed on the molecular mechanism(s) of retinal light damage. By administering N-acetyl-L-cysteine (L-NAC) in doses of 270-1000 mg/kg IP, protection was obtained against retinal light damage by 380 nm radiation (UVA), but not against damage by 470 nm radiation ('blue light damage'). Three hours after injection the protection had diminished. The stereoisomer D-NAC did not protect at all. L-NAC increased the minimum dose required to cause visible aberrations (threshold) approximately 60%. The difference between L-NAC and D-NAC is that D-NAC cannot be deacetylated and incorporated into glutathione (Sjodin *et al.*, 1989), due to the stereospecific synthesis. From this study we concluded that N-acetyl-L-cysteine protects stereospecifically against retinal damage in the UV, but not in the visible part of the spectrum. This suggests the protection is mediated by glutathione synthesis enhancement. The glutathione redox system protects against lipid peroxidation, and against several reactive oxygen intermediates. Thus the protection by L-NAC may suggest that retinal light damage by wavelengths of 320-440 nm is mediated by lipid peroxidation.

In Chapter 5 we evaluated whether the new metalloporphyrin Aeol 11201 offers protection against the 320-440 nm type of retinal damage. The catalytic antioxidant Aeol 11201 is a potent inhibitor of lipid peroxidation. Aeol 11201 was injected intra-venously (50 mg/kg) in Long Evans rats, and irradiations of small retinal patches were performed at 380 nm. Four small rectangles (5×6^{o}) of rat retina were simultaneously irradiated to four different doses. Irradiations lasting 15 minutes, started 30 to 180 minutes after intravenous injection of the compound. Three days after irradiation the threshold for funduscopically visible alterations was determined. At 30 to 60 minutes after injection the Aeol 11201 treated group showed a reduced sensitivity for 380 nm radiation. The sensitivity had decreased by 60% as compared to solvent injected controls. No protective effect was observed at later time points. This result suggests the involvement of lipid peroxidation in the process of retinal damage at high irradiation levels of short wavelengths. This fits well with the result in the NAC study, which also indicated lipid peroxidation could be the damaging mechanism.

On the mechanisms of retinal light damage

In the research for this thesis the development of two spectral types of retinal light damage is documented, and investigations were performed to obtain more information on the mechanisms in retinal light damage. One method to obtain more information on the mechanisms is to investigate where the first changes occur. Histologically no clear focus could be identified for damage by 380 nm. Shortly after irradiation changes could be observed throughout the photoreceptors. These morphological changes indicate that damage occurs (in a cellular element present) throughout the cell. The observed protection by L-NAC and Aeol 11201 against 380 nm radiation suggest that lipid peroxidation is involved in the damage process. Lipid peroxidation occurs in polyunsaturated fatty acids. Polyunsaturated fatty acids are major components of membranes throughout the cell. The combination of these findings suggests that the damage is mediated by lipid peroxidation of cellular membranes throughout the photoreceptors.

The lack of protection by L-NAC demonstrates that the molecular mechanism of the damage by 470 nm differs from the mechanisms of damage by 380 nm radiation. The suggestion is made that the damage is not mediated by lipid peroxidation like the damage by 380 nm. Because the first signs of damage after blue light irradiations are in the mitochondria of the RPE and the photoreceptors, the origin of the damage type appears to be in these organelles. Our results support the hypothesis that a mitochondrial enzyme is the chromophore for this type of damage (Lawwill, 1982). Additional evidence is provided by the fat that the action spectrum of RPE damage in cultures of bovine RPE cells resembles the absorption spectrum of one such enzyme, cytochrome C oxidase, peaking at 420 nm (Pautler, Morita and Beezley, 1990). Blue light (404 nm) inhibited this enzyme in vivo in rat retina (Chen, Soderberg and Lindstrom, 1992).

Implications of the findings for clinical practice

The limited histological data on light damage in humans demonstrate damage to the RPE as well as to the photoreceptors (Tso, 1989; Green, 1991). Similar damage morphology is found in rat after monochromatic irradiations at 470 nm (blue light). This could suggest the “blue light” damage type is the only type of light damage occurring in human retinae. Yet, in theory the other spectral damage type can also be very relevant for humans. The human ocular lens transmits wavelengths down to 400 nm (van Norren and Vos, 1974). Thus, the damage type which we studied at 380 nm, may occur in humans in the wavelength range of 400-440 nm.

