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The effect of single and repeated ultraviolet radiation on the anterior segment of the rabbit eye

Thesis for the degree philosophiae doctor

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Norwegian University of Science and Technology
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Effekt av en enkel og gjentagende UV-stråling på fremre segment i kaninøyet

I løpet av de siste to tiårene har uttynning av ozon i stratosfæren ført til økt ultrafiolett (UV) stråling på landjorda og den kumulative effekten av UV-stråling har fått en økende betydning for UV-induserte øye skader. Epidemiologiske studier kan generelt vurdere kroniske tilstander ved lavdose UV eksponering, mens man ved hjelp av dyreforsøk vanligvis undersøker akutte responser av høyere doser UV-stråling. I og med at observasjonsbetingelsene er forskjellige kan vi ikke uten videre anta at de to ulike eksperimentelle tilstandene nødvendigvis igangsetter de samme nedbrytnings eller reparasjons mekanismer. For å bedre korrelere resultatene både fra epidemiologiske studier og dyreforsøk, trenger man å utføre studier av gjentagende UV-stråling under spesielle eksperimentelle design. Hensikten med dette studiet var å sammenligne metabolske effekter i fremre del av kaninøyer som er eksponert for enkelt eller repetert UV-stråling av totalt den samme UV dosen i begge tilfeller.

Kaniner har blitt eksponert for en singel UV dose (312 nm, 3.12 J/cm^2) eller repeterte UV doser (312 nm, 3 x 1.04 J/cm^2) og prøver av cornea, kammervannet og linsen ble analysert med NMR spektroskopi. Grupperingsmønster mellom prøvene og relativ prosentvis forandringer i spesifikke metabolitter ble evaluert ved hjelp av statistiske analyse verktøy (Principal component analysis, One-way ANOVA, Independent sample t-test).

Det ble observert signifikante forskjeller mellom UV bestrålte og kontroll prøver. Spesielt ble det observert forandringer i antioksidanter (askorbate og GSH), metabolitter relatert til sukkermetabolisme (glukose og laktat), osmolytter (taurin, hypo-taurin, myoinositol, scylloinositol) samt forandringer i choline, fosfocholine og flere aminosyrer. En betydelig tilleggsfaktor ble observert for de repeterte UVB eksponeringene.

For første gang er det utført en sammenligning av metabolske effekter i kaninøyer mellom singel og repetert UV-stråling av totalt den samme UV dosen. Dette studiet viser at det er en kumulativ effekt av repetert UV-stråling i den fremre del av kaninøyet og viser til og med at 48 timers intervall mellom UV-strålingene ikke er nok for at helingsprosessen skal føre tilbake til normal metabolsk status i fremre segment i kaninøyet.

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Summary

Over the last two decades, depletion of stratospheric ozone has increased the flux of ultraviolet radiation (UVR) at the surface of the earth and the cumulative effect of UVR has become an important aspect of UV-induced eye damage. Epidemiological studies generally assess the chronic, low dose UVR exposure conditions while the laboratory animal experiments usually examine the acute response to high dose exposures. Thus, the study conditions are dissimilar and we are not free to assume that the two variant experimental settings necessarily trigger the same damage or repair mechanism. In order to correlate the results obtained from both experimental settings, laboratory studies of repeated UVR exposures under specific experimental design need to be conducted. The purpose of the present study was to focus on the comparison of the effects of single and repeated UVR-B exposures of the same overall doses on the metabolic profile of the anterior segment of the rabbit eye.

Rabbit eyes were exposed to single (312 nm, 3.12 J/cm^2) or repeated (312 nm, 3 x 1.04 J/cm^2) UVB irradiations and corneal, aqueous humour and lenticular samples were analysed by NMR spectroscopy. Special grouping patterns among the tissue samples and the relative percentage changes in particular metabolite concentrations were evaluated using advanced statistical methods (Principal component analysis, One-way ANOVA, Independent sample t-test).

The metabolic profiles of UVB irradiated and control samples were significantly different. Especially, alterations in the concentrations of antioxidants (ascorbate, GSH), compounds related to sugar metabolism (glucose, lactate), osmolytes (taurine, hypo-taurine, myoinositol, scylloinositol), choline-containing compounds (choline, phosphocholine) and amino acids were observed. A substantiall additivity of the repeated UVR-B exposures was revealed.

For the first time, a comparison of the effect of a single and repeated UVR exposure of the same overall dose on the metabolic profile of rabbit eye was conducted and described. This study reveals the cumulative effect of repeated UVB irradiation on the anterior segment of the rabbit eye and shows that even a 48 hours interval between subsequent UVR-B exposures is not sufficient for the healing process to restore normal metabolic status in the anterior segment of the rabbit eye.

List of papers

I. Fris M, Tessem MB, Čejková J, Midelfart A (2006) The effect of single and repeated UVB radiation on the rabbit cornea. Graefes Arch Clin Exp Ophthalmol. 244:1680-1687

II. Fris M, Tessem MB, Čejková J, Midelfart A (2007) Changes in aqueous humour following single or repeated UVB irradiation of rabbit cornea. Graefes Arch Clin Exp Ophthalmol. (DOI - 10.1007/s00417-007-0620-7)

III. Fris M, Čejková J, Midelfart A (2007) The effect of single and repeated UVB radiation on rabbit lens. Submitted to Graefes Arch Clin Exp Ophthalmol.

Additional studies were conducted in order to get experienced with the NMR techniques and statistical approaches used in the present UVR experiments. However, results and conclusions from the previous investigations are not included in the present study.

(IV.) Fris M, Tessem MB, Saether O, Midelfart A (2006) Biochemical changes in selenite cataract model measured by high-resolution MAS H NMR spectroscopy. Acta Ophthalmol Scand 84:684-692

(V.) Fris M, Midelfart A (2007) Postnatal biochemical changes in rat lens: an important factor in cataract models. Curr Eye Res 32:95-103

Abbreviations

ACGIH	American Conference of Governmental Industrial Hygienists
ANOVA	Analysis of Variance
ARVO	Association for Research in Vision and Science
CIE	Commission Internationale de l'Éclairage
COSY	Correlation Spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill (spin-echo pulse sequence)
D	Relaxation delay
FID	Free induction decay
^1H	Proton nucleus
H_2O	Water
H_2O_2	Hydrogen peroxide
HR-MAS	High-resolution magic angle spinning
ICNIRP	International Commission on Non-Ionizing Radiation Protection
J/m^2	Physical unit for the dose (1 kJ/m^2 = 0.1 J/cm^2) (1 J = 1 W×s)
JRES	J-resolved Spectroscopy
MAD	Maximum Acceptable Dose
NMR	Nuclear Magnetic Resonance
O_2^-	Superoxide anion
PC1/PC2	The first principal component/the second principal component
PCA	Principal component analysis
ppm	Parts per million
$R^\cdot$	Free radical species
ROS	Reactive Oxygen Species
T_1	Spin-lattice (longitudinal) relaxation
T_2	Spin-spin (transverse) relaxation
UVB1	First experimental group
UVB2	Second experimental group
UVR-A	Ultraviolet A Radiation (315-400 nm)
UVR-B	Ultraviolet B Radiation (280-315 nm)
UVR-C	Ultraviolet C Radiation (100-280 nm)
UVR	Ultraviolet Radiation
τ	Inter-pulse spacing

