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Disertační práce

Fyzikální fakory ovlivňující vznik poškození rohovky králičího oka UV zářením

Physical factors influencing the development of corneal damage of the rabbit eye with UV rays

Ing. Čestmír Čejka
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Děkuji především mému školiteli, panu profesorovi MUDr. J. Rozinovi, PhD, přednostovi Ústavu lékařské biofyziky a lékařské informatiky, Univerzita Karlova, 3. lékařská fakulta a děkanovi Fakulty biomedicinského inženýrství, ČVUT, za odborné vedení během mého doktorandského studia lékařské biofyziky a cenné připomínky k mé práci.

Paní profesorce MUDr.B.Brůnové,DrSc, mému školiteli konsultantovi, děkuji za odborné rady z oftalmologie.

Děkuji všem spolupracovníkům, kteří se podíleli na experimentální práci.

Děkuji též své rodině za trpělivost a podporu během mého studia.

Praha, 2011

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1.Introduction

1.1. The increased danger to the eye from UV rays related to the decrease in stratospheric ozone.

The health risks associated with ozone depletion are due to the enhanced UVA radiation (320-400 nm) in the environment and the penetration of UV radiation of shorter wavelength (between 280 nm and 320 nm, UVB rays) (Zigman, 1993, Tenkate, 1999, Ma et al. 2001, Kabuyama et al. 2002, Diffey, 2004, Norval et al., 2007). The anterior eye segment damage from UV exposure includes effects on the cornea, lens, iris, and associated epithelial and conjunctival tissues (Reddy et al. 1998, Merriam et al. 2000, Čejková et al., 2000, 2001, Midelfart et al., 2002, Di Girolamo et al., 2006). The most common acute effect of environmental ultraviolet radiation on the cornea is photokeratitis. Chronic eye conditions likely to increase with ozone depletion include cataract, squamous cell carcinoma, ocular melanoma, and a variety of corneal and conjunctival pathologies (Ringvold 1980, Longstreth et al. 1998, Ringvold 1998, Hockwin et al. 1999, West 1999, Balasubramanian, 2000, Berwick, 2000, De Gruil, 2000, Gallagher and Lee, 2006). Exposure to ultraviolet light has been postulated as a cause of age-related macular degeneration, perheps through the damage to the retinal pigment epithelium (Ohira et al., 2008).

1.2. Atmospheric UV absorption and scattering.

UVA, UVB and UVC (100-280 nm) - all three of these UV bands are present in the extraterrestrial solar spectrum, but due to atmospheric absorption and scattering, UV at ground level, which comprises 7% of total solar spectrum, does not extend beyond 280 nm (Menter and Hatch, 2003). UVC radiation is therefore completely absorbed by ozone and oxygen in the stratosphere but any potential thinning of the ozone layer will lead to an intensification of solar

UVC at ground level making it biologically relevant (de Gruil, 2000). The total solar UV at earth´s surface has been termed “global“ solar radiation.

1.3. The irradiance and dose of UV at the ocular surface.

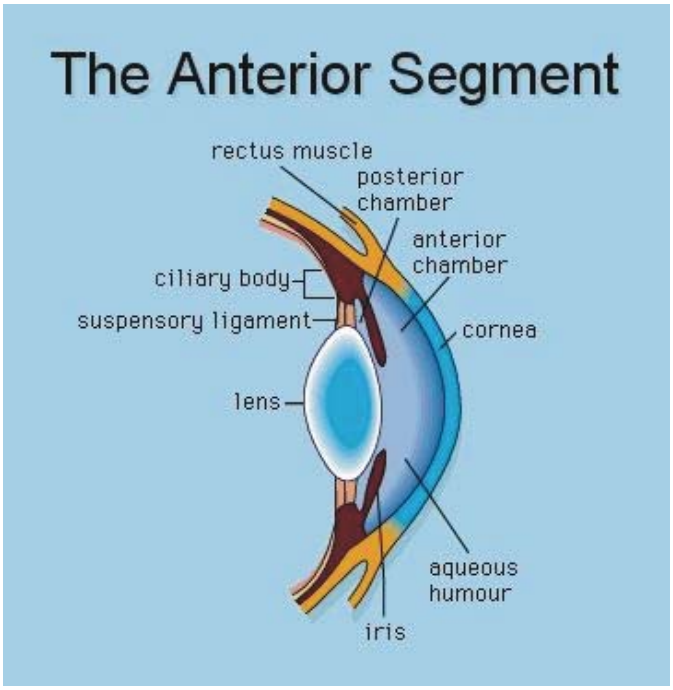
A number of different studies have shown that exposure of the ocular surface to UV varies considerably in diverse environments. The irradiance and dose of UV at the anterior surface have been examined by many researches using different methods. Of these, a number have used polysulphone film as a contact lens. This material degrades on exposure to UV and the degree of degradation is proportional to the received UV dose (McLaren et al., 1997). Narayanan et al. (1996) constructed model cornea assembly and placed it inside the orbit of a human skull that was exposed to sunlight. Sakomoto et al. (1997) attached 18 photodiodes to a mannequin head and recorded the UV intensity at specific location. Kwok et al (2003) placed UV sensor at the nasal limbus of a fabricated physical model eye, which was placed inside the orbit of manequin head and exposed the assembly to UV rays.

Zigman (1993) critically reviewed individual methods and UV doses presented in literature and reported average values of solar dose of UVA and UVB reaching the human cornea: 3.4 J cm^2 of UVA and 105 mJ/cm^2 of UVB during 1 hour exposure.

1.4. UV penetration into the eye.

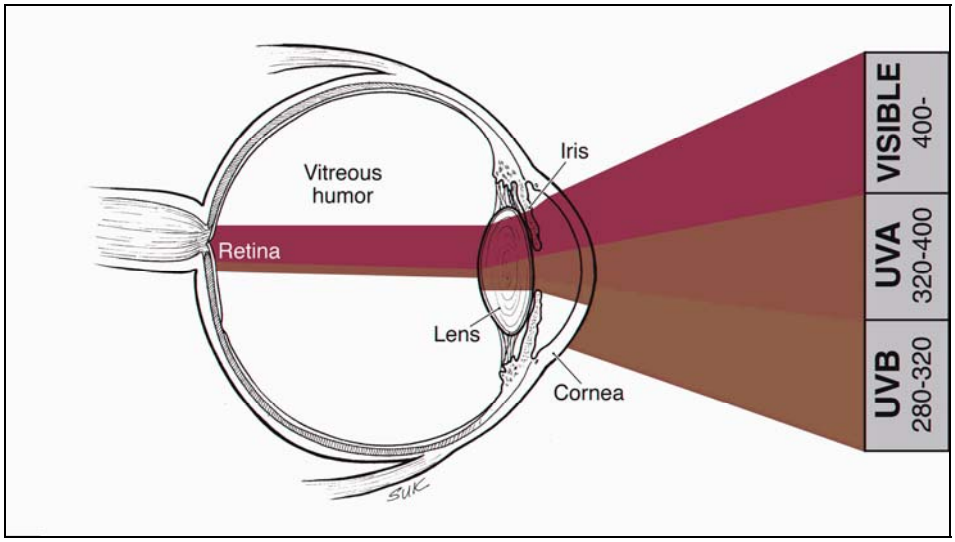
Of ocular tissues and fluids, the cornea absorbs approximately 34% of UVA rays and 80% of UVB rays. The aqueous humor, containing ascorbic acid, proteins and amino acids, is also responsible for some UVB absorption. The other ocular tissue (particularly the lens) absorb approximately 66% of UVA rays and 20% of UVB rays. Mainly the lens acts to filter UV rays from reaching the retina (Eaton 1994-1995).

The anterior eye segment



UV absorption in the eye

The cornea absorbs 34% of UVA radiation and 80% of UVB radiation. The other ocular tissues and fluids (particularly the lens) absorb 66% UVA a 20% of UVB radiation



1.5. Reactive oxygen species induced by UV rays.

One of the causes of the ocular damage induced by UV irradiation is the generation of reactive oxygen species (Riley 1988). Reactive oxygen species (hydrogen peroxide, singlet oxygen and oxygen free radicals such as superoxide anions and hydroxyl radicals) are a danger to biological systems. They might cause cellular damage by reacting with lipids, proteins and DNA (Chace et al. 1991, Kehrer 1993, Mehlhase and Grune 2002). Many pathologies have been attributed to the action of reactive oxygen species, and one of the dominant theories of aging contends that senescent changes are a consequence of the accumulated action of these toxic products (Emerit 1992, Stadtman 2001, Hensley and Floyd 2002). According to Ogura et al. (1991), reactive oxygen species are important mediators of lipid peroxidation in the epidermis exposed to UV light. Reactive oxygen species are suggested to play a major role in a number of specific pathological conditions of intraocular tissues, such as cataract formation and retinal degeneration (Mittag 1984, Ohira et al., 2008). In the cornea, direct cleavage of corneal glycosaminoglycans was found (Carubelli et al. 1990).

1.6. High molecular weight corneal protective mechanisms against oxidative damage.

Ocular tissues and fluids contain both low molecular weigh anti-oxidants (such as ascorbic acid, glutathione and alpha-tocopherol) and high molecular weight anti-oxidants (enzymes, such as catalase, superoxide dismutase, glutathione peroxidase) that have the key role in protecting against oxidative damage. Superoxide dismutase catalyses dismutation of superoxide to peroxide and molecular oxygen. Thus, this enzyme protects the ocular tissues from the superoxide radical. This enzyme has been identified in the normal rabbit corneal epithelium and corneal endothelium by Bhuyan and Bhuyan (1978). Glutathione peroxidase is a very

important enzyme scavenging hydrogen peroxide. Glutathione peroxidase was detected immunohostochemically by Atalla et al. (1988, 1990) in rats in the corneal epithelium and the corneal endothelium. Catalase protects ocular tissues from hydrogen peroxide and also protects superoxide dismutase from inactivation by hydrogen peroxide. Catalase was investigated biochemically in the normal rabbit corneal epithelium and corneal endothelium by Bhuyan and Bhuyan (1970) and immunohistochemically by Atalla et al. (1987). Cytosolic aldehyde dehydrogenase 3A1, the major water-soluble protein in the mammalian cornea, has several defense systems protecting the corneal epithelium against UV-oxidative injury, such as the detoxification of peroxidic aldehydes, the scavenging of free radicals, and the direct absorption of UV radiation (Abedinia et al., 1990, Downes et al., 1992, Uma et al., 1996, Pappa et al., 2001, Piatigorski, 2001, Estey et al., 2007, Lassen et al., 2008).

1.7. Low molecular weight corneal protective mechanism against oxidative damage.

Ascorbic acid is an important low-molecular weight scavenger of reactive oxygen species, known to be generated by UVB rays. The corneal epithelium absorbs UVB radiation mainly owing to its ascorbate content (also water-soluble proteins, RNA and DNA) (Ringvold 1997, 1998). Ringvold et al. (2000) analyzed the distribution of ascorbate in the anterior bovine eye. According to these authors, ascorbate is not equally distributed throughout the anterior eye segment. The highest ascorbate concentration was found in the corneal epithelium, where it is higher in the central part covering the pupillary area than in the peripheral areas. The authors suggeted that ascorbate might act as a UV filter shielding the internal structures from radiation damage. Brubaker et al. (2000) studied the ascorbic acid content in the human corneal epithelium and descibed that the concentration of ascorbate there, 1.33 +- mg/g wet weight, was 14 times higher than its concentration in the aqueous humor. The ascorbate content in the corneal

epithelium can be influenced by environmental changes (Ringvold et al., 2003). The authors suggested that the seasonal adaptation of the mammalian corneal epithelium in response to variations in radiation might be a natural strategy for counteracting radiation damage to the eye. Bilgihan et al. (2001) described that the protective role of ascorbic acid in the oxidative defences of the eye lies in its reducing properties. The molecule is likely to contribute an electron to any free radical species, resulting in the neutralization of the original free radical. Tessem et al. (2005 a,b) found that repeated irradiation of the cornea with UVB rays (not with UVA rays) significantly decreases the level of ascorbic acid in the cornea and aqueous humor.

Besides ascorbic acid, DL-alpha-tocopherol acetate (vitamin E) also protects against the harmful effects of free oxygen radicals (Fletcher et al., 2008). Vitamin E is a free radical scavenger and protects cells by breaking the chains of reactive oxygen species (Bilgihan et al. 2003).

1.8. Corneal light absorption properties (normal and after UV rays).

Until now, little has been known about corneal light absorption properties after the irradiation of the cornea with UVB or UVA rays. There was only one paper (Schive et al., 1984) dealing with corneal light absorption. These authors irradiated the cornea with UVB rays once with a rather low dose and examined animals following 18 hrs after exposure. Therefore, we decided to study this problem in detail to fill this gap. The rabbit cornea was repeatedly irradiated with UV rays of different wavelength (UVB, UVA) for one to five days and after sacrificing the animals changes of corneal hydration properties and corneal light absorption were examined. Moreover, the protective effect of UV filter on corneal hydration and light absorption was studied. Finally, the rabbit corneas were irradiated with UVB doses equivalent to 2.5 and 5 hrs

exposure of the human cornea to UVB rays from sunlight. The aim of this study was to examine, whether these UVB doses change corneal hydration and light absorption in rabbits.

1. 9. Hypothesis and Aims of the study.

We hypothesized that a new spectrophotometrical method, which we attempted to develope in experimental animals (rabbits, New Zealand white) for measuring the transmission of the light across the cornea, enabled us to examine qualitatively as well as quantitatively optical properties of the cornea, particularly corneal transparency changes.

The specific aims were:

*** To determine the light absorption properties in the rabbit cornea irradiated with UVB rays (once or repeatedly) by measuring of physical values of absorbance A and transmittance T as functions of wavelength λ.**

*** To investigate the absorption coefficient α as a function of wavelength λ.**

*** To evaluate the importance of this coefficient α for corneal light absorption properties.**

*** To determine the light absorption properties in the rabbit cornea irradiated with UVA by measuring physical values of absorbance A and transmittance T as functions of wavelength λ (to compare the effect of UVA with UVB rays).**

To achieve individual aims, the suitable spectrophotometrical method was developed (see chapter 3.5 and Paper 1 for details) and following experimets were performed:

a) Repeted irradiation of the rabbit cornea with UVB rays (daily dose 1.01 J/cm^2 during 5 days). After the end of experiments the animals were sacrificed and corneas examined spectrophotometrically and for corneal thickness (levels of hydration) (Paper 1 and Paper 2);

In these experiments physical values of absorbance A and transmittance T as functions of wavelength λ were examined as well as the absorption coefficient α as a function of wavelength λ was investigated. Moreover, the importance of this coefficient α for corneal light absorption properties was evaluated.

b) Repeated irradiation od the rabbit cornea with UVA rays (daily dose 1.01 J/cm^2 or 2.02 J/cm^2) during five days. Afterwards the animals were sacrificed. Corneas were examined spectrophotometrically (physical values of absorbance A and transmittance T as functions of wavelength λ). The effect of same doses of UVA and UVB rays were compared (Paper 3).

c) The rabbit cornea was irradiated with UVB rays (daily dose 1.01 J/cm^2) for one, two, three or four days. During the irradiation procedures corneal hydration was measured using an ultrasonic Pachymeter and after the end of irradiation the corneas were examined spectrohotometrically using the method deascibed in Paper 1. In individual time intervals

corneas were examined spectrophotometrically (physical values of absorbance A and transmittance T as functions of wavelength λ) (Paper 4).

d) The UV-protective effect of actinoquinol combined with hyaluronic acid (UV filter) was investigated. The rabbit corneas were irradiated with different doses of UVB rays during four days. During irradiation UV filter was dropped on the right eye, and buffered saline on the left eye. At the end of irradiations the animals were sacrificed and corneas investigated spectrophotometrically; corneal hydration was measured with a Pachymeter (Paper 5).

e) The effect of UVB doses on the rabbit cornea equivalent to 2.5 and 5 hours exposure to UVB rays reaching the human cornea from sunlight. For this reason the rabbit corneas were irradiated with the dose of 0.5 J/cm2 or 0.25 J/cm2 for four days and one day after the end of irradiations (on day five) the corneas were investigated spectrophotometrically (Paper 6).