The risk of retinal light damage during various procedures has been demonstrated by numerous case reports (Ross, 1984; Brod *et al.*; 1986, Kuhn *et al.*; Kingham, 1991; Lucke and Reme, 1984), and also in a prospective study (Byrnes *et al.*, 1992). During intra ocular surgery a lack of filtering can occur when the ocular lens is removed or bypassed. This will strongly increase the risk of the photoreceptor type of retinal light damage. In addition, high illumination levels are often used with the source close to the retina. The type of damage caused will highly depend on the spectral composition of the light reaching the retina. This depends on by the type of light source (xenon vs halogen), and the filters used. Thus these factors will determine the spectral type of damage.

In order to reduce the risk of retinal light damage during ocular surgery several precautions could be taken. The shortest wavelengths are the most harmfull. These could be blocked by using filters in illumination devices and operation microscopes. The exposure is the product of duration and illumination level. Thus the shorter the exposure, the better. Also, the spot could be moved during the operation, so that exposure time to an retinal location is limited. The spot should be aimed as perifer as possible, so that in case of damage this will result in a peripheral visual field defect, in stead of loss of visual acuity. The threshold for retinal light damage is temperature dependant (de Lint, 1992). If the temperature of the retina could be reduced, the threshold for light damage could increase. And eventually, pharmacological prophylaxis could be introduced.

L-NAC could be a candidate for pharmacological protection against retinal light damage, in particular with intra-ocular light sources. We observed protection by NAC against photoreceptor-damage. NAC is already widely used in humans for other applications (van Zandwijk, 1995; Cotgreave, 1997; Kelly, 1998). Yet, the lack of protection against 470 nm ('blue light') damage by NAC limits its usefulness. The lack of protection of the thiol L-NAC against blue light damage, could have implications for the promising results by thiols in other retinal light damage models. The protection by for example the thiols WR 77913 (Reme *et al.*, 1991) and DMTU (Organisciak *et al.*, 1992) might be similar to the protection we observed by NAC. Then these thiols might protect against the damage type which affects the photoreceptors, but they lack protecting properties against the other, and perhaps more important spectral light damage type. This underscores the importance of further research into the mechanism of 'blue light damage'.

Further research

Damage from 470 nm radiation causes subtle changes in the RPE from as early as one hour after irradiation. This suggests a chromophore is present in the RPE. An efficient chromophore will have a high absorbance somewhere in the range between 470-550 nm. In the retinal pigment epithelium substances are present that absorb light at 470 nm. The cytochrome C oxidase complex, with an absorbance peak at 420 nm, remains a candidate for the chromophore (Lawwill, 1982; Pautler, Morita and Beezley, 1990). But the RPE contains more potential chromophores. Upon visible light irradiation melanin can produce superoxide (Rozanowska *et al.*, 1997), and retinal lipofuscin can produce singlet oxygen (Burke *et al.*, 1998). However, there appears to be no essential role for melanin in the damage by blue light, because the two spectrally defined damage types occur in albino retinas as well (Gorgels and van Norren, 1998). The interesting ROX generating capacities of such compounds of the RPE, and their possible role as chromophores *in vivo* would be a good starting point for further research. (In particular because thiols like glutathione or NAC do not protect against singlet oxygen.) For example, protection by a scavenger of singlet oxygen could support this hypothesis.

The use of other specific antioxidants, could supply more information about the role of the substrates of these oxidants. For example, when superoxide dismutase mimics protect against retinal light damage, this strongly indicates the involvement of superoxide in the damage process.

Another interesting possibility would be the visualization or detection of reactive oxygen intermediates. Recently, in vivo visualization of reactive oxygen intermediates has become possible. This can be accomplished by the use of dichloro-diflourescein-acetate. This compound is non-fluorescent, but in the presence of hydrogen peroxide it becomes fluorescent (ref). Using such compounds, combined with flurophotometry or a scanning laser ophthalmoscope would visualize e.g. the formation of hydrogen peroxide. Other techniques to measure the formation of reactive oxygen intermediates would be the use of electro-spin resonance during irradiation.