List of metabolites

Ace	Acetate
Ala	Alanine
Asc/MDHA/DHA	Ascorbate/Monodehydroascorbate/Dehydroascorbate
ATP/ADP/AMP	Adenosine Triphosphate/Diphosphate/Monophosphate
Bet	Betaine
Cho	Choline
Cit	Citrate
GDP/GTP	Guanosine Diphosphate/Triphosphate
Glu	Glutamate
Gly	Glycine
GPcho	Glycerophosphocholine
GSH/GSSG	Glutathione, reduced/oxidised form
Hcy	Homocysteine
H-tau	Hypo-taurine
IgG	Immunoglobulin G
Lac	Lactate
Mal	Malate
Met	Methionine
Methyl-THF	Methyltetrahydrofolate
M-ins	Myoinositol
NAD	Nicotinamide adenine dinucleotide
NADPH/NADP	Nicotinamide adenine dinucleotide phosphate reduced/oxidized form
Phe	Phenylalanine
Pyr	Pyruvate
Pcho	Phosphocholine
PrSSGs	Protein-glutathione mixed disulfides
PtdCho	Phosphatidylcholine
SAM	S-adenosylmethionine
SM	Sphingomyelin
Sor	Sorbitol
Succ	Succinate
S-ins	Scylloinositol
Tau	Taurine
TSP	sodium-3'-trimethylsilyl-propionate-2,2,3,3-d_4
Val	Valine
α,β-Glc	α,β-glucose

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1 Introduction

1.1 The human eye

1.1.1 Basic and applied anatomy of the eye

The eye is the receptor organ of the visual system. Photons of light entering the eye are focused by the cornea and the accomodative lens onto the retina. The light energy produces changes in the specialized nerve cells in retina, the rods and cones. These changes result in nerve action potentials, which are subsequently relayed to the optic nerve and then to brain, where the information is processed and consciously appreciated as vision.

The eye is situated in the anterior part of the orbital cavity. It is approximately a sphere 2.5 cm in diameter with a volume of 6.5 ml. The eyeball consists of three basic layers. These are the fibrous coat, the uvea or uveal tract and the neural layer (retina). The outer, inelastic fibrous coat, comprising the transparent cornea and the opaque sclera, provides the necessary rigidity of the eye when distended by the intraocular pressure. The middle, vascular coat consists of choroid which is responsible for the nutrition of the outer part of retina, the ciliary body and the iris. The coats surround the contents of the eye, namely the avascular lens and the transparent media (aqueous humour and vitreous body). The eye anatomy with major components is illustrated in Figure 1.

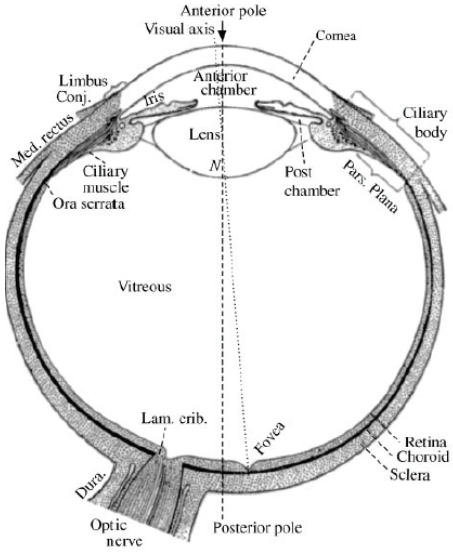

Figure 1 The globe (adapted from Smerdon).[98]

1.2 Structures of the anterior segment of the human eye

1.2.1 The cornea

The cornea is the major light-refractive structure in the eye and constitutes approximately one-fifth of the outer coat of the eye. The most important property of the cornea is its transparency, although due to its highly exposed position it presents also a tough physical barrier to trauma and infection. The corneal transparency is maintained by number of related factors, such as the regularity and smoothness of the covering epithelium, its avascularity and regular arrangement of the extracellular and cellular components in the stroma.[37] Moreover, the fluid-pump mechanism located in the endothelium plays a critical role in maintenance of corneal hydration and in this way also of its transparency.[37]

The normal cornea is smaller in the vertical diameter (10.6 mm) than in horizontal diameter (11.7 mm) and also thicker at the periphery (0.67 mm) than in the centre (0.52 mm). It is composed of five layers: corneal epithelium, Bowman's layer, corneal stroma, Descement's membrane and corneal endothelium. Corneal epithelium is a stratified, squamous nonkeratinized epithelium, 50-60 μm thick, consisting of five or six layers.[37] The outermost layer has microvilli, providing the stabilization of the precorneal film. Bowman's layer consists of fine, randomly arranged, collagen fibrils. The anterior surface is well delineated and is separated from the epithelium by the thin basal lamina, while the posterior boundary merges with stroma. The stroma constitutes 90% of the corneal thickness, containing a highly organised lamellar structure. These are collagen fibrils embedded in a matrix of proteoglycans, proteins and glycoproteins.[77] Between the lamellae lie extremely flattened, modified fibroblasts known as keratocytes. Descement's membrane is an 8-12 μm thin, homogenous, discrete layer between the posterior stroma and the endothelium. It is rich in basement membrane glycoproteins, laminin and type IV collagen. The corneal endothelium is a simple squamous epithelium on the posterior surface of the cornea.

All corneal layers have active metabolism, but the highest activity is in epi- and endothelium. Oxygen and all the important nutrients can reach the cornea from the tear film, limbal blood vessels and aqueous humour,[58] though the

aqueous humour is the main nutritive source. Cornea derives its energy predominantly by carbohydrate metabolism. Glucose is catabolised both via the anaerobic glycolysis, the pentose phosphate pathway and the citric acid cycle. The utilization of the citric acid cycle versus the glycolytic pathway is determined by the energy demands of the tissue. The endothelium has large energy requirements to sustain its pump mechanism and is about five times as active as the epithelium.[37] The constant renewal and desquamation of the epithelial cells requires a continuous supply of amino acids to synthesize the protein that is lost. These are met mainly by diffusion from the aqueous humour.

1.2.2 The aqueous humour

The aqueous humour is a transparent fluid which fills the anterior and the posterior chamber in the anterior segment of the eye and is formed by blood plasma and secreted by the nonpigmented ciliary epithelium. After its secretion into the posterior chamber, it circulates through the pupil into the anterior chamber. The majority of the aqueous humour leaves the anterior chamber through the trabecular meshwork and Schlemm's canal. Balance between formation and drainage of the aqueous humour is responsible for maintaining of the intraocular pressure.

The aqueous humour contains all the essential nutrients for supplying the avascular lens and cornea, and also removes the waste from the tissues (Table 1). It has a very low concentration of proteins which is maintained by

Table 1 Composition of aqueous humour compared with plasma (adapted from Forrester).[37]

Component	Aqueous	Plasma	Units
Glucose	2.7-3.9	5.6-6.4	$mmol/dm^3$
Lactate	4.5	0.5-0.8	
Ascorbate	1.1	0.04	
Albumin	5.5-6.5	3400	mg/dl
Transferrin	1.3-1.7		
Fibronectin	0.25	29	
IgG	3.0	1270	

the blood-aqueous barrier[1] and is crucial for the optical clarity of the fluid.

1.2.3 The lens

The lens is an avascular tissue packed with proteins which provides the refractive index necessary to focus image on the retina. While it has less refractive power than the cornea, the lens has the ability to change shape, under the influence of the ciliary muscle, and thus alter its refractive power. The transparency of the lens is due to the shape, arrangement, internal structure, and biochemistry of the lens cells or lens fibres.[37]

The lens is a biconvex, ellipsoid structure lying behind the iris and in front of the vitreous body. It is held in its position by the zonular fibres which arise from the ciliary processes and attach to the lens capsule at the equator. The lens comprises three parts: the capsule, lens epithelium and lens fibres. The lens capsule is a thickened basement membrane produced by the lens epithelium and lens fibres. It completely envelops the lens and possesses elastic properties important for the accommodation process. Lens epithelium is a single cuboidal epithelium restricted to the anterior surface of the lens. These cells divide and migrate to the equator where they elongate to fibre cells. Each lens fibre is only a 4 x 7-µm hexagonal prismatic band in cross-section, however, it may be up to 12 mm in length. The fibres are meridionally oriented extending the full length of the lens, and converge to anterior and posterior sutures. The continual growth of the lens, by addition of superficial strips of new cells, produces a series of concentrically arranged laminae. The deeper and older lens fibres are anucleate and form the lens nucleus. The outer cortex has a softer consistency than the hard central nucleus.[37]

The avascular lens relies on the aqueous humour as the main nutritive source of oxygen, glucose, and other nutrients needed to support its normal metabolic activity. About 80% of glucose is consumed by the lens via anaerobic glykolysis. The residual glucose may be metabolized via the pentose phosphate pathway (10%), the citric acid cycle and the sorbitol pathway.[37] Enzymes necessary for the two latter metabolic pathways are present mainly in the epithelium which is thus the energetic centre of the lens. The specific lens proteins, crystallins which make up 90% of the water-soluble

proteins of the lenticular tissue are produced by the lens fibre cells. Any perturbation in the lenticular water balance or highly organised arrangement of the crystallins may lead to the defect in the lens clarity. To buffer the effects of oxidants constantly present in the aqueous humour and lens itself, the lenticular tissue, especially the epithelium, contains high levels of glutathione. Glutathione is produced from the interaction between glutamate and cystein in the lens cells and more than 95% of its concentration is in the reduced state.