2. Materials and Methods

2.1. Experimental animals.

Female New Zealand White rabbits weighing 2.5-3.0 kg were used in our experiments. The procedures used in or experiments were consistent with the ARVO resolution on the Use of Animals in Research, according to the World Medical Association Declaration of Helsinki, Finland, 1964 and revised by the World Medical Assembly in Hong Kong in 1989.

2.2. Anesthesia and sacrificing animals.

The rabbits were anesthetized by an i.m. injection of Narkamon and Rometar (Narkamon 5%, 1ml/1kg weight + Rometar 0.2% 0.2ml/1kg weight). For sacrificing animals the rabbits were anesthetized with Narkamon + Rometar and sacrificed with Thiopental injection.

2.3. Measuring equipment.

Special equipment for determination of radiation properties of the radiation source and for accurate estimation of the radiation dose was employed. We used the Cole Parmer VLX3W radiometer equipped with the interchangable probes (sensors) for measuring of UVB (312 nm) and UVA (365 nm) radiation in two modes of employ.

At first, this radiometer was used for exact measuring of the intensity of radiation in given distance from the source (location of the sample - eye surface). The values of the intensity were possible to read directly on the display (in mV/cm^2). At second, the radiometer had an important role in the exact estimation of the total irradiation dose. This total energy of radiation was measured using the UVA, UVB sensors respectively, located in the same place (distance of the radiation source) where the eye surface was placed. The radiometer with the probe was on power for the whole time of exposure and it computed the total dose using the integration of the differential doses for the whole time of exposure. This enabled us to determine the really exact total dose because it was not possible to forcast the constant performance of the source of radiation during the whole time of exposure. The values of the total dose (in mJ/cm^2) were red on the display at the end of exposure time.

2.4. Irradiation.

Both eyes of the rabbits were irradiated. The eyes were open and the corneas (the other parts of the eye were covered with a sterile cotton gauze) were irradiated with Bioblock UV lamps (Scientific, Illkirch, Cedex, France, 6W), which generate UV rays of 312 nm wavelength

(UVB rays) or 365 nm (UVA rays) for 1 to 5 days. The daily dose of UVB was 1.01 J/cm^2, UVA 1.01 J/cm^2 or 2.02 J/cm^2 (Paper 1, Paper 2, Paper 3, Paper 4). The efficacy of UV filter was examined in the rabbit irradiation model using different doses of UVB rays (312 nm, 0.5 J/cm^2, 1.01 J/cm^2) (Paper 5). UVB doses in the rabbit irradiation model equivalent to 2.5 and 5 hrs exposure of the human cornea to UVB rays from sunlight were studied in Paper 6.

2.5. Spectrophotometry of corneal buttons by measuring of physical values of absorbance A and transmittance T as functions of wavelength λ in following experiments:

a) The corneas were irradiated with UVB rays, daily dose 1.01 J/cm^2 during one to five days (Paper 1, Paper 2, Paper 4);

b) the corneas were irradiated with UVA rays, the daily dose 1.01 J/cm^2 or 2.02 J/cm^2 for five days (Paper 3);

c) the corneas were irradiated with UVB rays (daily dose 0.5 J/cm2 or 1.01 J/cm^2 for four days), during irradiation UV filter was dropped on the right eye (actinoquinol combined with hyaluronic acid, manufactured by Laboratoires Thea, Clermont-Ferrand, France) and buffered saline was applied on the left eye (Paper 5).

d) The corneas were irradiated with the UVB dose equivalnet to 2.5 and 5 hours exposure to UVB rays reaching the human cornea from sunlight (Paper 6).

Immediately after the death of the animals the corneas were excised from the sclera. From the center of each cornea a circle of 6 mm diameter was cut out using a special circular knife. Each of these circles was placed into a 6 mm diameter hole in a stainless steel sheet insert, covered on both sides with quartz glass, and the whole assembly was placed between two parts - halves of the insert (made of acryl glass) designed to fit inside a standard quartz cuvette. The insert also contained a 6.0 mm hole that coincided with the measuring light beam of the

spectrophotometer, and instrumental light entered the measured piece of cornea from the epithelial side, i.e. from the same direction as with cornea in situ.

The thickness of the stainless sheet insert varied according to the central thickness of the normal and irradiated (hydrated) corneas. In previous experiments with 10 normal and 10 UVB irradiated corneas, the central thickness of the corneas was measured, using two methods. The first method used an optical device (JCF Soft Contact Lens Dimension Analyser, Optimec Ltd., Worcestershire, England) where the cornea was placed in 340 mOsm kg^{-1} PBS (phosphate-buffered saline) and the thickness of the corneal button was examined by optical projection. This device was originally engineered as a measuring equipment of the thickness of contact lenses. In the similar way as the contact lens the corneal button was placed into the device, bathed with PBS and using visible light, the optical projection on the screen began. The corneal button was shifted in vertical direction till the moment when the contact of mechanical sensor appeared. The thickness was red on the scale of the meter. In the second method a special micrometrical watch (Clock gauge, Lambda Engineering, England) was employed. The sample – corneal button was inserted between two branches of the micrometrical watch and using the binocular optical device the moment was determined when the contact of these two branches with the sample occurred. The thickness was red on the scale of the measuring device. The mean thickness values for both groups of corneas were computed and served as the basis for manufacturing the stainless sheet inserts. We used the sheet thickness values 0.4 mm, and 1.2 mm for normal and UVB irradiated corneas, respectively.

Control measurements of the thickness of spectroscopically analysed corneas were made similarly as described above. The thickness of each cornea was measured before the spectroscopic analysis. The variation in thickness values was within 10%.

Later, in Paper 4 – Paper 6, the central corneal thickness of anesthetized rabbits was measured using Ultrasonic Pachymeter SP-100, Tomey Corporation, Noritake-shinmachi, Nishi-ku, Nagoya, Japan. For every spectrophotometrical measurements, a matching thickness of the stainless steel were manufactured in advance in ascending series of thickness (0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3 mm). Also in these papers for the spectrophotometrical measurements, the insert with the same thickness as the thickness of the normal or irradiated cornea or the nearest higher sheet insert thickness was used.

For the spectrophotometrical measurements, the corneas (with the whole assembly described above) were submersed into 340 mOsm kg^{-1} PBS in the measuring cuvette. The reference spectrophotometrical measurements (with metal sheets for normal and hydrated corneas) were conducted with the same assembly bathed in the same solution (only without the samples). The absorbance readings were made over the range of wavelengths 190-650 nm using a HELIOS b 84021 v4.55 scanning spectrophotometer (Spectronic Unicam, Cambridge, UK), with wavelength resolution (step size) 1 nm. The obtained data were expressed either as spectrum of transmittance $\mathbf{T = T(\lambda)}$, or absorbance $\mathbf{A = A(\lambda).}$

$$A = - \log T$$

For the absorbance the following formula is valid: $\mathbf{A = \alpha \cdot d}$,

where α [mm^{-1}] is coefficient of absorption, and **d** [mm] is thickness of the light absorbing sample layer. Indexes **N**, **I,** when used in conjunction with **A**, **T**, α, and **d,** designate the values for normal, and irradiated corneas, respectively.

2.6. Spectrophotometry of the corneal lysates (cornea irradiated with UVB rays for five days) by measuring of physical values of absorbance A and transmittance T as functions of wavelength λ (Paper 1).

The whole normal or irradiated rabbit corneas were cut into pieces and transferred to 1 ml of ice-cold deionized water, combined with 2 ml of 0.15 M NaCl, 0.2 ml 7.6 M NaOH, and incubated in 60˚C water bath for 30 minutes with occasional shaking. The lysates were then cooled on ice, centrifuged (2590 g 10 min.) to remove any particulate material and allowed to equilibrate to room temperature prior to spectrophotometry. The protein concentration was measured by the biuret method; utilizing bovine serum albumin as the standard.

The incubation of corneas in 0.475 M NaOH at 60˚C for 30 minutes was sufficient to dissolve the tissue completely, yielding lysates free from any turbidity. Solubilization had to result from partial cleavage of corneal collagen, as the lysates remained clear after neutralisation (intact collagen is insoluble in neutral solutions). Running the lysates on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed a smear and one prominent band with MW about 15 kDa. On the other hand, the treatment was not able to hydrolyse proteins completely to amino acids: a separate test with rat tail collagen subjected to the same procedure indicated that the decrease in number of peptidic bonds, as detected by the biuret assay, is negligible.

The UV-VIS spectra of the corneal lysates, suitably diluted with deionized water or 0.1 N NaOH, were recorded on a diode-array spectrophotometer HP 8452A (Hewlett-Packard Co., Palo Alto, CA) against deionized water over the spectral range 190-820 nm, with step size 2 nm. A blank sample consisting of the same solutions, but without any corneal tissue, was always processed in the same way as the samples, and subtracted from the sample spectral data. The indexes **N**, **I** again refer to the absorbance values for normal, or irradiated corneas, respectively.

2.7. Statistics for corneal hydration (measured with Pachymeter) and for light absorption and transmission.

The data are generally presented as mean ± SD. The repeated measures ANOVA with the Bonferroni's post-test was used for statistical evaluation of corneal thickness measurements. For the transmitance and absorbance data, the Mann-Whitney test (non-parametric t-test) was employed. Both tests were calculated using InStat ver. 3.06 (GraphPad Software, Inc., San Diego, CA).

3. Results and Discussion.

3.1. The light absorption in the whole cornea (corneal buttons) irradiated with UVB rays (daily dose 1.01 J/cm^2 for five days) (Paper 1, Paper 2).

During the irradiation rabbit corneas turned grayish and first signs of vascularization at the limbus were observed. The irradiated corneas were hydrated (swollen) to more than two-fold of normal tissue weight (204.3 ± 60.8 mg versus 80.4 ± 3.5 mg, N=8 for each group, $p<0.001$), and to three-fold of normal thickness (1.208 ± 0.009 mm versus 0.398 ±0.009 mm, N=8-12, $p<0.001$)

The shapes of our absorbance curves obtained with the whole normal or irradiated rabbit corneal buttons (Fig. 1A) strongly suggest that recording of sample optical properties is possible throughout the visible (VIS) and UVA spectral regions up to UVB, but ends at about 285 nm for normal corneas (A>2.5) and at about 300 nm for UVB irradiated ones (A>3). The absorbance

traces continue for shorter wavelengths but reflect the instrumental stray light error rather than sample optical properties (Perkampus, 1992) (and data below).

Within these technical limits, nevertheless, the data expressed as corneal transmittance (Fig. 1B) (page 19) or as % of absorbed light for selected wavelengths (Table) (Page 22) clearly show that the cornea hydrated after irradiation with UVB absorbs considerably more light throughout the whole measurable spectral range.

Figure 1 *- Results of the spectrophotometry of the whole cornea (corneal buttons). Comparison of light absorption of the normal and irradiated rabbit corneas, expressed either as spectrum of absorbance A = A (λ)* ***(Fig. 1A)****, or transmittance T = T (λ)* ***(Fig. 1B).*** *The spectral curves are means from measurements of 7 normal corneas, and 7 irradiated ones.*

Figure 1 A

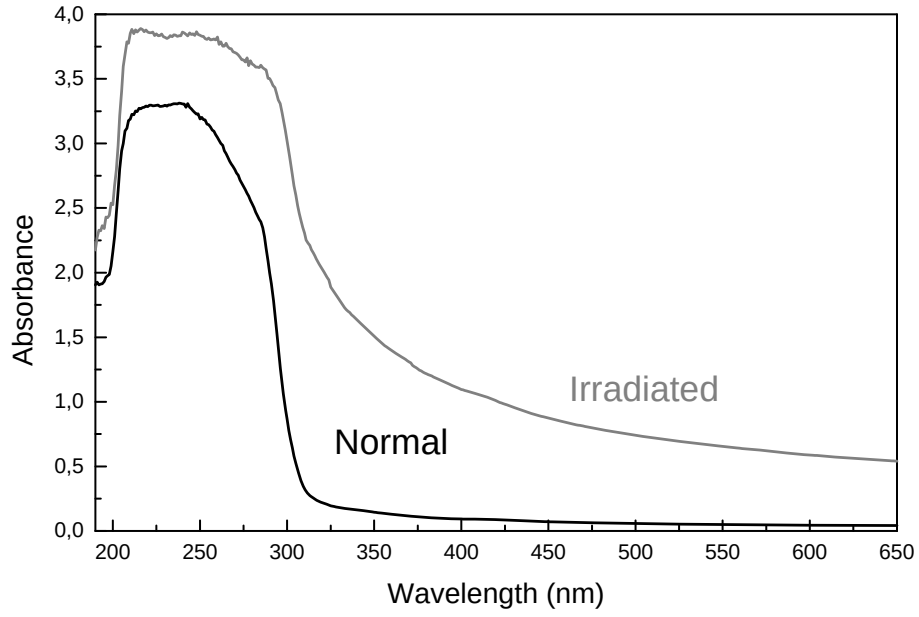
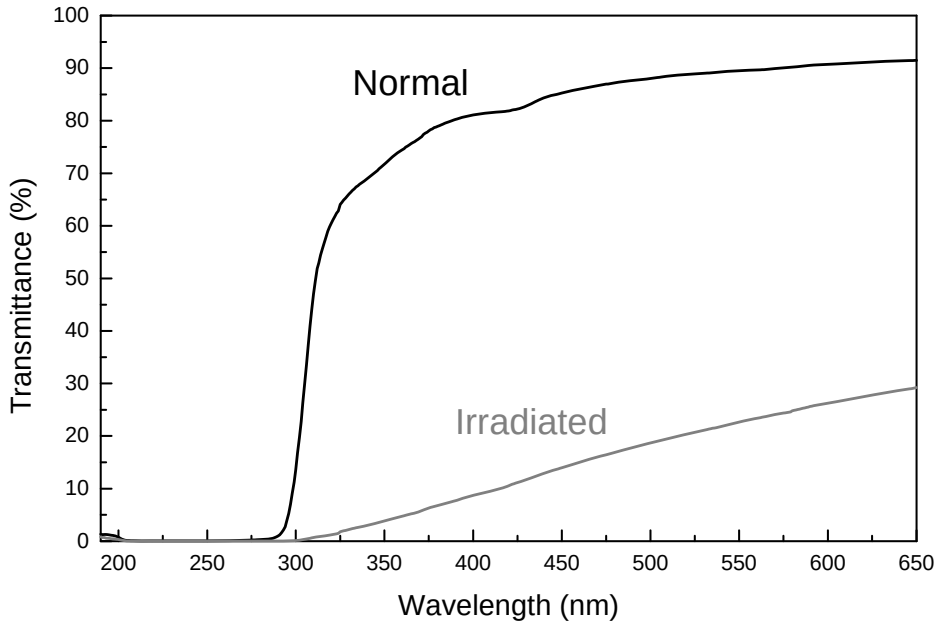


Figure 1 B



Next, the absorbance values were divided with corneal thickness and plotted as absorption coefficients α (Fig. 2, see below). For the measurable spectral range >300 nm the $\alpha_I > \alpha_N$ with the difference being maximal at about 310-320 nm (Fig. 2, inset). For 316 nm for instance, the $\alpha_N = 0.6186 \pm 0.0723$ mm^{-1} (N=7), while $\alpha_I = 1.7611 \pm 0.2938$ mm^{-1} (N=7), i.e., 185 % more (statistically significant, $p<0.0001$). It indicates that higher absorbance of UVB irradiated cornea results not only from increase in thickness, but also from a change in its chemical properties/composition.