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Chapter 6

Nederlandstalige samenvatting

Dit proefschrift gaat over fotochemische lichtschade aan het netvlies. Bij fotochemische lichtschade veroorzaakt lichtenergie schade, zonder dat er sprake is van noemenswaardige temperatuurs-verhoging. Het mechanisme loopt dus niet via opwarming van het weefsel. De benodigde hoeveelheid licht is relatief bescheiden. Fotochemische schade aan het netvlies kan zelfs optreden onder "normale", zij het langdurige, laboratorium omgevingslicht omstandigheden, zoals beschreven door Noell cs. in 1966. Zij vonden dat groen licht het meest efficient het netvlies beschadigde. Onder omstandigheden met hogere licht intensiteiten vonden diverse onderzoekers een ander actiespectrum: korte golflengtes zijn dan het schadelijkst (Ham *et al.*, 1979; Ham, Mueller and Sliney, 1982; Gorgels and van Norren, 1995). Wanneer men de hoeveelheid licht verder vergroot komt men in het gebied van de thermische schade. De hoogste retinale irradiantie in de experimenten in dit proefschrift is ongeveer 300 mW/cm^2. Dat lijkt vrij veel, maar het is onvoldoende om de temperatuur meer dan 10^o C te doen toenemen, (wat de algemeen aanvaarde veiligheidsgrens is (Ham *et al.* 1979)). Een ander argument tegen thermische schade is de duur van de expositie. Thermische schade treedt op binnen een seconde, of in het geheel niet (Ham *et al.*, 1979; van Norren, Keunen and Vos, 1998). In de experimenten in dit proefschrift waren langere blootstellingen (van 8 to 100 minuten) nodig om schade te veroorzaken. De experimenten in dit proefschrift hebben dus allen betrekking op fotochemische en niet op thermische schade.

De doelen van dit promotieonderzoek waren:
(1) het documenteren van de de ontwikkkeling van twee spectraal verschillende typen fotochemische lichtschade aan het netvlies.
(2) Het verkrijgen van meer informatie over het mechanisme van deze twee typen lichtschade, en het onderzoek naar mogelijke beschermers tegen netvliesschade door licht.

In hoofdstuk 2 wordt een vergelijking gemaakt tussen twee spectraal verschillende typen netvliesschade door licht. In alle experimenten werden scherp begrensde rechthoeken geprojecteerd op het netvlies, en deze bestraalde stukken werden vergeleken met het omliggende controleweefsel. De drempeldosis was omschreven als de minimale dosis om juist zichtbare veranderingen te veroorzaken.
De drempeldosis voor schade door 380 nm straling is een factor 1000 lager dan die voor schade door 470 nm licht. Voor beide schadetypen correlleert de drempeldosis voor funduscopisch zichtbare afwijkingen zeer goed met de drempeldosis voor licht-microscopisch zichtbare schade. Deze correlatie kan worden gebruikt ter beperking om de hoeveelheid werk, en de hoeveelheid dieren, nodig om de bescherming van stoffen tegen lichtschade te evalueren. Immers, de histologische analyse is niet meer nodig voor het bepalen van de drempeldosis. Histologische analye is in zulke experimenten alleen nodig om te verifiëren of de schade in behandelde en controlegroep morfologisch verschilt, en om de toxiciteit van de stoffen te beoordelen.

Wanneer de morfologie van de twee schadetypen wordt vergeleken op hun respectievelijke drempeldoses voor funduscopische afwijkingen, is er een duidelijk verschil. Bij twee maal de drempeldosis, is de schade door 380nm straling beperkt tot de staafjes-fotoreceptoren; de kegeltjes blijven relatief gespaard. Een uur na bestraling met 470 nm met een vergelijkbare dosis (twee maal de drempeldosis), waren afwijkingen in de mitochondria in de fotoreceptoren en in het retinale pigment epitheel zichtbaar.
Drie weken na beschadiging met 470 nm straling blijft er een lesie in de fotoreceptorlaag, terwijl het onderliggende retinale pigmentepitheel is hersteld tot een verminderd gepigmenteerd eenlagig epitheel. Behalve de hypopigmentatie, ziet zo'n lesie er vergelijkbaar uit met de schade na 380nm bestraling. Voor geen van beide schadetypen vonden we een duidelijk verschil in morfologie tussen de schade na drie weken en de schade na twee maanden.