1.3 Human versus rabbit anterior segment of the eye

1.3.1 The cornea

The anatomy of the rabbit cornea is very similar to the human cornea. Among the few differences, we can mention the corneal thickness (rabbit 0.41 mm; man - 0.52 mm), absence of a distinct Bowman's membrane in the rabbit and larger intercellular spaces among the rabbit endothelial cells.[51] Moreover, the rabbit cornea has been found to be a good model tissue to study human transcorneal penetration of drugs *in vitro.*[124] Chemical composition of the rabbit cornea with respect to the concentration of the low-molecular-weight metabolites is shown in Table 2.

1.3.2 The aqueous humour

The formation and circulation of the rabbit aqueous humour is similar to man. Minor differences are found in the anatomy of the ciliary body resulting in the small modifications in the process of the aqueous drainage. Thus, variation in responses to pharmaceutical agents between human and rabbit may be present.[6] Table 2 shows concentrations of some low-molecular-weight compounds in the rabbit aqueous humour.

1.3.3 The lens

The rabbit lens is not as flat as the human lens, however, the general structure and growth pattern are found to be comparable to the human lenses.[111] Concentrations of some low-molecular-weight compounds in the rabbit lens are shown in Table 2.

Table 2 Composition of the rabbit cornea, the aqueous humour and the lens (adapted from Midelfart and Gribbestad).[45,64,65]

Components [mM]	Cornea	Aqueous	Lens
Alanine	0.59 ± 0.08	-	0.86 ± 0.22
Lactate	4.27 ± 0.66	12.1 ± 1.9	9.12 ± 0.86
Valine	0.11 ± 0.01	0.40 ± 0.012	0.28 ± 0.05
Acetate	0.25 ± 0.02	0.56 ± 0.14	0.16 ± 0.02
Glutamate	0.34 ± 0.04	-	1.93 ± 0.35
Succinate/pyruvate	0.12 ± 0.02	-	-
Hypo-taurine	0.33 ± 0.04	-	-
Choline	0.37 ± 0.05	-	-
Formate	1.27 ± 0.20	-	0.34 ± 0.06
Ascorbate	-	1.42 ± 0.40	-
Citrate	-	0.30 ± 0.13	-
Glucose	-	6.10 ± 1.1	-
3-Hydroxybutyrate	-	0.25 ± 0.053	-
Taurine	-	-	2.07 ± 0.33
Glycine	-	-	0.95 ± 0.11
Scylloinositol	-	-	0.49 ± 0.10
Myoinositol	-	-	3.25 ± 0.29
Tyrosine	-	-	0.23 ± 0.06
Histidine	-	-	0.12 ± 0.03
ATP/ADP	-	-	0.98 ± 0.13
NAD	-	-	0.17 ± 0.05

1.4 Ultraviolet radiation and the eye

Ultraviolet radiation refers to wavelengths from 1-400 nm. The waveband 1 to 100 nm is usually referred to as far UVR or vacuum UVR. According to the Commission Internationale de l'Éclairage (CIE), UVR in the waveband 100 to 400 nm can be divided into types A, B, and C (UVR-A, 315-400 nm; UVR-B, 280-315 nm; UVR-C, 100-280nm). The sun is quantitatively the most important source of UVR, electric and welding arcs being the second largest potential sources. The irradiance of UVR in different wavebands reaching the surface is largely dictated by the temperature of the sun, its distance from the earth and the composition of the atmosphere.[7] As sunlight passes through the atmosphere, all UVR-C wavelengths and approximately 90% of UVR-B are normally absorbed by ozone, water vapour, oxygen and carbon dioxide. The less energetic UVA radiation is not absorbed by ozone and reaches ground level without much attenuation through a clear atmosphere. Therefore, the average UVR reaching the surface of the earth is largely composed of UVR-A (97%) and small component of UVR-B (3%). The level reaching the earth is however strongly influenced by environmental factors such as sun height, season, latitude, altitude, ozone and cloud cover.[123]

1.4.1 Factors affecting the amount of UVR reaching the eye

1.4.1.1 Ozone and UVR

The thickness of the atmospheric ozone is closely related to the intensity of solar UVR-B on the earth. The ozone found in the earth's atmosphere is formed by an interaction between oxygen molecules (O_2), composed of two atoms of oxygen, and UVR. When an oxygen molecule absorbs UVR, the oxygen molecule breaks apart into single atoms of oxygen (Equation 1)

Equation 1

$UVR + O_2 \rightarrow O + O$

These single atoms of oxygen are very reactive, and a single atom combines with a molecule of oxygen to form ozone (O_3), which is composed of 3 atoms of oxygen (Equation 2)

Equation 2

$2O + 2O_2 \rightarrow 2O_3$

Although the ozone layer is spread out from 10 to 50 km in the stratosphere, it is only 3 mm thick when compressed to ground level pressure.[34] Ozone might be destroyed by industrially produced chlorine- or bromine-containing substances such as chlorofluorocarbons. A depletion of stratospheric ozone has over the last two decades been discovered in temperate and polar climate zones.[26,76] The consequence of a depleted amount of the ozone layer is an increased flux of UVR at the surface of the earth, and especially UVB radiation.

1.4.1.2 Environment

The exposure to UVR and visible light constantly changes during the day. When the sun is overhead at noon, the level of UVR at a wavelength of 300 nm is ten times greater than at either three hours before or three hours after local solar noon.[97] Approximately 60% of effective UVR falls on the Earth between the hours of 10:00 AM and 2:00 PM.[34] When the sun is low in the sky, the amount of the UVR dose reaching the Earth is much lower due to a longer atmospheric pathlength. Much more UVR and blue sunlight is scattered, and the sun, which is white at noonday becomes yellow and then orange as less UVR and blue light are present in the direct rays.[97] Similar situation applies for latitude and seasonal effects on the amount of the incident UVR. As the latitude increases, the atmospheric pathlength for the UV rays becomes longer and the total UVR irradiance is lower. Moreover, at each latitude, the maximum intensity is reached in summer and the minimum in winter. The UVR dose reaching the earth is also dependent on the altitude. An increase of 300 m results in an UVR increase by 4%.[34] Clouds do not completely block UVR, but they do influence the spectral composition of the solar radiation reaching ground level. Clouds may serve to scatter and re-distribute UVR to the horizon sky, potentially leading to increased UVR exposure along the line of sight. The eyes may therefore experience a greater UVR dose on an overcast day than on a bright sunny day.[19,73,75,127]

1.4.1.3 Surface

People seldom look directly overhead at the sun when it is very hazardous to view, and the sun is not very hazardous to view when the sun is sufficiently

low in the sky to fall within our field-of-view. When we look straight forward, the field-of-view extends upward from the horizontal only about +10º to +20º on an overcast day, and this angle is further reduced by squinting on a bright day. From this simple analysis, it becomes clear that the geometry of ocular exposure precludes dangerous eye expositions except when the ground reflectance exceeds approximately 15%.[97] Reflectance of UVR-B from various terrain surfaces is shown in Table 3.