Figure 2 *- Main diagram: Absorption coefficients of normal (αN) and irradiated (αI) corneas plotted as functions of wavelength for the measurable spectral range 300-650 nm. The values of a were obtained by dividing the absorbance data (the same as shown in Fig. 1) with thickness of each cornea (mm). The data are averaged measurements of 7 normal corneas, and 7 irradiated ones. The columns show standard deviations (SD) for selected wavelengths. Inset: The difference between absorption coefficient for irradiated (λI) and normal (λN) corneas plotted for the spectral region 300-400 nm.*

Figure 2

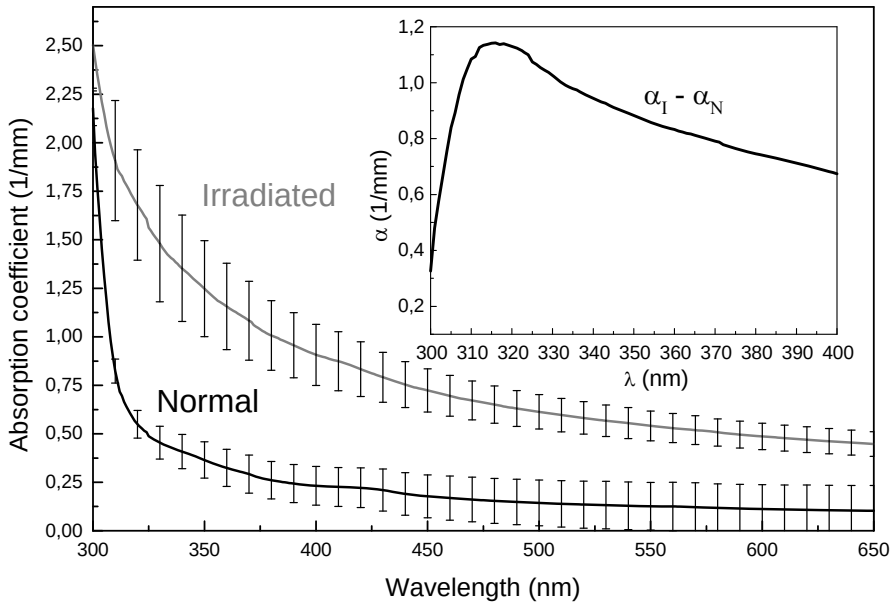


Figure 3 - A *: Absorption spectra of one selected corneal sample, serially diluted with ultrapure deionized water as indicated, against the deionized water; in this way the sample spectra actually include the UV-absorbing impurities in solvents and reagents, omnipresent except for the ultrapure water.* ***B*** *: The same spectral data replotted as function of sample dilution for selected wavelengths. The deviations from linearity (the Lambert-Beer law) indicate where the stray light error prevails over the useful light detection. For instance, at 240 nm the recording is linear for A<3.0, while at 210 nm only for A<2.0.*

Figure 3A

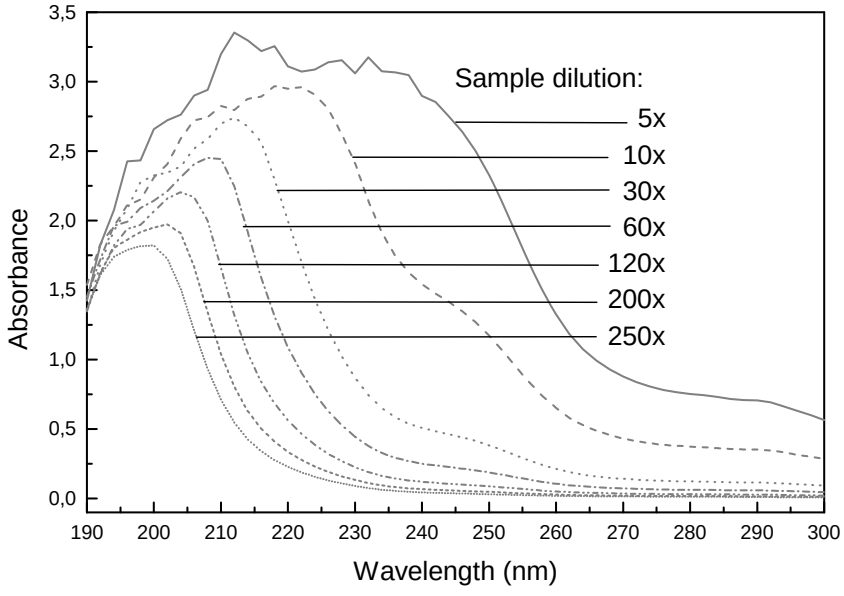


Figure 3B

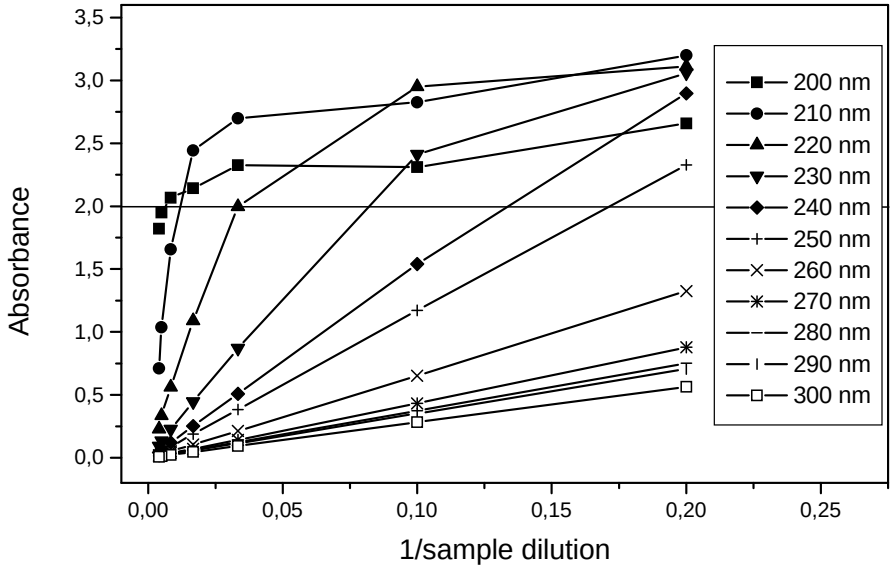


Table : *Amount of light absorbed by normal rabbit cornea (N=7), compared to cornea hydrated due to UVB irradiation (N=7) at various wavelengths. In the range 300-650 nm all the differences are statistically significant (p<0.0001).*

Wavelength	% of light absorbed	
	Normal cornea	UVB irradiated cornea
285 nm (UVC-UVB)	99.59 ± 0.03	>99.9
300 nm (UVB)	86.1 ± 1.0	99.89 ± 0.07
312 nm (UVB)	48.1 ± 3.6	99.2 ± 0.7
365 nm (UVA)	24.4 ± 6.5	94.8 ± 3.3
400 nm (VIS violet)	18.9 ± 7.1	91.2 ± 4.1
555 nm (VIS yellow-green)	10.4 ± 9.7	77 ± 5.4
650 nm (VIS red)	8.5 ± 10.4	70.8 ± 5.6

3.2. The light absorption in the corneal lysates (corneas irradiated with UVB rays (daily dose 1.01 J/cm^2 for five days) (Paper 1).

Likewise the spectra of whole corneas described above, the lysates from hydrated corneas displayed higher absorbances than samples from normal corneas at all the wavelengths examined (Fig. 4, p.20). In the VIS region >400 nm the absorption of lysates from iradiated corneas was negligible except for a faint shoulder at about 410 nm (Fig. 4A) that could perhaps represent traces of heme from hemoglobin (the Soret band). The most pronounced spectral alterations were found in the UVB region: at 290 nm the absorbance of UVB irradiated corneal lysates was 149 % of the normal values (Fig. 4A). Higher dilution of the samples was necessary to extend spectral analyses down to 210 nm (Fig. 4B). It revealed that spectral difference between UVB irradiated and normal corneal samples continued to the UVC region forming another maximum at 228 nm (Fig. 4B inset) where the irradiation-associated increase represented 10.4 % of normal values.

Proteins in general are well-known to absorb UVB light, chiefly because of their tyrosine and tryptophan residues (Murray et al., 2000). Estimation of protein separately in the corneal epithelium and the corneal stroma revealed that the actual protein exudation into the UVB irradiated and hydrated stroma could reach about 2 mg, or 15 % of the normal protein content, while simultaneously the irradiated epithelium suffered a dramatic reduction. Nevertheless, the total amount of protein obtained from UVB irradiated corneas was only slightly and not significantly higher (about 1 mg on average, or 7 %) than the protein yield from normal corneas. Accordingly, some difference in absorbance of lysates made from iradiated vs. normal corneas persisted even if the values were related to the corneal protein. For example, at 290 nm the absorbance was 0.4481±0.0225 per 1 g/l protein for the normal corneas (N=8) and 0.6168±0.0617 per 1 g/l protein for the irradiated ones (N=6), i.e. 38 % more (statistically significant, p=0.0007).

Figure 4 - A *: Absorption spectra of normal (AN, solid line) and irradiated (AI, dash line) corneas dissolved in NaOH as described in the Methods section. The spectra are means from recordings of 8 normal and 6 hydrated corneal samples, diluted 5x. The inset shows the same data replotted as difference spectrum AI - AN for 260-460 nm.*

Figure 4 A

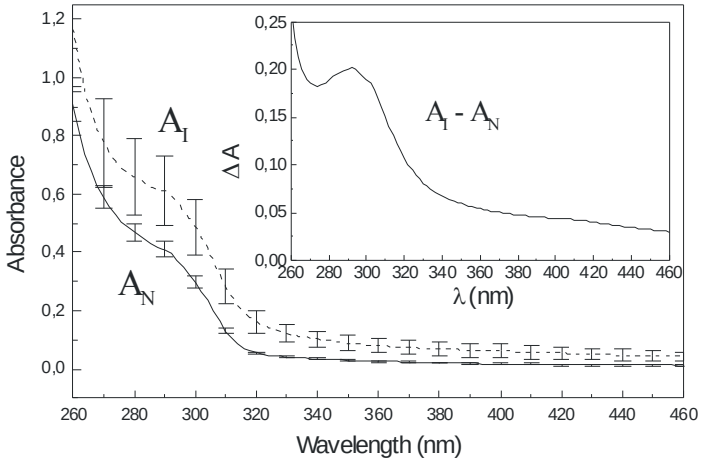
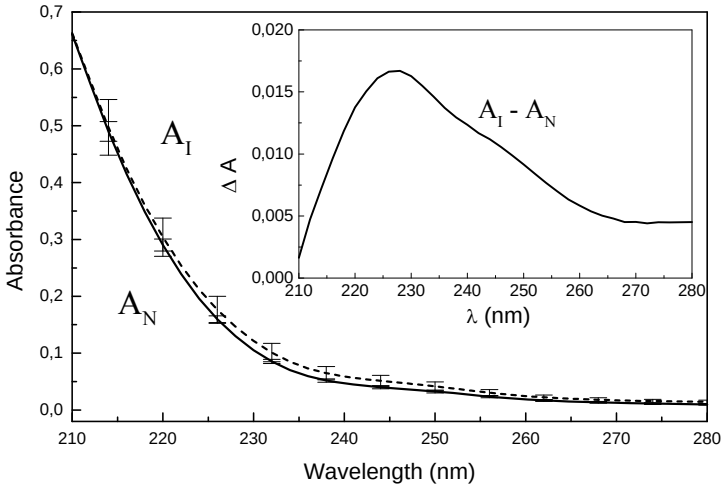


Figure 4 - B *: Averaged spectra of 4 normal (AN, solid line) and 4 irradiated (AI, dash line) corneal lysates, recorded at dilution 200x, which enabled measurement down to 210 nm. The inset shows again the same data replotted as difference spectrum AI - AN for 210-280 nm.*

Figure 4 B



3.3. The light absorption in the whole cornea (corneal buttons) irradiated with UVA rays (daily dose 1.01 J/cm^2 or 2.02 J/cm^2 for five days) (Paper 3).

As compared to the normal cornea, UVA rays (1.1 J/cm^2 per day, once a day, for 5 days) as well as UVA rays at a two-fold higher dose (2.2 J/cm^2 per day, once a day, for 5 days) do not significantly change corneal light absorption properties (Figs. 1, 2, p. 26). Also, the thickness of corneal centers after irradiation with UVA rays (1.1 J/cm^2 per day or 2.2 J/cm^2 per day) is not significantly changed as compared to normal corneas. Corneal transparency is unchanged after UVA irradiation. In contrast to UVA rays, the cornea repeatedly irradiated with UVB rays (1.1 J/cm^2 per day, once a day, for 5 days) absorbs more light throughout the whole spectrum than the normal cornea. This is due to changes in corneal transparency (the cornea turns grayish), increased corneal thickness (elevated hydration), (see Table below), which shows that the thickness of the UVB irradiated corneal centers is approximately three times greater than the thickness of the normal corneal centers) and altered chemical properties of the cornea (an increase in proteins) (Paper 1).