Het lot van defecten in de fotoreceptorlaag was het onderwerp van de studie beschreven in hoofdstuk 3. Straling van 380 nm in een beperkte dosis kan een snel, specifiek en totaal verlies van fotoreceptoren veroorzaken, zonder duidelijke veranderingen aan andere lagen in het netvlies. Een dag na bestraling met zo'n dosis zijn alle kernen van de staafjes fotoreceptoren pyknotisch. Drie weken later zijn praktisch alle beschadigde fotoreceptoren verdwenen. Na het ontstaan van zo'n scherp begrensd fotoreceptordefect schuiven de aangrenzende fotoreceptoren snel de lesie in. De capaciteit voor deze redistributie is echter beperkt. Binnen drie weken neemt de diameter van 400 en 800 µm brede lesies af, maar alleen de lesies van 200 µm breed werden volledig opgevuld. Tussen drie weken en zes maanden na bestraling zagen wij geen verdere afname van de diameter van de defecten. De verplaatsing van de fotoreceptoren naar het midden van het defect heeft ook een effect op het netvlies dat de lesie omringt. De uiteinden van de buitensegmenten zijn tot 200 µm opgeschoven, de binnenste kernen van de buitenste kernlaag beduidend minder. Als gevolg van dit verschil in afgelegde afstand, zijn de fotoreceptoren schuin geörienteerd. Deze schuine orientatie kan worden aangetroffen tot enkele honderden micrometers buiten het bestraalde gebied. Dit proces zou de verklaring kunnen zijn voor de veranderde orientatie van fotoreceptoren rond gebieden van fotoreceptorverlies zoal bijvoorbeeld rond gebieden van choroidea atrofie (Bedell *et al.*, 1981). Het proces zou kunnen bijdragen aan een verminderde functionaliteit van de omringende fotoreceptoren.

Hoofstukken 4 en 5 beschrijven studies naar het moleculaire mechanisme van lichtschade aan het netvlies. In hoofdstuk 4 wordt beschreven dat N-acetyl-L-cysteine (L-NAC) in doses van 270-1000 mg/kg lichaamsgewicht beschermde tegen schade door 380 nm straling (UVA), maar niet tegen schade door 470 nm (blauw licht). Deze bescherming was drie uur na injectie. De stereoisomeer N-acetyl-D-cysteine (D-NAC) beschermt niet tegen de schade door 380 nm. L-NAC verhoogde de drempel voor schade door 380 nm straling met ongeveer 60%. Het verschil tussen D-NAC en L-NAC is dat D-NAC niet kan worden gedeacetyleerd en worden ingebouwd in glutathion. (Sjodin *et al.*, 1989) als gevolg van de stereospecifieke synthese. Uit deze studie concluderen we dat N-acetyl-L-cysteine stereospecifiek beschermt tegen schade door straling met een golflengte van 380 tot 440nm, maar niet tegen schade door straling met een golflengte van 470 to 550nm. De resultaten suggereren dat de bescherming wordt veroorzaakt door een verhoogde glutathion synthese. Het glutathion redox systeem beschermt tegen vetzuurperoxidatie en tegen verschillende zuurstofradicalen.

De resultaten van deze studie geven dus aan dat vetzuurperoxidatie mogelijk betrokken is bij schade aan het netvlies door straling van 350 tot 440 nm.

In hoofdstuk 5 is een studie beschreven waarin wordt onderzocht of Aeol 11201 beschermt tegen netvliesschade van het type dat veroorzaakt wordt door straling van 320-440nm. We laten zien dat de catalytische antioxidant Aeol 11201 een krachtige remmer is van vetzuurperoxidatie. Aeol 11201 werd intra-veneus toegediend aan Long Evans ratten, en kleine stukjes netvlies werden bestraald met 380 nm. Vier kleine rechthoeken van 5 bij 6º werden simultaan bestraald met vier verschillende intensiteiten, leidend tot vier verschillende doses. De bestralingen duurden 15 minuten, en begonnen 30 tot 180 minuten na de toediening van het middel. Tijdens de bestralingen die 30 en 60 minuten na injectie van Aeol 11201 begonnen, waren de drempeldoses verhoogd, terwijl dat bij de controlegroep niet het geval was. De gevoeligheid van het netvlies was in de met Aeol behandelde groep 60% verlaagd in vergelijking tot de controle dieren die alleen het oplosmiddel toegediend kregen. Langer na injectie vonden we geen beschermend effect. De resultaten van deze studie, in combinatie met de resultaten van hoofdstuk 4, wijzen er sterk op dat vetzuurperoxidatie betrokken is bij netvliesschade door UVA straling en licht met een golflengte van minder dan 440nm.

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