1.4.1.4 Personal behaviour

Personal behaviour can have 18-fold impact on the ocular dose of UVR-B, far greater than the 4-fold global difference in the UVB-irradiance by latitude. The ocular dose depends on the amount of time spent outdoors and the use of ocular protection such as brimmed hat, or eye wear (Table 4).

Table 3 Reflectance of ACGIH-effective solar UV-B from terrain surfaces (adapted from Sliney).[97]

Terrain surface	**Diffuse reflectance of ACGIH-weighted solar UV-B [%]**
Green mountain grassland	0.8-1.6
Dry, parched grassland	2-3.7
Wooden boat dock	6.4
Black asphalt	5-9
Concrete pavement	8-12
Atlantic beach sand (dry)	15-18
Atlantic beach sand (wet)	7
Sea foam (surf)	25-30
Aged, ‘dirty’ snow	50
Fresh snow	88

Table 4 Effect of behaviour on relative personal UVR-B exposure (adapted from McCarty).[59]

Behaviour	Relative UVR-B dose [%]
Indoor	4
Outdoor wearing brimmed hat and sunglasses	8
Outdoor wearing sunglasses	17
Outdoor wearing brimmed hat	47
Outdoor with no ocular protection	72

1.4.2 Biological effects of UVR

Ultraviolet radiation has the ability to damage organic molecules such as nucleic acids, proteins and other molecules within the living cells, which absorb UVR and may be structurally altered, cleaved or react with other molecules. Such alterations can cause changes in cell function, mutations or cell death.[123] Additionally, UVR can induce generation of reactive oxygen species (hydrogen peroxide, singlet oxygen and free radicals such as superoxide anions and hydroxyl radicals), agents that present a great danger for biological systems and might cause serious cellular damage.[14]

1.4.3 Defensive system of the eye against the effects of UVR

Ocular tissues and fluids need an effective protecting system against the enhanced UVR-induced oxidative stress. The main natural antioxidative agents include water soluble antioxidants (ascorbate, glutathione), lipid soluble antioxidants (tocopherols, retinols), low-molecular weight UV filters (kynurenine, 3-hydroxykynurenine, 3-hydroxykynurenine *O*-β-D-glucoside), specific enzymes (superoxide dismutase, catalase, glutathione peroxidase and reductase) and metal-binding proteins (transferring, ceruloplasmin and albumins). Figure 2 shows the cooperative function of the particular antioxidative agents and the complexity of the defensive system.

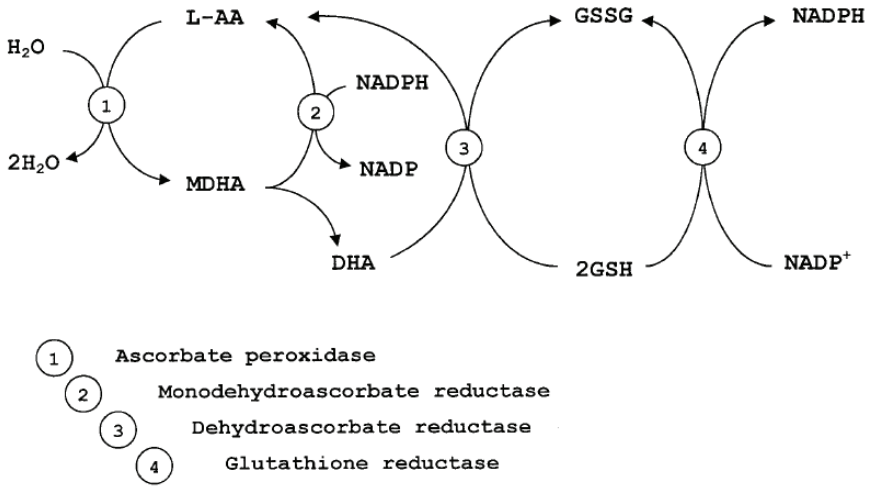

Figure 2 The ascorbate-glutathione cycle. DHA, Dehydroascorbate; GSH/GSSG, Glutathione reduced/oxidised form; H_2O, Water; H_2O_2, Hydrogen peroxide; L-AA, L-ascorbate; MDHA, Monodehydroascorbate; $NADP^+$/NADPH, Nicotinamide adenine dinucleotide phosphate oxidised/reduced form.[25]

Ascorbate is present in high concentrations in the cornea and the aqueous humour and is supposed to be the most effective low-molecular-weight antioxidant in the eye. It can protect the eye by several mechanisms. First of all, ascorbate can scavange the free radicals species in the eye.[93] Additionally, this substance is to large extent responsible for the UVR-absorption and suppression of the protein and tryptophane fluorescence and finally, it can also protect the eye by shifting the high-energy UVR-B into less biotoxic radiation of longer wavelength.[88] As shown in Figure 2, a sufficient amount of ascorbate is sustained mainly by an enzymatic process involving GSH and NADPH.

Glutathione has been found in high concentrations particularly in the lenticular tissue.[93] The redox-couple GSH/GSSG maintains lens protein thiols in the reduced state, protects membrane –SH groups, and is a cofactor in the detoxification of H_2O_2. The enhanced oxidative stress in the ocular tissue might lead also to the formation of the protein-glutathione mixed disulfides. This mechanism is necessary for the inhibition of disulfide-linked light scattering protein aggregate formation.[130] Sufficient concentrations of GSH further plays an important role in the protection of the lens proteins from the modification by UVR filters.[110]

In the primate lens, low-molecular-weight compounds formed by an enzymatic transformation of tryptophane play the role of UVR filters.[120,131,132] These compounds possess an absorption band between 300 and 400 nm. They are characterized by short fluorescence time and low quantum yields of fluorescence, triplet state formation, and active forms of oxygen generation.[32,54,121] Due to such photochemical properties, these compounds protect the retina and the lens itself from the UVR-induced damage.

The protective role of some specific enzymes in the eye is apparent from Figure 2. Superoxide dismutase catalyses dismutation of superoxide to peroxide and molecular oxygen and thus, protects the ocular tissues from the superoxide radicals.[14] Inactivation of superoxide dismutase by hydrogen peroxide is prevented by catalase, an enzyme catalysing the decomposition of hydrogen peroxide to water and oxygen. Another important enzyme scavenging hydrogen peroxide is glutathione peroxidase. The activity of all of these enzymes was previously reported in various eye tissues in rabbit and rat[2,3,8,84] and was found to be crucial for preventing the oxidative damage of the eye.

1.4.4 UVR path through the anterior segment of the eye

When UVR reaches the eye, the proportion absorbed by different structures depends on the wavelength of the radiation. A diagrammatic representation of UVR attenuation in the eye is shown in Figure 3. The cornea absorbs most of the harmful high energy radiation of the wavelengths bellow 300 nm.[89]

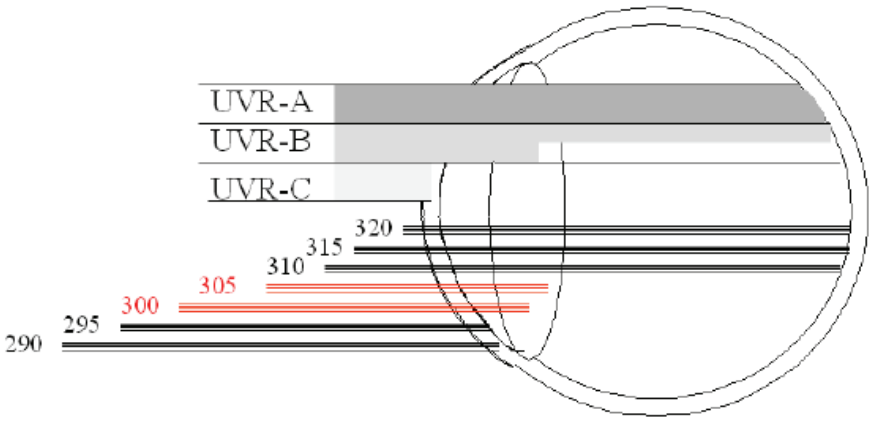

Figure 3 UVR transmission to the eye (adapted from Dong).[34]