Description to the Table below:

The thickness of the center of UVA irradiated cornea does not significantly change as compared to the thickness of the center of normal cornea. In contrast, the thickness of UVB irradiated corneal centers is approximately three times greater than the thickness of normal corneal centers.

	normal [mm]	**UVA** 1.1 J/cm^2 [mm]	**UVA** 2.2 J/cm^2 [mm]	**UVB** 1.1 J/cm^2 [mm]
CORNEAL CENTER THICKNESS	0.39	0.40	0.41	1.21
	0.40	0.39	0.39	1.20
	0.40	0.39	0.39	1.20
	0.39	0.40	0.41	1.22
	0.41	0.41	0.40	1.20
	0.40	0.38	0.38	1.21
	0.38	0.38	0.39	1.20
	0.39	0.39	0.39	1.20
mean[mm]	0.395	0.393	0.395	1.205
SD [mm]	0.009	0.01	0.01	0.007

Description to the Figure 1 and Figure 2 (below)

*Results of the spectrophotometry of the corneal centers, expressed either as the spectrum of transmittance T = T (λ) (**Fig. 1**), or absorbance A = A (λ) (**Fig. 2**). The spectral curves are means from measurements of 14 normal corneas, 7 irradiated with UVA rays (1.1 J/cm^2 per day, once a day, for 5 days), 6 irradiated with a UVA double dose (2.2 J/cm^2 per day), and 7 irradiated with UVB (1.1 J/cm^2 per day, once a day, for 5 days). Note that for wavelengths shorter than about 300 nm, the spectra show the instrumental stray light error rather than the corneal optical properties. The corneas repeatedly irradiated with UVB rays absorb more light than the normal corneas throughout the whole measurable spectral range. In contrast, no significant differences between normal corneas and corneas irradiated with UVA rays were found (tested at 320, 380, and 550 nm by one-way ANOVA with Dunnett´s post-test).*

Figure 1

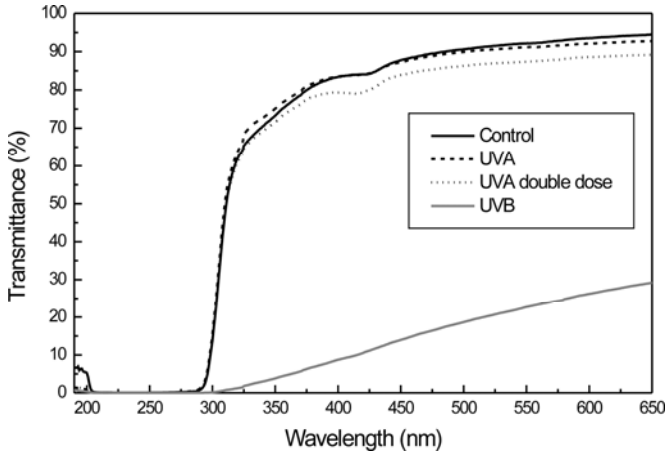
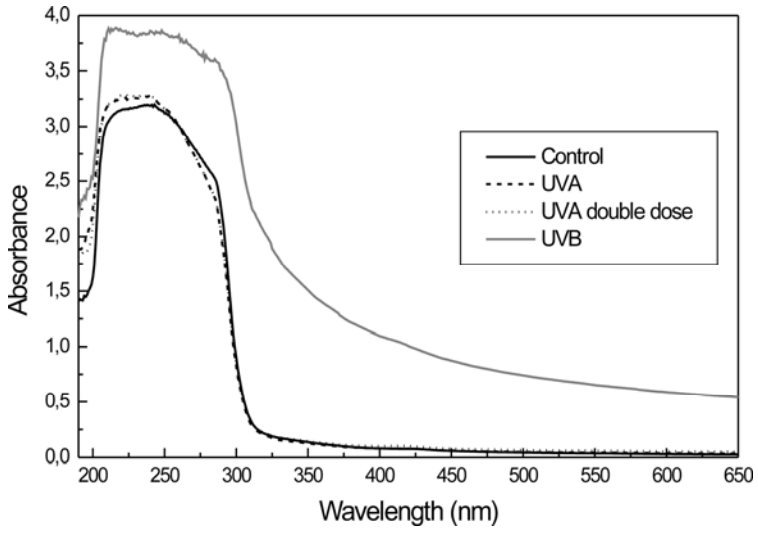


Figure 2



The transparency of the cornea is a consequence of the detailed ultrastructure of the tissue and has been attributed to the narrow, uniform diameter collagen fibrils, and to the regularity of their lateral packing (Maurice 1957, Twersky, 1974). The transparency of the corneal stroma is critically dependent on the hydration of the tissue; if the cornea swells, light scattering increases. (This was found in our study after the irradiation of the cornea with UVB rays). This scattering has been ascribed to the disruption caused to the arrangement of collagen fibres; changes in refractive index of the extracellular material make only a small contribution to the increase in light scattering when the cornea swells (Meek et al., 2003).

In UVB irradiated corneas, morphological disturbances (the thinning of the corneal epithelium, death of some keratocytes, Čejková et al., 2001) went parallel with enzymatic disorders, particularly in antioxidants. Corneal antioxidants cleave and detoxify reactive oxygen species generated by UVB rays and thus protect the inner eye against the oxidative injury. Repeated exposure of the rabbit cornea to UVB rays led to the significant decrease in activities of glutathine peroxidase (an enzyme cleaving hydrogen peroxide) and superoxide dismutase

activities (an enzymatic scavenger of superoxide) in the epithelium (Čejková et al., 2000). Very similar results were obtained with superoxide dismutase activity in in vitro experiments with rabbit cornea-derived cells irradiated with UVB rays (Lodovici et al., 2003). Moreover, decreased activity of this enzyme in the cornea after photorefractive keratectomy was demonstrated (Bilgihan et al., 2003). Corneal aldehyde dehydrogenase 3A1 has an important role in the detoxification of ultraviolet-induced peroxidic aldehydes and in corneal UVB absorption (Abedinia et al., 1990, Downes et al., 1992, Uma et al., 1996, Pappa et al., 2001, Piatigorski 2001, Estey et al., 2007, Lassen et al., 2007). UVB irradiation of the mice cornea dramatically reduced the activity of this enzyme (Downes et al., 1993, Manzer et al., 2003).

Of non-enzymatic antioxidants, ascorbic acid is an important scavenger of reactive oxygen species. The protective role of ascorbic acid in the oxidative defense of the eye lies in its reducing properties (Bilgihan et al., 2001). After repeated UVB exposure, a significant decrease in ascorbic acid in the cornea and aqueous humor was demonstrated (Tessem et al., 2005 a, b).

From the above-mentioned studies, it follows that in the normal cornea antioxidants, together with tissue components, absorb and detoxify UVB radiation and reactive oxygen species generated by them. However, from these studies it also appears that UVB irradiation leads to a pronounced reduction in antioxidants, which might initiate oxidative injury to the internal parts of the eye, particularly the lens. Our previous studies showed that this does not necessarily occur. The lens might remain undamaged (Tessem et al., 2005a), even if after UVB irradiation of the cornea significant metabolic disturbances in the cornea and aqueous humor were found (Čejková et al., 2000, Čejková et al., 2001, Čejková et al., 2005, Tessem et al., 2005 a,b). The results of the present study (Paper 1) suggest that the lens could be protected from oxidative injury by the increased light absorption capacity of the UVB irradiated cornea which has increased hydration and changed chemical properties.

3.4. Changes of hydration and light absorption of the cornea irradiated with UVB rays (daily dose 1.01 J/cm^2 for one, two, three or four days) (Paper 4).

Already after one UV dose, the corneal thickness increased together with the elevation of corneal absorption capacity. The protection of the cornea in UVB region was apparent after single UVB irradiation and highly increased from two time (and more) irradiation. The corneal absorption gradually increased together with the increase in corneal thickness. In all cases of irradiation the irradiated corneas absorbed more light throughout the whole measurable spectral range.

Figure 1: *Thickness of normal corneas and corneas irradiated with UVB rays (daily dose 1.01 J/cm^2) once daily for one, two, three or four days. The corneal thickness was measured by an ultrasonic pachymeter at the beginning of each experiment, before each irradiation, and before sacrificing the animals (the total number of measurements is indicated under each column). *** ... statistically significant (p <0.001) when compared to normal corneas.*

Figure 1

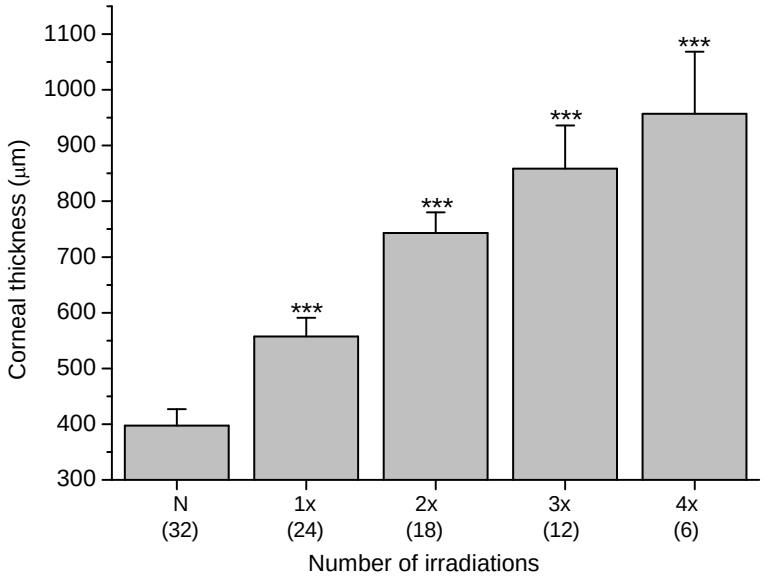


Figure 2 (below): *Results of the spectrophotometry of the corneal centers, expressed either as the spectrum of transmittance T = T (λ) (**A**), or absorbance A = A (λ) (**B**) of normal corneas and corneas irradiated with UVB rays (1.01 J/cm^2) once daily for one (1x), two (2x), three (3x) or four (4x) days. The spectra are averaged from 16 traces for normal corneas and 6 traces for each group of irradiated ones. Significant differences of T respectively A in selected wavelengths (312 nm, 320 nm, 550 nm) between normal corneas and corneas irradiated with UVB rays (1x, 2x, 3x, 4x) by one-way ANOVA with Dunnett´s post-test were found with the exception of A in 312 nm. (Note that for wavelengths shorter than about 300 nm, the spectra show instrumental stray light error rather than the corneal optical properties).*

Figure 2 A

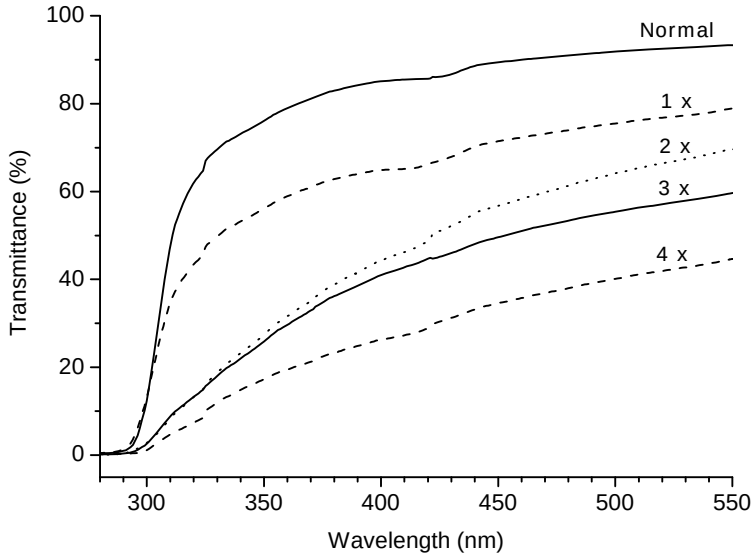


Figure 2 B

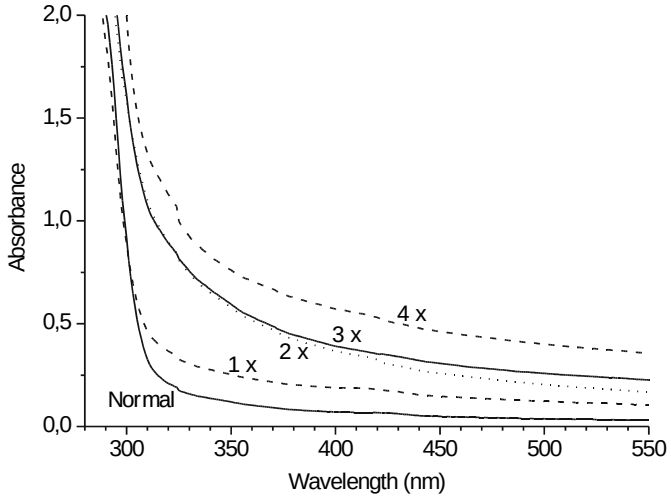


Figure 3: *Corneal optical properties of normal and UVB irradiated corneas shown as the extinction coefficient α=A/d. (Corneal thickness was taken one day after the end of individual irradiations, before sacrificing the animals for spectrophotometrical examinations). The data were converted to % of the mean of normal values for each selected wavelength. N=16 for normal corneas, and 6 for each group of irradiated ones. Statistically significant differences between the irradiated values and the corresponding normal values are marked with * ($p<0.05$) or ** ($p<0.01$).*

Figure 3

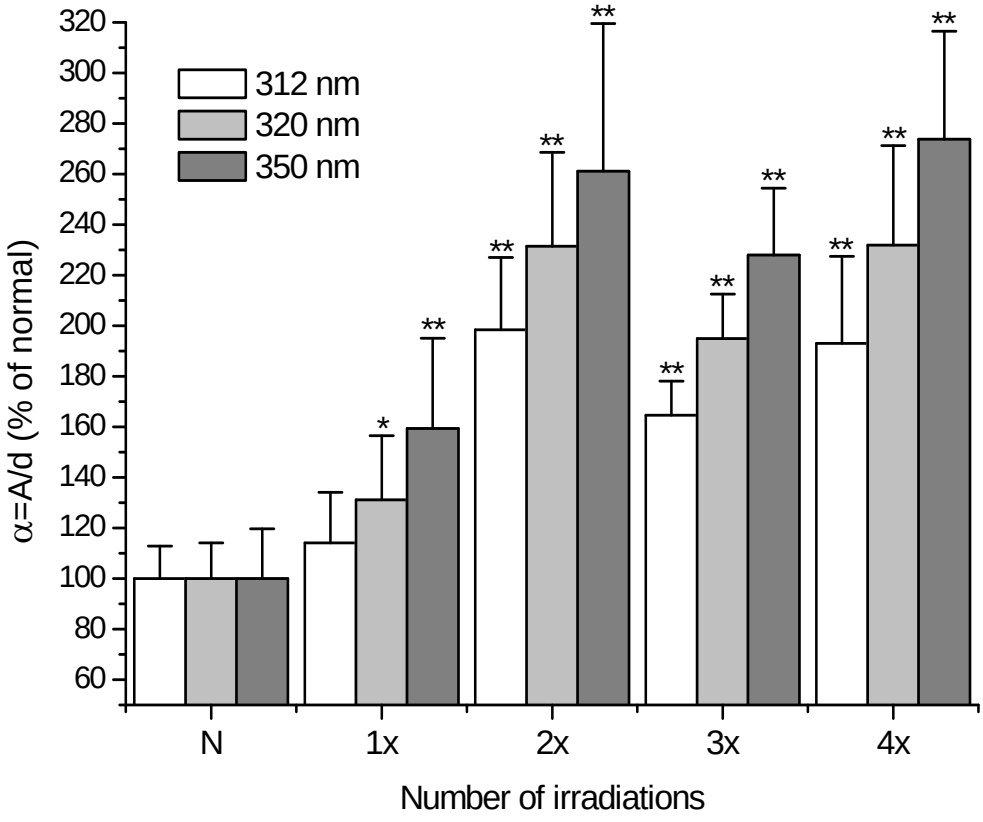


Fig. 4*.(below) Macroscopical picture of the normal rabbit cornea and rabbit corneas irradiated with UVB rays. Photographic images were done one day after the end of individual irradiations, immediately after sacrificing the animals.*