A 62-year old human cornea transmits 0% of UVR at 290 nm, 10% at 300 nm, and 63% at 380 nm. Increasing corneal transmittance is seen when comparing human (10%), rabbit (13%), rat (32%) and mouse (37%) samples at 300 nm,[33] indicating that transmittance strongly depends on the corneal thickness. There also is a considerable variability among individuals,[94] and some reports further indicate that transmittance of UVR decreases with age.[9]

The UV radiation penetrating the cornea passes through the aqueous humour where it is partly absorbed. The absorption varies considerably with wavelength from 6-16% of the incoming UVR.[9]

In the lens, transmission of UVR is not only wavelength dependent, but also is age dependent. Absorbance of UVR in the lens increases with age.[9,31,33] The young lens attenuates UVR primarily between 300 and 400 nm, but it exhibits a small window of transmission centred at 320 nm. The aged lens absorbs UVR throughout the entire spectrum and also in the visible region to at least 550 nm.[33] The human lens attenuates almost all the UVR-B and UVR-A that passes through the cornea. This radiation is thus potentially harmful to the lens. Only about ≤1% of UVR-A reaches the retina.[9]

1.4.5 UVR limits for the anterior segment of the eye

The UVR limits are usually expressed in the form of threshold radiant exposure data for the cornea and the lens.[81] Radiation thresholds are generally derived for limited acute (short-term) effects and represent the dose below which there is no significant effect of the radiation on the biological response of interest. Conversely, a dose above the threshold limits always results in a significant response. The action spectra of radiant exposure for corneal and lens thresholds for the pigmented rabbits, based on the slit lamp microscopy examination,[81] are shown in Figure 4.

In order to quantify cataract, Söderberg developed an objective method for measurement of the overall intensity of forward light scattering in the lens and introduced the concept of maximal acceptable dose (MAD).[104,105] Cataract is defined as an opacity in the normal transparent crystalline lens of the eye that impairs normal light transmittance through the lens and may or may not produce an impairment of vision in humans. The concept of MAD is based on the principle that there is a continuous dose-response function for

UVR-induced cataract and the intensity of forward light scattering in the lens continuously increases with a higher dose.[63] The concept of the estimation of the MAD is shown in Figure 5.

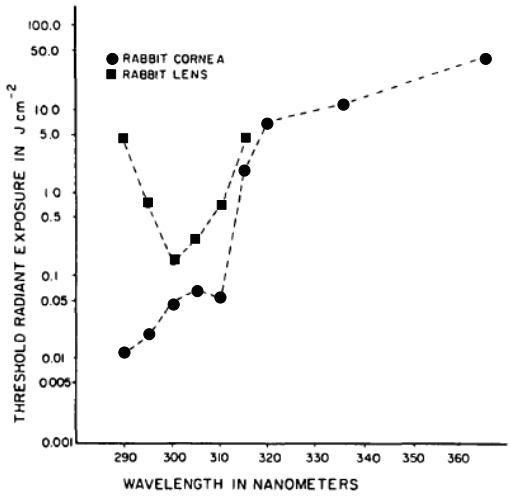

Figure 4 The action spectra of radiant exposure for corneal and lens thresholds for the rabbit. The symbols are as follows: dashed lines represent the rabbit cornea threshold (●); rabbit lens thresholds are represented by (■). The rabbit lens threshold is reversible damage (adapted from Pitts).[81]

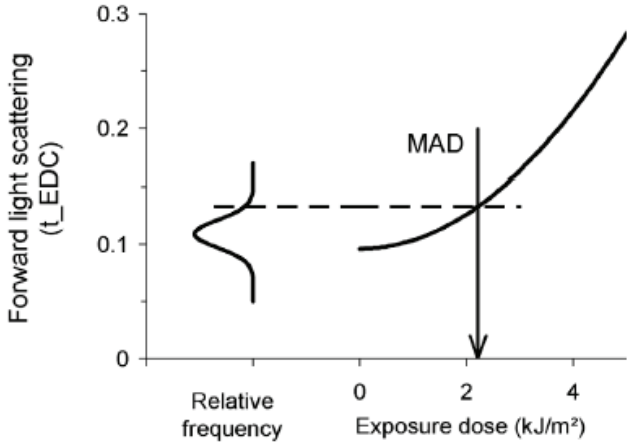

Figure 5 Estimation of $MAD_{0.975}$. The limit for pathological forward light scattering derived from the 20 non-exposed lenses (left: relative frequency) is projected (dashed line) onto the dose–response function from the 20 exposed contralateral lenses of the same animals (right). The intersection gives the MAD, here 2.2 kJ/m^2 (arrow) (adapted from Söderberg).[106]

1.4.6 The effect of acute and chronic ultraviolet radiation exposure on the anterior segment of the eye

When comparing the effects of acute and chronic UVR exposures, one has to be aware of certain dissimilarities in the study design. First of all, it should be remembered that the epidemiological study generally assesses the chronic, low dose UVR exposure condition, while the laboratory study usually examines the acute response to high dose exposures. Thus, the study conditions are dissimilar and we are not free to assume that the two variant experimental settings necessarily trigger the same damage or repair mechanism.[7] Individual case reports and epidemiological studies share a common shortcoming in that there is a little opportunity to control other factors that may contribute to or influence the manifestation of the response evaluated. Therefore, well-controlled animal studies are invaluable in identifying causative factors when studying specific conditions. In order to correlate the conditions of acute and chronic UVR exposures, laboratory studies of repeated UVR exposures of specific experimental design need to be conducted.

1.4.6.1 The cornea

The UV radiation bellow 290 nm is fully absorbed by corneal epithelium and thus, exceeding the UVR threshold radiant corneal exposures results in the most common acute reversible injury, photokeratitis.[126] Early studies described corneal epithelial cell changes and death, and quantified the cellular exfoliation and recovery following a supra-threshold exposure to UVR.[18,22] However, the corneal epithelium regenerates quickly (within 5 days)[41,60] and therefore, this painful condition was generally not regarded as a serious threat to corneal health. The UVR with longer wavelengths (310 nm) may penetrate much deeper, inducing significant damage and cell death among keratocytes (source of stromal collagens and proteoglycans) and endothelial cells. Unlike the epithelial damage, changes in the endothelium are permanent.[126] The alterations in the corneal structure by a supra-threshold UVR has further functional consequences. Endothelial dysfunction leads to fluid imbalance and abnormal corneal hydration, resulting in thickening of the cornea.[18,23,35,87] The inhibition of the endothelium may be caused by increased

permeability or reduced fluid pump function. The UVR exposure is further capable of severe disruption of corneal metabolism. In the previous studies, decrease in corneal oxygen uptake, reduction in phosphocreatine, increase in glucose and elevation in glycogen concentrations were reported.[55,56]

Chronic UVR exposition of the eye, likely to increase with ozone depletion, may be associated with a variety of corneal and conjunctival pathologies, including pterygium (a non-malignant growth on the conjunctiva),[126] pinguecula (a benign degenerative tumour normally seen on the bulbar conjunctiva)[15,109] and keratopathy (degenerative condition of the corneal stroma).[44]

During the last decades, studies comparing the effects of single and repeated UVR doses under specific experimental conditions were conducted, and the cumulative effect of multiple short pulses or repeated long UVR exposures was found as an important factor in assessment of the risks of the corneal UVR damage.[21,82,136,137] Based on biomicroscopic observations of the corneal tissue, two threshold exposures, separated less than 8 hours, produced more extensive damage than a single threshold exposure.[21] Moreover, separation by only 4 hours resulted in a more severe corneal response than that produced by a single double-threshold exposure.[21] The secretion of hyaluronan in the corneal stroma (a compound produced in cells surrounding the damaged tissue) after single and repeated UVR exposures was examined by Podskochy.[82] Fourteen days after the last irradiation, the corneal stroma exposed to the repeated UVR revealed substantially higher deposits of hyaluronan. Such a production and accumulation of hyaluronan may be a sign of long-term changes in the cornea that may lead to corneal haziness and, eventually, to development of corneal degeneration.[82] The alterations in the rabbit corneal metabolism following five repeated doses of UVR-A and UVR-B were examined by Tessem.[114] The UVA irradiation of the rabbit cornea did not result in any alterations of the metabolic profile. However, the dose of 5 x 0.589 J/cm^2 daily, used in the study, was far bellow the threshold corneal dose (42.5 J/cm^2) and changes in the corneal biochemistry could be hardly expected. On the other hand, repeated supra-threshold dose of UVB irradiation resulted in alteration of several metabolites (Figure 6). However,

comparison of the effects of single and repeated supra-threshold doses was not examined in these experiments.