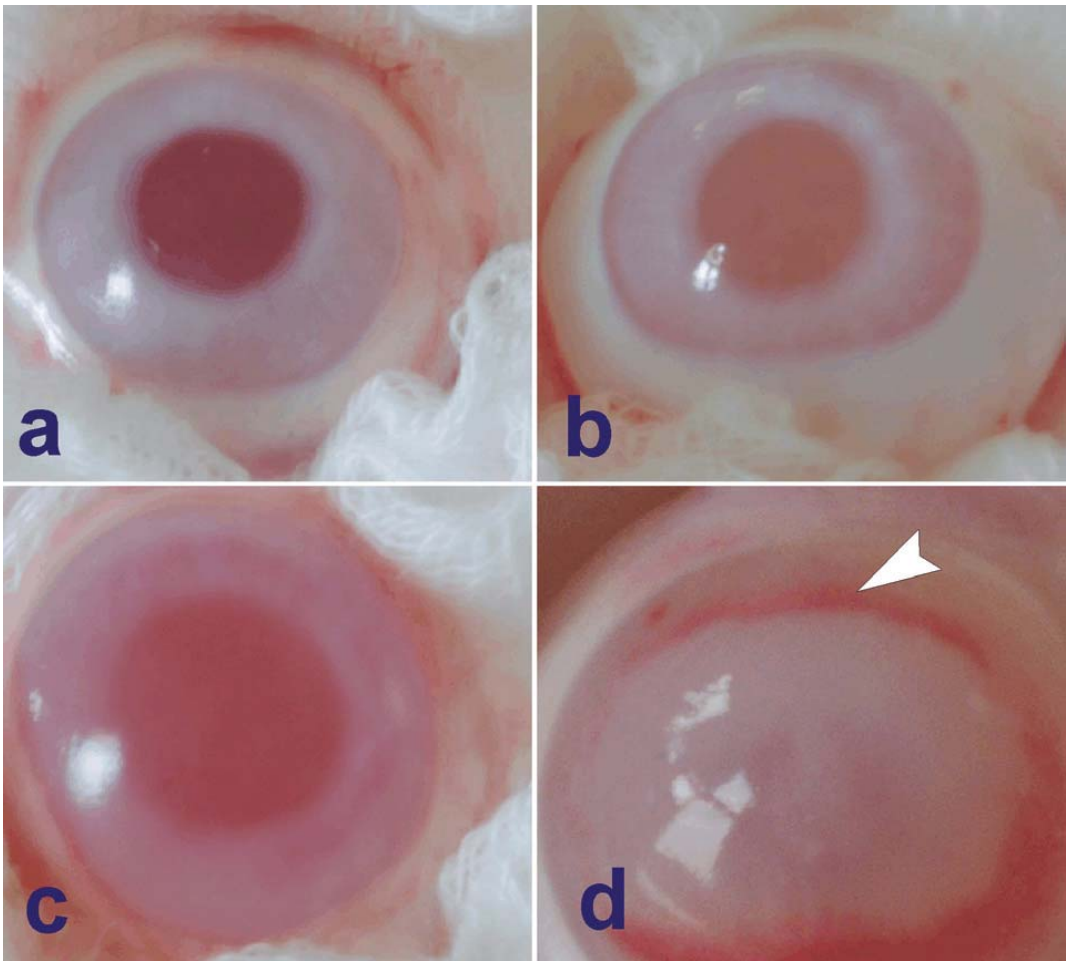
a – normal cornea;

b – cornea irradiated once. No changes of corneal transparency can be seen;

c – cornea irradiated two times. The corneal transparency is slightly changed (cornea becomes opalescent);

d – cornea irradiated four times. The changes of corneal transparency are highly pronounced. Corneal neovascularization appears at the limbus (arrow).

Fig. 4



3.5. The protective effect of actinoquinol/hyaluronic acid (UV filter) on corneal hydration and light absorption (Paper 5).

The examination of UV filter on the normal cornea (corneal thickness, hydration and spectrophotometry in UV region)

and in UVB irradiated model of the rabbit cornea (corneal thickness, hydration and spectrophotometry in UV region)

Figure 1: *Thickness of normal rabbit corneas measured by an ultrasonic Pachymeter before and after 5 days of application of either actinoquinol-hyaluronic acid or buffered saline (N=6). No significant differences were found.*

Fig. 1

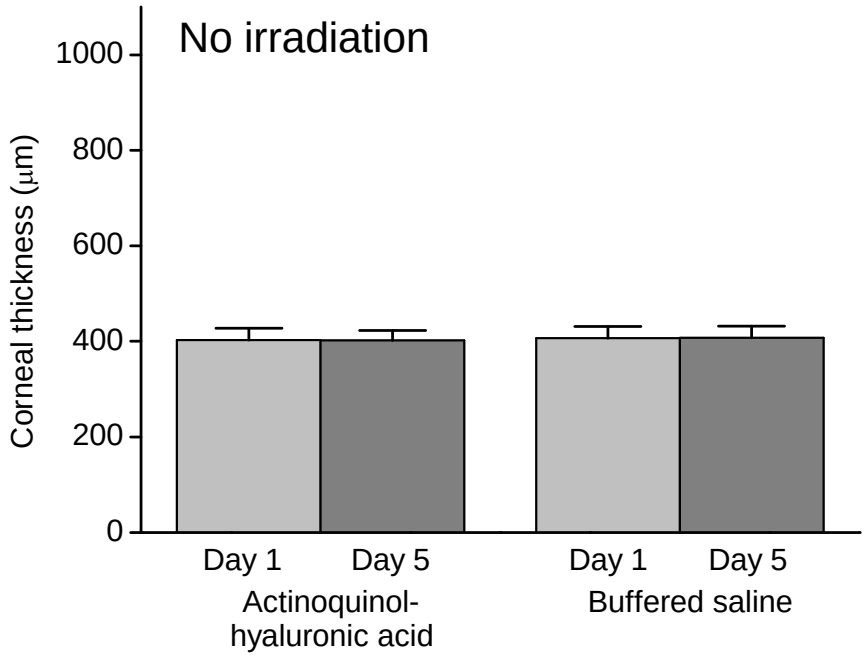


Figure 2: *Averaged absorption spectra of rabbit corneas, expressed either as the transmittance T = T (λ) (**A**), or absorbance A = A (λ) (**B**), after 5 days of application of either actinoquinol/hyaluronic acid or buffered saline (N=6). The spectrum of normal rabbit corneas (mean from 16 measurements) without any treatment is also included for comparison. Note that for wavelengths shorter than about 300 nm, the spectra show the instrumental stray light error rather than the corneal optical properties.*

Figure 2 A

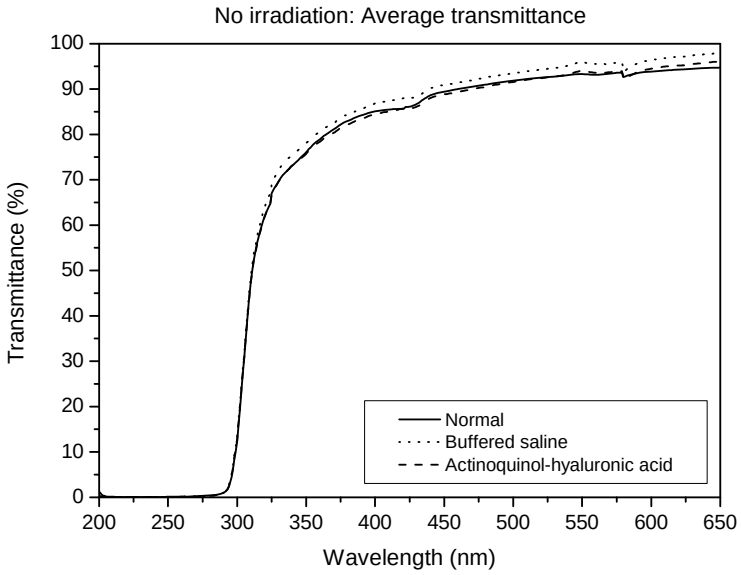


Figure 2 B

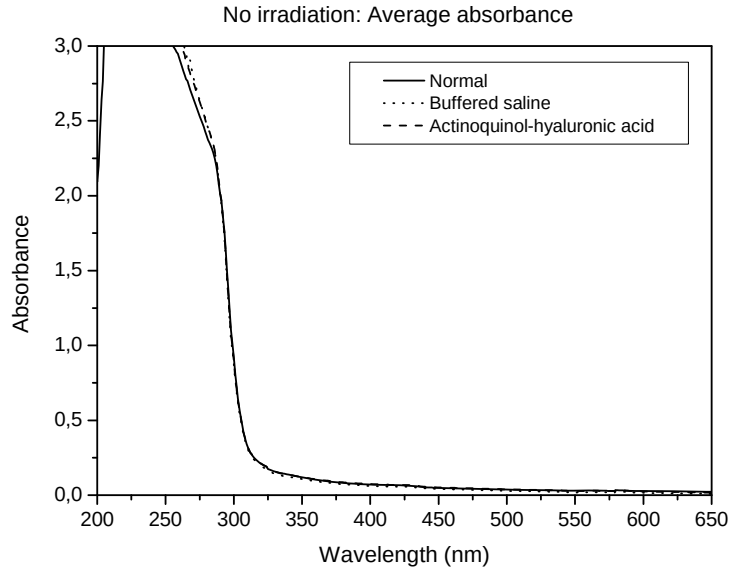


Figure 3: *Thickness of rabbit corneas measured by ultrasonic Pachymeter before and after 5 days of irradiation with UVB rays (daily dose 0.5 J/cm^2), together with the application of either the UV filter or buffered saline (N=12). The difference between the UV filter- and saline-treated corneas is statistically significant (*** ... p <0.001)*

Figure 3

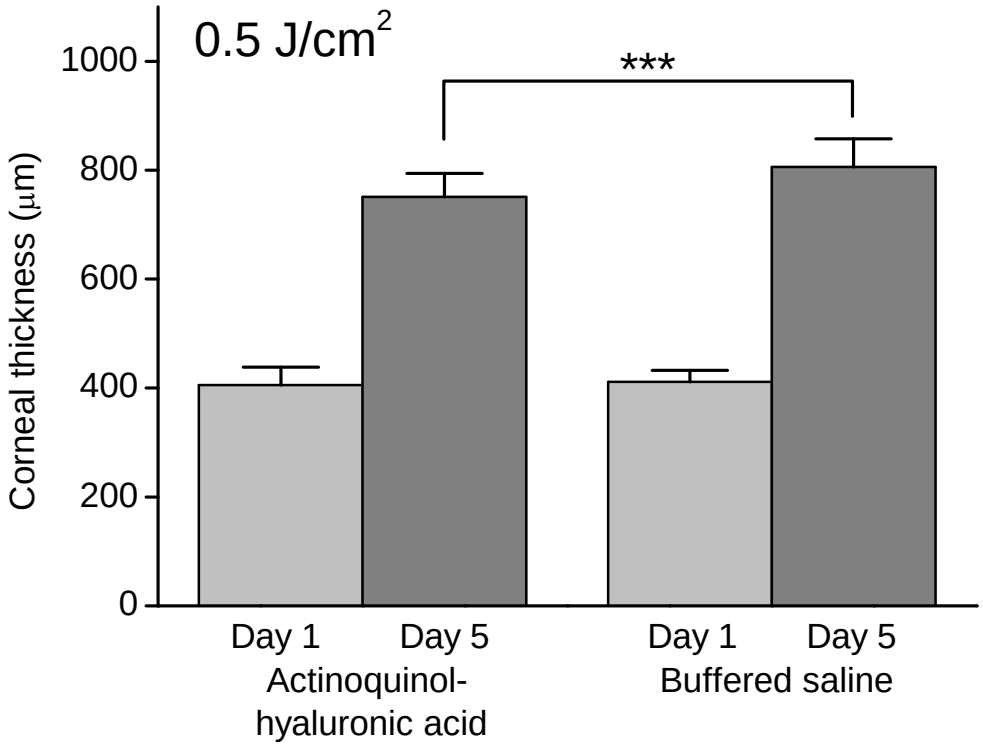


Figure 4: *Averaged absorption spectra of rabbit corneas, expressed either as the transmittance T = T (λ) (**A**), or absorbance A = A (λ) (**B**), after 4 days of irradiation with UVB rays at a daily dose of 0.5 J/cm^2, together with the application of either actinoquinol/hyaluronic acid or buffered saline (N=12). The spectrum of normal rabbit corneas (mean from 16 measurements) without any treatment or irradiation is also included for comparison. Note that for wavelengths shorter than about 300 nm, the spectra show the instrumental stray light error rather than the corneal optical properties***.**

Figure 4 A

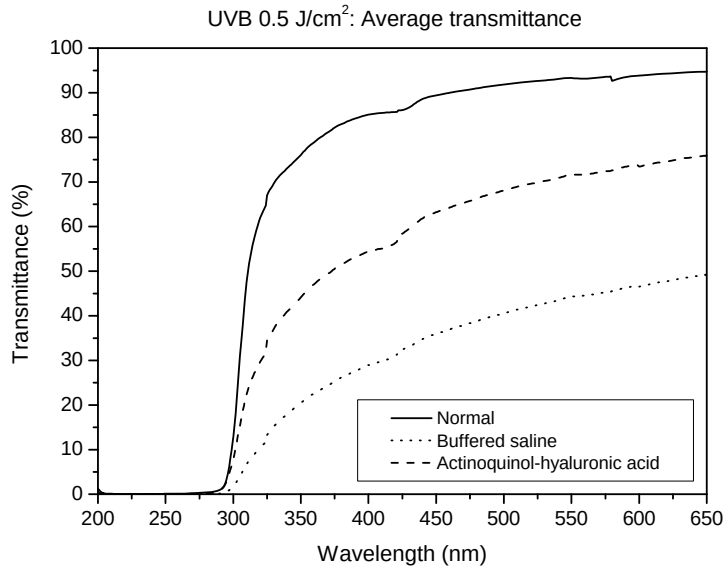


Figure 4 B

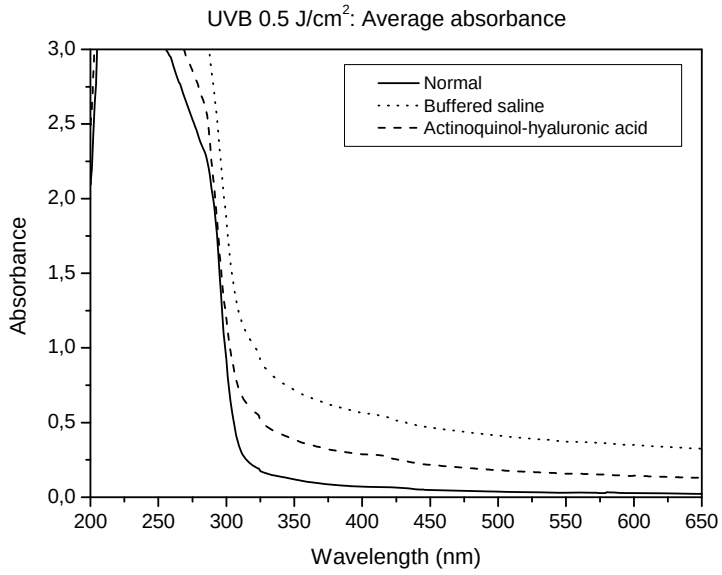


Figure 5: *Thickness of rabbit corneas measured by an ultrasonic Pachymeter before and after 5 days of irradiation with UVB rays (daily dose 1.01 J/cm^2), together with application of either actinoquinol/hyaluronic acid or buffered saline (N=6). The difference between the UV filter- and saline-treated corneas is statistically significant (** ... p <0.01)*

Figure 5

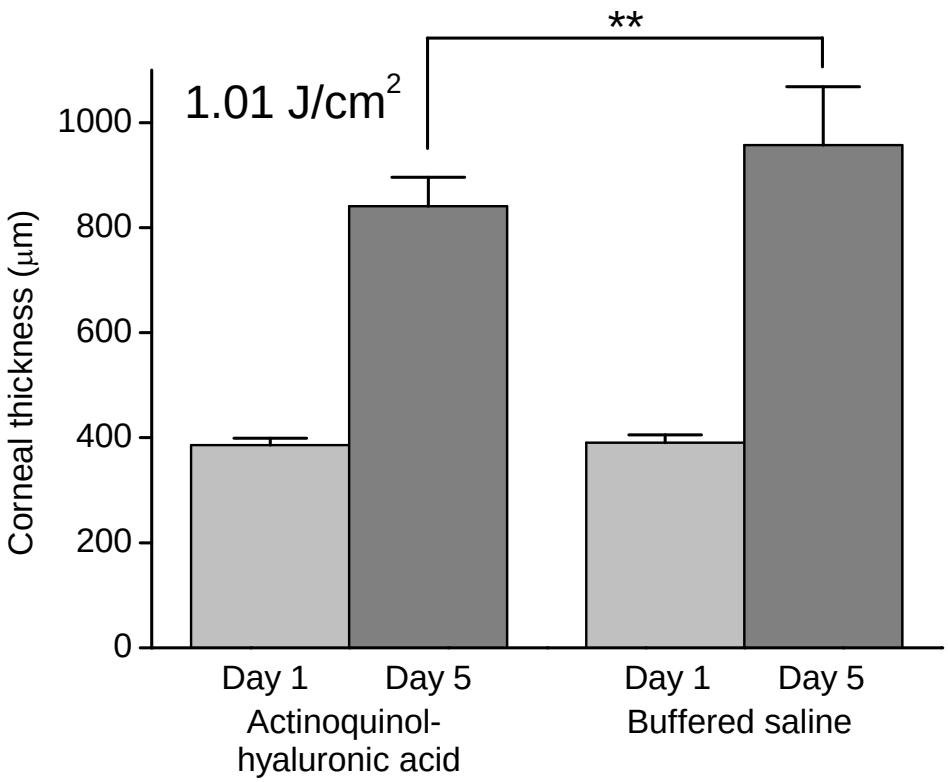


Figure 6 *(below): Averaged absorption spectra of rabbit corneas, expressed either as the transmittance T = T (λ) (**A**), or absorbance A = A (λ) (**B**), after 4 days of irradiation with UVB rays at a daily dose 1.01 J/cm^2, together with the application of either the UV filter or buffered*

saline (N=6). Spectrum of normal rabbit corneas (mean from 16 measurements) without any treatment or irradiation is also included for comparison. Note that for wavelengths shorter than about 300 nm, the spectra show the instrumental stray light error rather than the corneal optical properties.

Figure 6 A

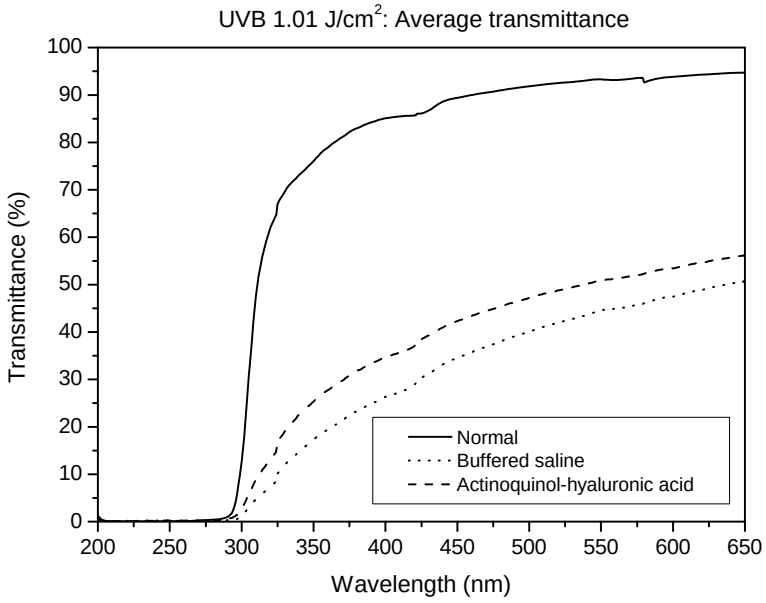


Figure 6 B

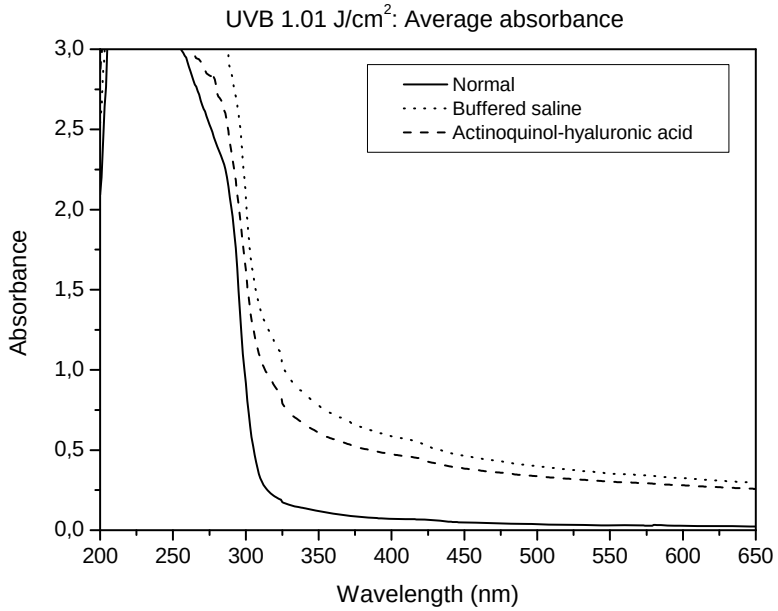


Figure 7: *Thickness of rabbit corneas measured using an ultrasonic Pachymeter before and after 4 days of irradiation with UVB rays (daily dose of 0.5 J/cm^2) together with application of either actinoquinol-hyaluronic acid or hyaluronic acid (N = 6). The difference between actinoquinol-hyaluronic acid and hyaluronic acid is statistically significant (***... p <0.001).*

Figure 7

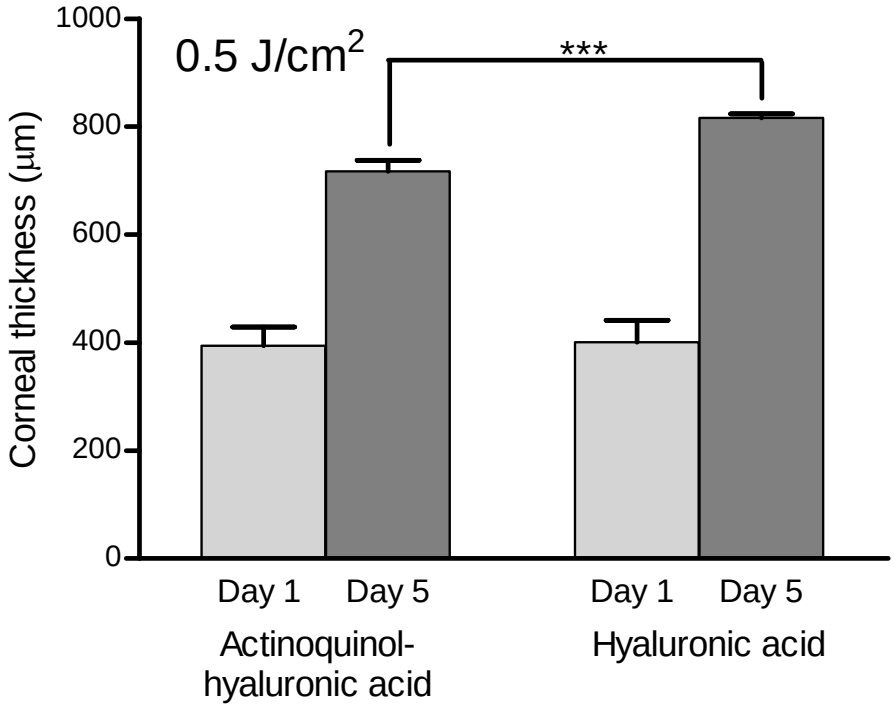


Figure 8 *(below): Averaged absorption spectra of rabbit corneas, expressed either as the transmittance T = T (λ) (**A**), or absorbance A = A (λ) (**B**), after 4 days of irradiation with UVB rays at a daily dose 0.05 J/cm^2, together with the application of either actinoquinol-hyaluronic acid or hyaluronic acid (N=6). Spectrum of normal rabbit corneas (mean from 16 measurements) without any treatment or irradiation is also included for comparison. Note that for wavelengths shorter than about 300 nm, the spectra show the instrumental stray light error rather than the corneal optical properties.*

Figure 8 A

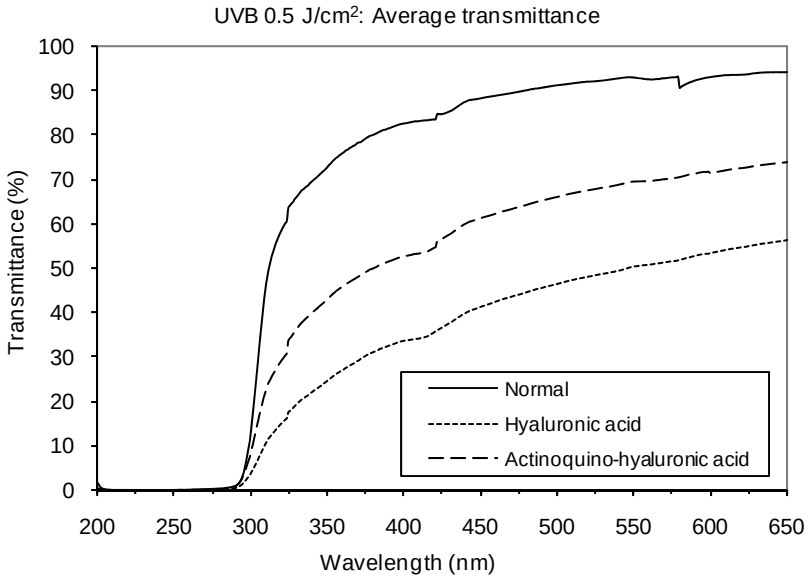


Figure 8 B

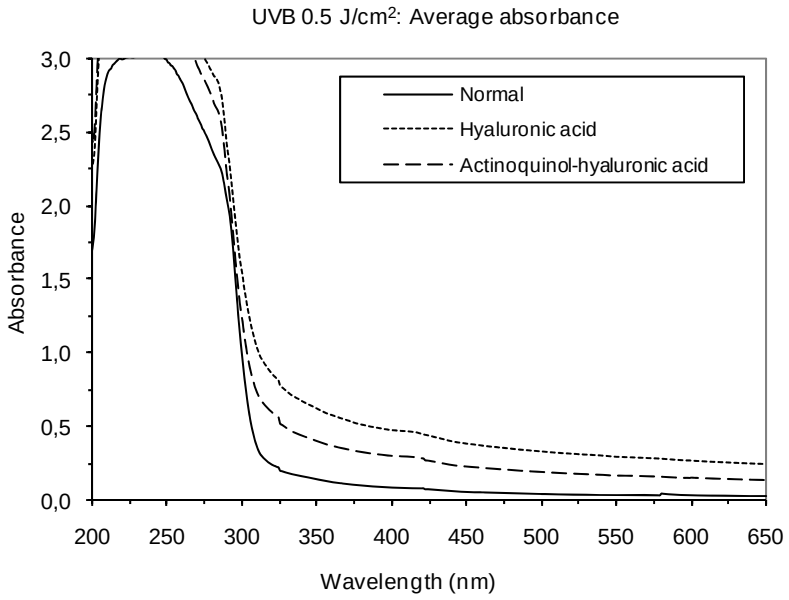


Figure 9: *In vitro UV absorbance of actinoquinol in water (10 µg/ml). Spectrum between 190 and 400 nm.*

Figure 9

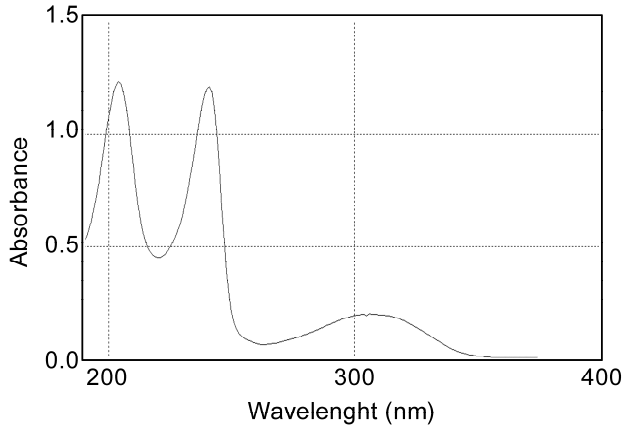


Figure 10 *(below):The eyes of rabbits after the repeated irradiation of the cornea with UVB rays (daily dose of 0.5 J/cm^2 during four days). During the irradiation buffered saline or hyaluronic acid were dropped on the left eye and actinoquinol-hyaluronic acid on the right eye. On day 5 the rabbits were sacrificed and the eyes immediately documented photographically .*

(a) Using buffered saline dropwise, the cornea became opalescent and neovascularization appeared at the limbus (arrows).

(b) The corneal neovascularization after buffered saline application in detail (arrow).

(c) After actinoquinol-hyaluronic acid treatment the changes of corneal transparency were less pronounced and cornea was without vessels. Vessels were only apparent at the corneal-scleral transition (arrow).

(d) Corneal-scleral region with the vessels (arrows) in detail (after actinoquinol-hyaluronic acid treatment).

(e) After hyaluronic acid application changes of corneal transparency were more pronounced than after actinoquinol-hyaluronic acid treatment, however, less than after buffered saline application. The neovascularization was suppressed (arrow). Compare with buffered saline treatment (a) and actinoquinol-hyaluronic acid application (c).

(f) The corneal neovascularization after hyaluronic acid application in detail (arrows).

(g, h) Normal cornea.