1.4.6.2 The aqueous humour

The aqueous humour is a transparent liquid and thus, alterations caused by acute or chronic UVR exposure has been examined mainly as the changes in its biochemical profile.[100,112] Following severe UVR-induced photokeratitis, cells and protein 'flare' were observed in the anterior chamber of the eye as manifestation of anterior uveitis (inflammation of iris and ciliary body).[81] Structural disorders induced by UV radiation in the iris, ciliary body and trabecular meshwork may result in changes in aqueous humour dynamics and lead to the breakdown of the blood-aqueous barrier.[78] Consequently, the levels of aqueous proteins would be elevated.[78] Furthermore, transport of the low-molecular-weighted compounds down their concentration would increase. It concerns particularly glucose[112] and lactate concentrations. Other possible pathways capable of inducing changes in the metabolic profile of aqueous humour might be a direct photochemical reaction with absorptive agents (ascorbate, amino acids, GSH) or perturbations in the metabolism of the surrounding tissues. Alterations in the aqueous humour composition following chronic low dose UVR exposure conditions have yet not been described. Tessem et al[112] recently reported severe alterations in the biochemical profile of aqueous humour subjected to 5 subsequent supra-threshold UVB irradiations. A significant decrease in ascorbate concentration was accompanied by elevation in glucose, betaine, formate, valine and isoleucine contents. Reversely, exposure to 5 sub-threshold UVR-A under the same experimental design did not cause any significant changes in the aqueous biochemistry.[112]

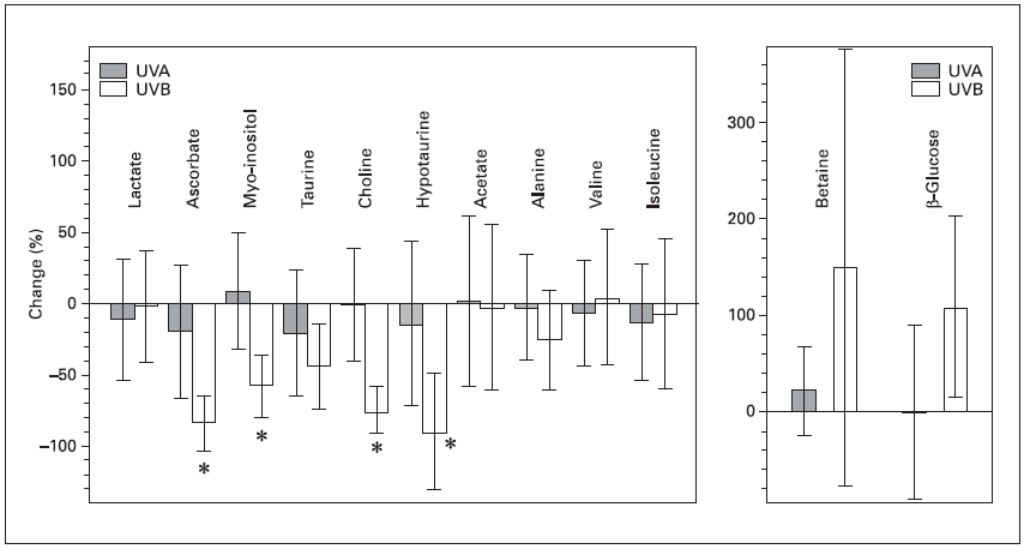

Figure 6 Changes in metabolite concentrations (%) between exposed (UVA and UVB) and non-exposed corneas. The error bars represent 95% confidence intervals for the mean difference. * $p < 0.05$: significant both for the UVB group compared to the control group, and the UVB group compared to the UVA group. No significant differences were detected in the UVA group versus the control group (adapted from Tessem).[114]

1.4.6.3 The lens

The effects of acute and chronic UVR exposures of the lens are usually assessed in the form of an increased level of lenticular opacity, a clinical syndrome known as cataract. Cataract is the leading cause of blindness in the world today[10] and the epidemiological studies have shown that the high prevalence of cataract is strongly associated with factors increasing the amount of daily ocular exposure (environment – high altitude, equator, summer; surface reflectance – fresh snow; personal behaviour – outdoor workers).[7]

Lenticular damage criteria induced by acute UVR ocular dose were established by Pitts.[81] Using a slit lamp microscopy, Pitts observed a loss or reduction of 'orange peel' appearance of the anterior capsule and an increased prominence of the vertical anterior suture line, as the first biomicroscopic signs of lenticular damage. As the radiant exposure approached the threshold lenticular dose, many small, discrete white dots appeared in the anterior subcapsular epithelium of the lens. Following the supra-threshold exposures, the fine discrete opacities coalesced and migrated posteriorly into the anterior cortex of the lens. At the same time, an increase in

the anterior lens cortical haze was detected. The opacities became permanent only when at least double-threshold lenticular UVR doses were used.[81] Microscopically, the cortical opacities correspond to swelling of lens epithelial cells and cortical fibres until they rupture and thus caused vacuolization of the cortical area.[9,20,24,46,49,102,103,107,108,116,134,135] The swelling has been associated with a transient increase of lens water[102] which is related to the impairment of the energy-dependent Na^+-K^+ ATPase, responsible for maintenance of the Na^+-K^+ balance over lens cells membranes.[116]

Recently, Söderberg developed a new method for cataract quantification, based on the measurement of the overall intensity of forward light scattering in the lens.[104] This method ignores the location of the cataract. Applying this method, it was demonstrated that the intensity of forward light scattering in the lens continuously increases with a higher UVR dose.[63,91] Moreover, it was shown that young rats are more sensitive to UVR-B than old rats and that there is no difference in sensitivity to UVR with regard to sex.[57] Investigation of the effects of variations in the exposure time at an equivalent *in vivo* dose of UVR revealed, that exposures around 15 minutes provoke more light scattering than shorter or longer exposures.[5] Risa et al demonstrated that the UVR impact on the metabolic profile of rat lens does not follow the same relationship as the development of light scattering.[90] After a single threshold UVR exposure, the light scattering peaked at 25 hours post exposure, however, most significant changes in the endogenous metabolites were observed after 125 hours. Thus, an apparent delay between the formation of lens opacity and alterations in metabolic profile of UV irradiated rat lenses was observed.[90]