Figure 10

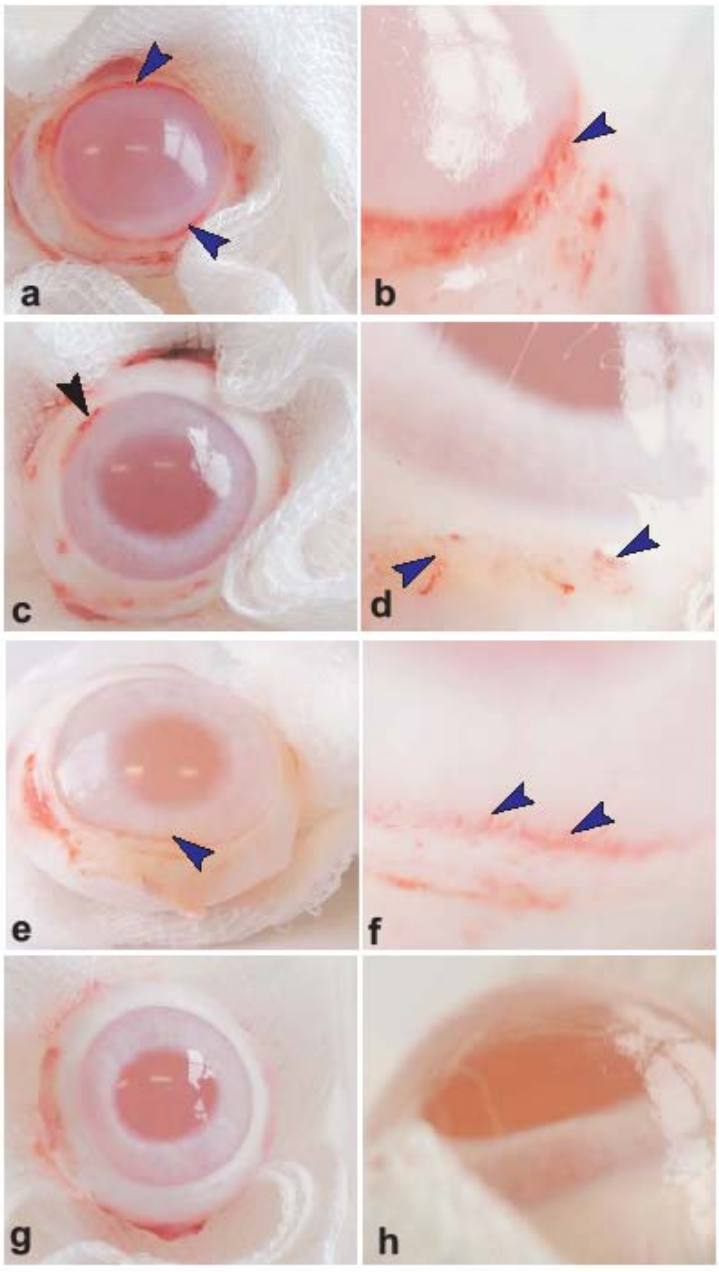


Table 1: *Statistical evaluation of transmittance data depicted in figure 2, figure 4 and figure 6 A for three wavelengths selected from the UV region. Results obtained with actinoquinol-hyaluronic acid were compared with those obtained with saline by means of the Mann-Whitney test (non-parametric t-test). NS... not significant, * ... p<0.05, ** ... p<0.01 ,***... p<0.001.*

Condition	*Wave-length*	*Transmittance*		*Statistical significance* Mann-Whitney test
		Actinoquinol-hyaluronic acid	*Buffered saline*	
No UVB	312 nm	52.15 ± 6.23 (6)	53.62 ± 2.91 (6)	NS
	320 nm	61.93 ± 5.66 (6)	64.00 ± 3.10 (6)	NS
	350 nm	75.74 ± 4.77 (6)	77.99 ± 2.67 (6)	NS
UVB 0.5 J/cm^2	312 nm	24.68 ± 12.32 (12)	7.732 ± 3.454 (12)	***
	320 nm	29.68 ± 13.83 (12)	10.45 ± 4.47 (12)	***
	350 nm	44.11 ± 15.91 (12)	20.43 ± 7.20 (12)	***
UVB 1.01 J/cm^2	312 nm	9.946 ± 3.289 (6)	5.355 ± 2.356 (6)	**
	320 nm	13.18 ± 4.22 (6)	7.417 ± 2.869 (6)	**
	350 nm	25.32 ± 7.01 (6)	17.29 ± 4.53 (6)	*

Table 2: *Statistical evaluation of absorbance data depicted in figure 2, figure 4 and figure 6 B for three wavelengths selected from the UV region. Results obtained with actinoquinol-hyaluronic acid were compared with those obtained with saline by means of the Mann-Whitney est (non-parametric t-test). NS... not significant, * ... p<0.05, ** ... p<0.01 ,***... p<0.001.*

Condition	*Wave-length*	*Absorbance*		*Statistical significance* Mann-Whitney test
		Actinoquinol-hyaluronic acid	*Buffered saline*	
No UVB	312 nm	0.2854±0.0521 (6)	0.2712±0.0237 (6)	NS
	320 nm	0.2096±0.0393 (6)	0.1943±0.0210 (6)	NS
	350 nm	0.1214±0.0276 (6)	0.1082±0.0148 (6)	NS
UVB 0.5 J/cm^2	312 nm	0.6656±0.2481 (12)	1.1524±0.2029 (12)	***
	320 nm	0.5784±0.2331 (12)	1.0193±0.1995 (12)	***
	350 nm	0.3871±0.1847 (12)	0.7188±0.1775 (12)	***
UVB 1.01 J/cm^2	312 nm	1.0198±0.1308 (6)	1.3199±0.2463 (6)	**
	320 nm	0.8962±0.1247 (6)	1.1674±0.2158 (6)	**
	350 nm	0.6090±0.1106 (6)	0.7781±0.1368 (6)	*

Table 3*: Statistical evaluation of absorbance data depicted in figure 8 A for three wavelengths selected from the UV region. Results obtained with actinoquinol-hyaluronic acid were compared with those obtained with hyaluronic acid by means of the Mann-Whitney test (non-parametric t-test) ***... p<0.001.*

Table 3

Condition	*Wave-length*	*Transmittance*		*Statistical significance* Mann-Whitney test
		Actinoquinol-hyaluronic acid	*Hyaluronic acid*	
UVB 0.5 J/cm^2	312 nm	24.52 ± 11.33 (6)	11.580± 4.430 (6)	***
	320 nm	29.72 ± 12.82 (6)	14.84 ± 5.123 (6)	***
	350 nm	43.89 ± 15.84 (6)	24.486± 6.423 (6)	***

Table 4: *Statistical evaluation of absorbance data depicted in figure 8 B for three wavelengths selected from the UV region. Results obtained with actinoquinol-hyaluronic acid were compared with those obtained with hyaluronic acid by means of the Mann-Whitney test (non-parametric t-test) ***... p<0.001.*

Table 4

Condition	*Wave-length*	*Absorbance*		*Statistical significance* Mann-Whitney test
		Actinoquinol-hyaluronic acid	*Hyaluronic acid*	
UVB 0.5 J/cm^2	312 nm	0.6554±0.2385 (6)	0.9773±0.2326 (6)	***
	320 nm	0.5672±0.2322 (6)	0.8610±0.2043 (6)	***
	350 nm	0.3975±0.1742 (6)	0.6277±0.1411 (6)	***

3.6. Hydration and transparency of the rabbit cornea irradiated with UVB-doses equivalent to 2.5 and 5 hours exposure to UVB rays reaching the human cornea from sunlight (Paper 6)

The aim of this paper was to examine whether UVB dose of 2.5 J/cm^2 or 5.0 J/cm^2 equivalent to 2.5 and 5 hours exposure to UVB rays reaching the human cornea from sunlight evoke changes in corneal optics evaluated by changes in hydration and light absorption.

Results showed that changes in corneal optics appeared after the repeated exposure of the cornea to a UVB dose of 0.25 J/cm^2 and massively increased after the repeated exposure to a UVB dose of 0.5 J/cm^2. The first significant changes in corneal hydration occurred after a single exposure of the cornea to a UVB dose of 0.25 J/cm^2.

Figure 1: *Thickness of rabbit corneas measured by an ultrasonic Pachymeter before irradiation with UVB rays (daily dose of 0.5 J/cm^2 or 0.25 J/cm^2) from day 1 to day 4 and on day 5 before sacrificing the animals (N = 12). The difference between normal and UVB irradiated corneas is statistically significant: after the first UVB dose, measured on day 2 of the experiment (** ... p <0.01), after four daily UVB doses, measured on day five of the experiment (*** ... p <0.001).*

Figure 1

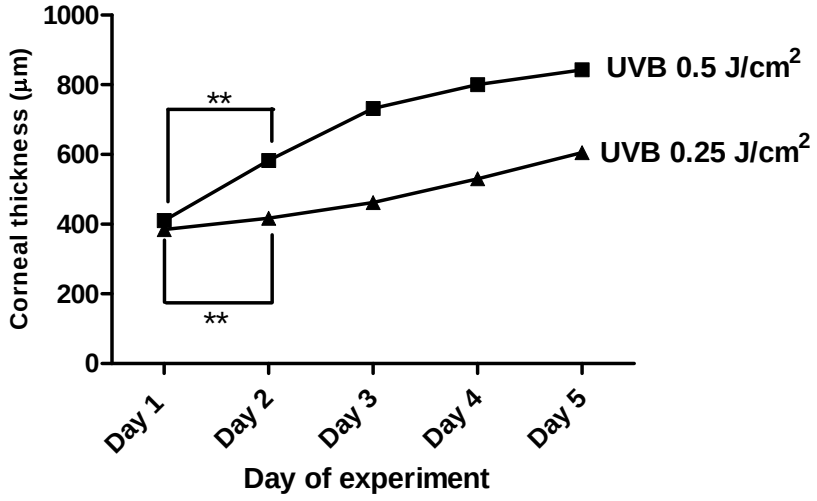


Figure 2: *Averaged absorption spectra of rabbit corneas, expressed as the transmittance* ***T = T (λ)*** *(**A**) or absorbance* ***A = A (λ)*** *(**B**), after 4 days of irradiation with UVB rays at a daily dose of 0.25 J/cm^2, (N=12). The rabbits were sacrificed one day after the end of irradiations (on day five) and the corneas measured spectrophotometrically. The average spectra of irradiated rabbit corneas were compared with the average spectra of normal rabbit corneas (mean from 16 measurements). Note that for wavelengths shorter than about 300 nm, the spectra show the instrumental stray light error rather than the corneal optical properties.*

Figure 2 A

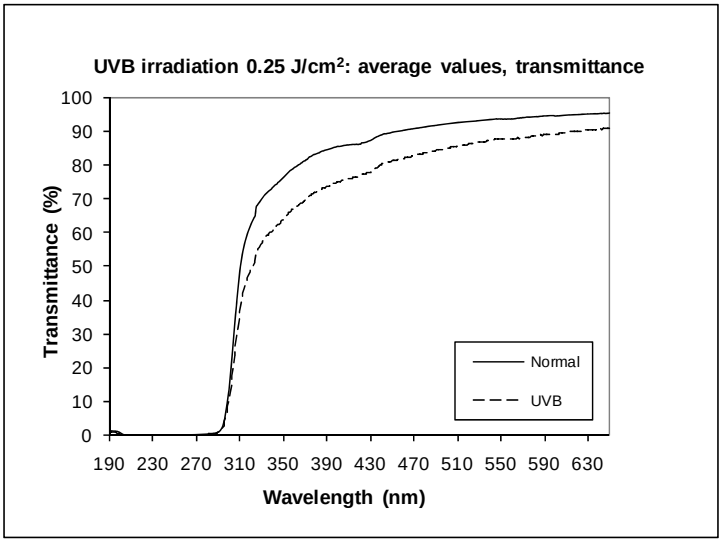


Figure 2 B

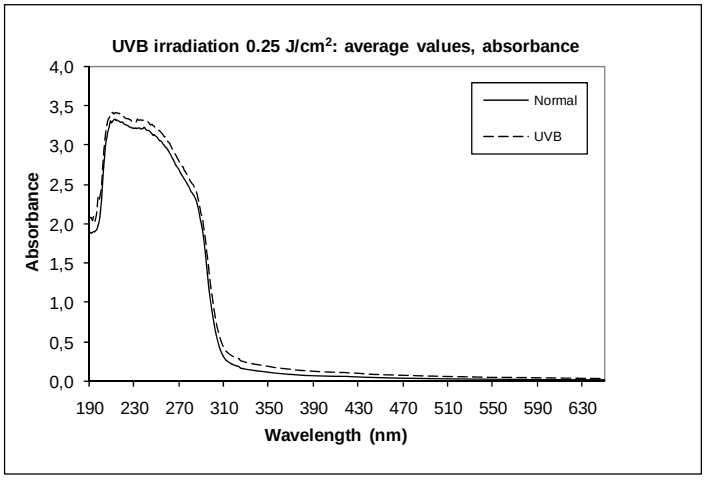


Figure 3: *Statistical evaluation of the transmittance (A) and absorbance data (**B**) depicted in Figure 2 for three wavelengths selected from the UV region. Results obtained with a dose of 0.25 J/cm^2 were compared with those obtained in normal corneas (significant differences, ***... p<0.001).*

Figure 3 A

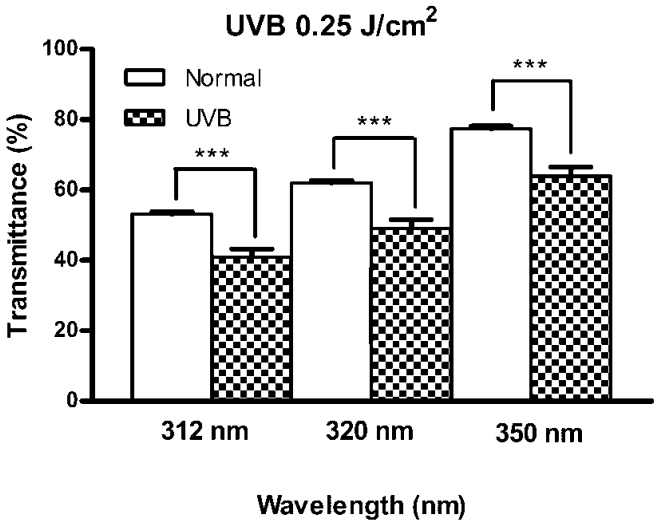


Figure 3 B

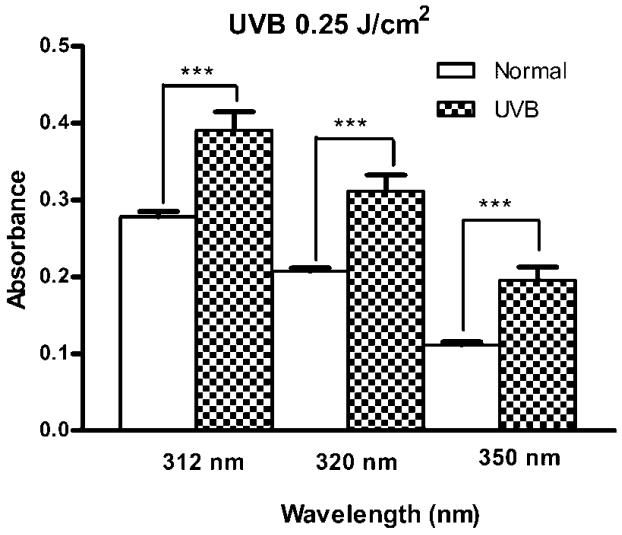


Figure 4: *A : Averaged absorption spectra of rabbit corneas, expressed as the transmittance* ***T = T (λ)*** *(**A**) or absorbance* ***A = A (λ)*** *(**B**), after 4 days of irradiation with UVB rays at a daily dose of 0.5 J/cm^2, (N=12). The rabbits were sacrificed one day after the end of irradiations (on day five) and the corneas measured spectrophotometrically. The average spectra of irradiated rabbit corneas were compared with the average spectra of normal rabbit corneas (mean from 16 measurements). Note that for wavelengths shorter than about 300 nm, the spectra show the instrumental stray light error rather than the corneal optical properties.*