The alterations in the metabolic profile of rabbit and rat lenses under different experimental designs were previously investigated in our laboratory in cooperation with Swedish and Czech research groups.[90,91,100,113,114] Risa et al revealed a significant decrease in the concentration of rat lens low-molecular-weight compounds following a single UVR-B exposure.[91] However, even the light scattering of the rat lens rose with increasing UVR-B dose (Figure 7), no concomitant dose response in the metabolic profile was found (Figure 8). Tessem et al investigated the differences in the level of various metabolites in particular lenticular segments of the rat lens (anterior and posterior cortex,

equator, nucleus) and their response to the UVR damage[113]. The UVB irradiation led to reduction of several compounds especially in the anterior cortex and decreased the natural variance in metabolite concentration among the various lens compartments. Surprisingly, studies exploring the alterations in the rabbit lenticular metabolism following five repeated doses of UVR-A and UVR-B did not show any significant changes, while the corneal metabolic profile was significantly altered by UVR-B.[114] Combination of the UVR-B exposure with a long-term steroid treatment resulted in GSH, taurine and myoinositol depletion and a concomitant elevation in glucose and sorbitol concentrations.[100] UVR induced cataract after single exposure has been extensively studied,[5,57,63,81,90,91,101,104] however, scarce information were found related to lens damage after repeated UV irradiation. Previously, repeated UVR exposures with interval less than 24 hours have been reported to have an additive effect on the lenticular tissue.[36] Moreover, Ayala et al introduced a novel nomenclature showing that the effect of repeated exposures could add together in different ways.[4] There could be pure additivity, when the resultant effect is mathematical sum of effects of the exposures (1+1=2), synergistic additivity (1+1=3), or partial additivity (1+1=1.5). In the light scattering study,[4] pure additivity was found when the interval between two threshold UVR exposures did not exceed 24 hours. Increasing the time interval between separate exposures, the lens opacity rose with a peak at three days separation. Thus, clear synergistic additivity was observed. For time intervals between exposures from 3 days to 1 month, the damage decreased inversely proportionally to the time separation between exposures.[4]

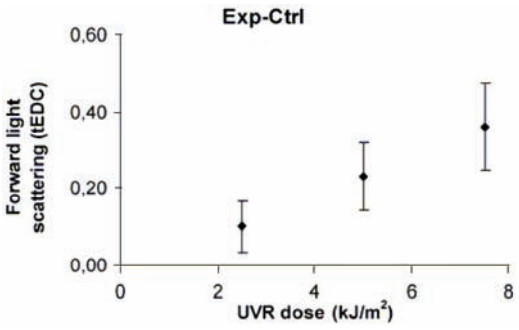

Figure 7 Difference in intensity of forward light-scattering between exposed and nonexposed rat lenses 1 week after a UV dose of 2.5 (*n* = 8), 5.0 (*n* = 11), or 7.5 (*n* = 8) kJ/m^2. The bars represent 95% confidence intervals for the paired-sample mean differences. $_t$EDC represents the transformed equivalent diazepam concentration (adapted from Risa).[91]

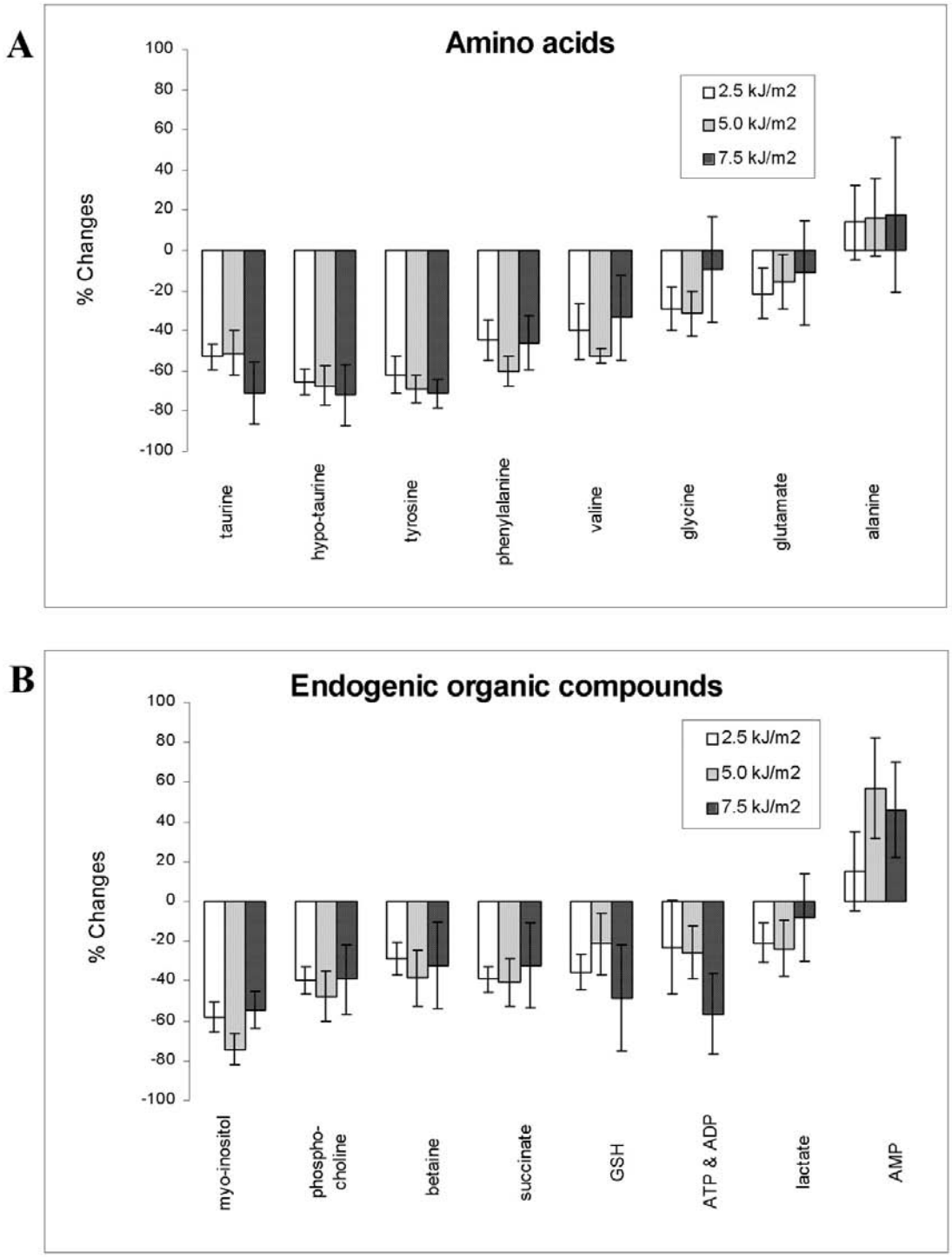

Figure 8 Relative differences in metabolite concentrations between exposed and nonexposed contralateral rat lenses 1 week after a UVB dose of 2.5 (n = 7), 5.0 (n = 8), and 7.5 (n = 6) kJ/m^2, respectively. Data were calculated as (exposed lens – control lens)/control lens. (**A**) Relative changes of detectable amino acids in the NMR spectra. (**B**) Relative changes of other quantifiable metabolites visible in the NMR spectra. The bars represent 95% confidence intervals for the mean differences. ATP/ADP/AMP, Adenosine triphosphate/diphosphate/monophosphate (adapted from Risa).[91]

2 Aims of the study

The aims of the present study were to:

- Utilize HR-MAS ^{1}H NMR spectroscopy as a non-destructive analytical method in order to investigate the alterations in the metabolic profiles of UVR-B exposed corneal and lenticular tissues
- Improve the assignment and quantification of the ^{1}H NMR spectra of rabbit aqueous humour with the help of Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse sequence
- Compare the effects of single and repeated UVB irradiations of the same overall doses on the metabolic profiles of rabbit cornea, aqueous humour and lens
- Investigate possible damaging mechanisms responsible for the alterations in the biochemical profiles of UVR-B exposed structures of the anterior segment of the rabbit eye

3 Methods

3.1 Experimental animals

All the animal investigations conformed to National Institutes of Health Guidelines on the Care and Use of Laboratory Animals in Research and to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. As the experimental animals, adult New Zealand white rabbits (3.0-5.0 kg) were chosen. The experiments were performed in the laboratory of our collaborators at the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague.

3.2 Exposure to UVR

3.2.1 UV lamp

As the UVB source, a 6-W mercury arc lamp (Bioblock Scientific, Illkirch Cedex, France; 312 nm wavelength) was used. The irradiance peaked at 312 nm (Figure 9) and was quantified with a radiometer (VLX-3W; Cole-Parmer, Vernon Hills, IL, USA) equipped with UVB sensor 312 nm.