Figure 4 A

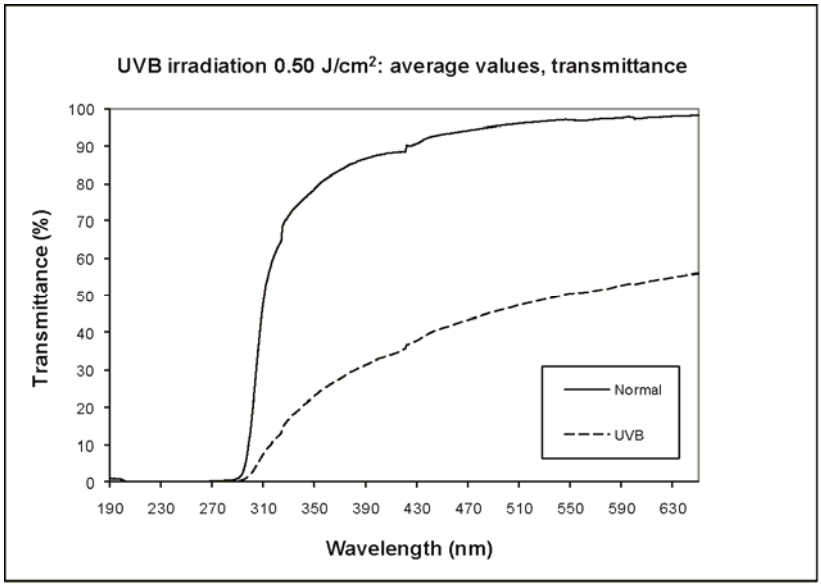


Figure 4 B

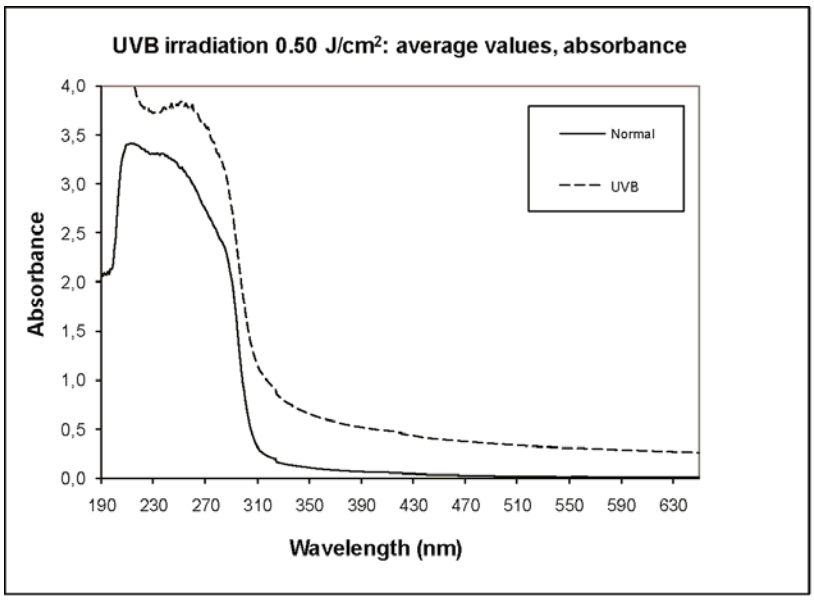


Figure 5: *Statistical evaluation of the transmittance (**A**) and absorbance data (**B**) depicted in Figure 2 for three wavelengths selected from the UV region. Results obtained with a dose of 0.5 J/cm^2 were compared with those obtained in normal corneas (significant differences, ***... p<0.001).*

Figure 5 A

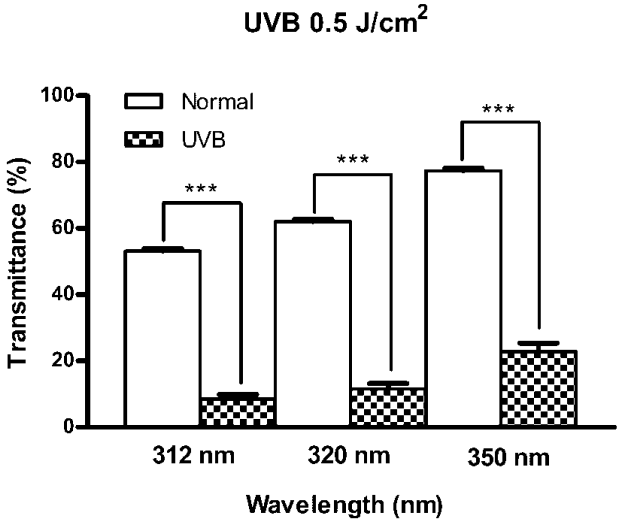
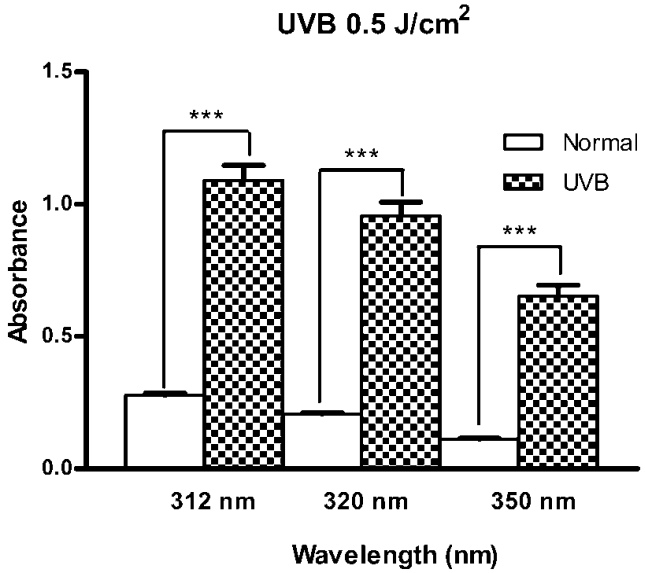


Figure 5 B



4. Summary and Conclusions

As compared to the normal cornea, UVA rays (1.1 J/cm^2 per day, once a day, for 5 days) as well as UVA rays at a two-fold higher dose (2.2 J/cm^2 per day, once a day, for 5 days) do not significantly change corneal light absorption properties. Also, the thickness of corneal centers after irradiation with UVA rays (1.1 J/cm^2 per day or 2.2 J/cm^2 per day) is not significantly changed as compared to normal corneas. Corneal transparency is unchanged after UVA irradiation, the corneas remain undamaged (Paper 3).

In contrast to UVA rays, the cornea repeatedly irradiated with UVB rays (1.01 J/cm^2 per day, once a day during five days) absorbs more light throughout the whole spectrum than the normal cornea. This is due to the increased corneal hydration and later also changes in corneal transparency (the cornea turns grayish) and altered chemical properties of the cornea (slight increase in proteins) (Paper 1, Paper 2). In the latter studies of the optical properties of the UVB-irradiated cornea, two methodological approaches that complemented each other were employed. Spectroscopy of the entire corneal centers provided valuable information about the absorbance of undissolved tissue, but the instrumental stray light error invalidated measurements below 300 nm. On the other hand, dissolution of the corneal tissue in NaOH and suitable dilution extended the spectroscopic analysis down to 210 nm, but some of the tissue´s optical properties could have been lost in the process of dissolution. Both approaches nevertheless showed that the UVB-induced hydration of the rabbit cornea increased the corneal absorption of light from the UVC to the VIS spectral region. This increase in corneal absorbance was still evident when the data were corrected for the hydration-induced change in corneal thickness, which suggested that a change in chemical properties could account for the greater absorption. The spectroscopic analysis of

dissolved corneas conclusively indicated that a change in the optical properties of corneal proteins is one cause. The proteins isolated from hydrated corneas displayed a greater absorption in the UVB-UVC region, most prominently around 280 nm, per number of peptide bonds (which is what the biuret protein assay measures) than the proteins from normal corneas. Even if further studies are necessary in this field, it appears that this difference can be explained by the inflammatory exudation of proteins into the corneal stroma of the UVB irradiated cornea.

Thus, even if the corneas after UVB irradiation are damaged and without any antioxidants (Čejkova et al. 2001, Tessem et al. 2005 a,b) and increased corneal hydration is a pathological event, from the view point of the damaging effect of UVB rays, it might serve as the an emergency mechanism protecting the inner eye against oxidative stress. This mechanism may be important for animals living outdoors and exposed to an increased amount of UVB rays due to the thinned ozone layer or even ozone hole, because changes in corneal hydration and transparency can be restored totally or at least partially and thus serve the vision, whereas oxidative changes of the lens and retina are irreversible and lead to the partial or total loss of vision.

To examine how early the corneal light absorption will be changed after UVB irradiation, the rabbit corneas were irradiated with UVB rays (daily dose 1.01 J/cm^2) once, two times, three times and four times (Paper 4). In this paper it was found that the corneal light absorption was increased very early after the irradiation and the increase of corneal light absorption was gradually more pronounced along with the number of irradiations.

We also examined in the rabbit irradiation model the efficacy of UV filter in eye drops on corneal hydration and light absorption. Results show that UV filter containing actinoquinol combined with hyaluronic acid, effectively protects the rabbit cornea against harmful changes of its optical properties (Paper 5).

Finally, changes of corneal hydration and light absorption were detected after the irradiation of the rabbit cornea with UVB rays equivalent to 2.5 and 5 hrs exposure to UVB rays reaching the human cornea from sunlight. Because changes in corneal hydration and light absorption appeared already after the exposure of the rabbit cornea to a single dose equivalent to 2.5 hours of solar UVB radiation reaching the human cornea from sunlight, the danger for the human eye from even a short stay in sunlight is supposed (Paper 6).

5. Conclusions related to the Hypothesis and Aims of the study.

Our results show that the repeated irradiation of the rabbit cornea with UVB rays significantly increases the value of absorbance A over the whole measurable wavelength λ (beginning UVB through VIS), whereas the repeated irradiation with UVA rays does not bring significant changes in absorbance A (as compared to the normal eye).

Also the values of absorption coefficient α are similarly increased after the repeated UVB irradiation and after UVA irradiation these values are not significantly changed (Paper 1, Paper 4).

The increase in absorbance A (after UVB) is evoked soon after the irradiation due to the increase in corneal hydration (it means physically the increase in corneal thickness), and later - during the irradiation - due to changes in corneal transparency and the slight increase in protein content. In contrast, the changes in values of absorption coefficient α are not dependent on the changes of corneal thickness.

In Paper 1 - Paper 6, following important new approaches and findings were presented:

a) new spectrophotometrical method for the examination of corneal light absorption properties was developed. This method serves for the investigation of quantitative as well as qualitative changes of the cornea causing the changes of corneal light absorption (resp. light transmission).

b) comparing the same doses of UVA or UVB rays, AVA rays do not change corneal light absorption as well as corneal hydration. In contrast, UVB rays evoke harmful changes of corneal optical properties.

c) The irradiation of the cornea with UVB rays increases corneal light absorption which – on one side cause changes in corneal optical properties and on the other side contribute to the protection of the inner eye against the damaging effect of UVB rays which is important for animals living outdoors.

d) UV filter containing actinoquinol combined with hyaluronic acid effectively protects the rabbit cornea against UV rays and changes of corneal light absorption properties.

e) The repeated exposure of the cornea to a UVB dose of 0.25 J/cm^2 equivalent to 2.5 hours of solar UVB rays reaching the human cornea from sunlight, was already sufficient to a significantly changed corneal optical properties in rabbits. From these results it follows that even a short stay in sunlight is potentially dangerous to the human eye and therefore effective protection of the eye against UV rays is necessary. Moreover, because there are striking differences in the severity of changes in corneal optics between one dose of 0.25 J/cm^2 and one of 0.5 J/cm^2, it is necessary to point out that as the time spent in sunlight is prolonged (from 2.5 hours to 5 hours), the danger to the eye from UVB rays dramatically increases.

In conclusion, UVB rays induce reactive oxygen species generation in the cornea. The cornea is damaged, the mechanism maintaining the normal (optimal) corneal hydration is disturbed, cornea swells. The increase in corneal hydration changes the transparency of the cornea due to the increased scatter in the corneal stroma. In the thesis, the new spectrophotometrical method was developed enabling us to measure (quantitatively as well as qualitatively) the light transmission across the cornea. Using this method we described at first in literature that UVB rays decrease the transmission of the light across the cornea dependent on the UVB dose. The results obtained in experimental animals are important because photokeratitis in humans is evoked by UVB rays from sunlight.

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7. Papers and other activities related to PhD thesis

Papers

Paper 1

Čejka Č., Pláteník J., Guryca V., Širc J., Michálek J., Brůnová B., Čejková J.: Light absorption properties of the rabbit cornea repeatedly irradiated with UVB Rays. Photochem Photobiol 83: 652-657, 2007. ***I.F. 2.253***

Paper 2

Čejka Č : Čím se oči zvířat chrání před UV zářením? Vesmír, 87: 364-365, 2008.

Paper 3

Čejka Č., Pláteník J., Buchal R., Guryca V., Širc J., Vejražka M., Crkovská J., Ardan T., Michálek J., Brůnova B., Čejková J. : Effect of two different UVA doses on the rabbit cornea and lens. Photochem Photobiol 85, 794-800, 2009. ***I.F. 2.253***

Paper 4

Čejka Č., Pláteník J., Širc J., Ardan T., Michálek J., Brůnová B., Čejková J.: Changes of corneal optical properties after UVB irradiation investigated spectrophotometrically. Physiol Res 59, 591-597, 2010***. I.F. 1.430***

Paper 5

Čejka Č., Luyckx J., Ardan T., Pláteník J., Širc J., Michálek J., Čejková J.: The effect of actinoquinol with hyaluronic acid in eye drops on the optical properties and oxidative damage of the rabbit cornea irradiated with UVB rays. Photochem Photobiol 86, 1294-1306, 2010. ***I.F***. ***2.253***

Paper 6

Čejka Č., Ardan T., Širc J., Michálek J., Beneš J., Brůnová B., Rosina J.: Hydration and transparency of the rabbit cornea irradiated with UVB-doses of 0.25 J/cm2 and 0.5 J/cm2 compared with equivalent UVB radiation exposure reaching the human cornea from sunlight. Curr Eye Res 36, 607-613, 2011. ***I.F. 1.519***

Patent application

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Grants

Čejka Č, main investigator of grant from Grant Agency of Charles University No. 47/2006

Light absorption in ocular tissues and fluids with special attention to the cornea (Absorpce světla v očních tkáních a tekutinách se zvláštním zřetelem na rohovku)

7.1. Presentations on Conferences (related to the Thesis)

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Papers related to the Thesis