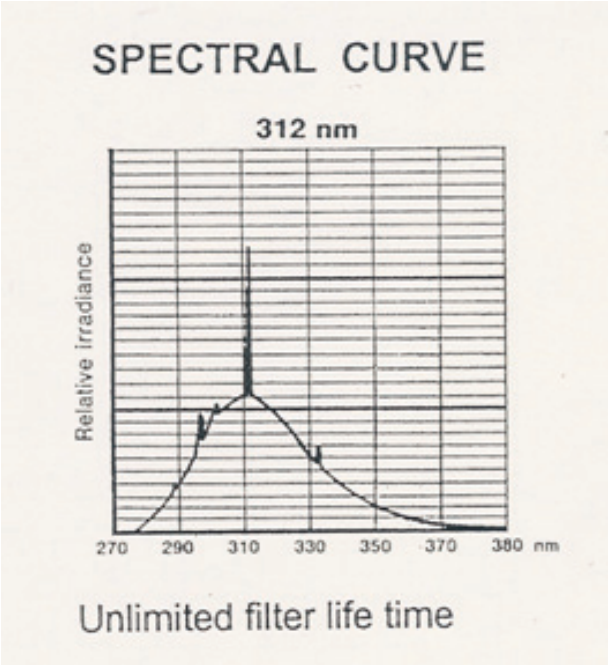

Figure 9 Spectral distribution of the radiation used in the present study.

3.2.2 Ocular exposure

The adult New Zealand white rabbits were divided into three groups of four animals. The first two groups were exposed to UVB radiation, while the third served as an untreated control group. Before the UVR exposure, the animals were intramuscularly anesthetized (2% Xylazinum hydrochloricum, Rotemar, 0.2 ml/kg, and 5% Ketaminum hydrochloricum, Narkamon, 1 ml/kg; Spofa, Prague, Czech Republic). Both eyes of the treated animals were exposed to UVB irradiation from a distance of 0.05m. Only the corneal surface was exposed to UV rays and the rest of the eye was protected. In the first group (UVB1), the animals were irradiated with a single dose 3.12 J/cm^2 (21 minutes) of UVB radiation reaching the cornea. Rabbits in the second group (UVB2) were three times irradiated for 7 minutes every second day (dose of 1.04 J/cm^2; days 1, 3, 5) to give the same overall dose (3.12 J/cm^2). All the experimental animals were sacrificed using intravenous thiopental anaesthesia (thiopentalum natricum, Spofa, Prague) one day after the last treatment (UVB1, UVB2), or on day 3 (control animals). After the animals were killed, the eyes were enucleated. The samples of aqueous humour were aspirated and the cornea and the lens were dissected free from the remnants of surrounding tissues. Finally, the samples were frozen and stored at -80 ˚C before NMR spectroscopy.

3.3 The NMR spectroscopy

3.3.1 NMR basics

NMR spectroscopy exploit the magnetic properties exhibited by nuclei with nuclear spin (I≠0) when placed in a uniform magnetic field. The nuclear spins are then oriented in 2I+1 different energy levels by equilibrium processes, and a radio frequency energy is applied to induce transmission between the different energy states. When the excited nuclei return to equilibrium via longitudinal (T_1) and transversal (T_2) relaxation processes, the NMR signal is observable as a free induction decay (FID). This time dependent decay is acquired and Fourier transformed into a frequency dependent spectrum. The appearance of a specific peak depends on the molecular environments of the originating nuclei and physical, chemical, and biological properties of the

studied sample can be revealed from the NMR spectra.[27]

3.3.2 CPMG spin echo pulse sequence

The spectral baseline might be to a large degree influenced by signals from macromolecules as proteins, lipids etc. In order to attenuate these signals, spectral editing techniques can be utilized. In the present study, CPMG spin echo pulse sequence[61] was used to attenuate resonances with relatively short T_2 relaxation times and to enhance the signals from low-molecular-weight metabolites (Figure 10).

3.3.3 HR-MAS ^{1}H NMR spectroscopy

HR-MAS ^{1}H NMR spectroscopy is a novel method, which is potentially bridging the divide between ^{1}H NMR spectroscopy of tissue extracts and *in vivo* NMR spectroscopy.[16,17,71,74] Avoiding the major drawbacks of these two techniques, especially extraction procedures in ^{1}H NMR spectroscopy and low peak resolution in *in vivo* NMR spectroscopy, this method is nowadays preferably used to produce high resolution spectra of small unprocessed tissues samples and intact cells.

By spinning the sample at a rate of rotation comparable to the NMR line-width of the material in static conditions (typically 4000-5000 Hz) ,and at the magic angle θ = 54.7º with respect to the direction of the static magnetic field, the normally broad lines become narrower, increasing the resolution for better identification and analysis of the spectrum. The major line-broadening factors in *in vivo* NMR spectroscopy are a result of dipole-dipole interactions, chemical shift anisotropy and magnetic field inhomogeneities. The chemical shift anisotropies and dipolar couplings have an angular dependence of $(3\cos^2\theta - 1)$, where $3\cos^2(54.7º) - 1 = 0$. Therefore, the development of HR-MAS solves the problem of line-broadening by averaging these factors to zero. In this study, HR-MAS ^{1}H NMR spectroscopy was used for the analysis of rabbit cornea and lens. The spinning rate of the zirconia 4-mm diameter HR-MAS rotor was set to 5000 Hz.

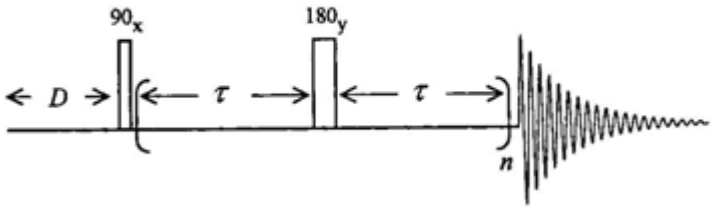

Figure 10 The CPMG pulse sequence (Carr-Purcell-Meiboom-Gill). After a 90^{o}_{x} excitation pulse, refocusing 180^{o}_{y} pulses are repeated *n* times with inter-pulse spacing 2τ. *D*, relaxation delay (adapted from Sæther).[99]

3.3.4 Assignment of the metabolites in the NMR spectra

Because of the complexity of the NMR spectra in the present study (large amount of peaks, spectral overlap), both one- and two-dimensional (2D) ^{1}H NMR techniques in combination with previously reported data[39,40,45,64,65,90,91,100,101,112-114] were used to assign the metabolites. A 2D NMR experiment involves a series of one-dimensional experiments. Each experiment consists of a sequence of radio frequency pulses with delay periods in between them. It is the timing, frequencies, and intensities of these pulses that distinguish different NMR experiments from one another. During some of the delays, the nuclear spins are allowed to freely precess (rotate) for a determined length of time known as the evolution time. The frequencies of the nuclei are detected after the final pulse. By incrementing the evolution time in successive experiments, a two-dimensional data set is generated from a series of one-dimensional experiments. In the present experiment, homonuclear correlated spectroscopy (^{1}H-^{1}H-COSY) and J-resolved spectroscopy (JRES) was used to assign complicated coupling patterns.

3.3.5 Quantification

There are several approaches for quantification of the NMR data.[16,69,91,101,112,115] However, in the recent ophthalmic NMR studies, absolute quantification method using a signal of sodium-3'-trimethylsilyl-propionate-2,2,3,3-d_4 (TSP) as an internal standard[45,64-68,112] and a relative quantification procedure established for the ophthalmologic experiments by Sæther,[101] were the most commonly used quantification concepts.

TSP is generally used as a chemical shift reference compound (δ=0) in biomedical ^{1}H NMR spectroscopy studies.[83,112,125] However, in the HR-MAS ^{1}H NMR experiments, one has to be aware of possible drawbacks when using this compound as an internal quantification standard. First of all, the volume of standard TSP solution added to each sample (cornea, lens) may vary because of variations in the individual volume of the sample. Furthermore, TSP possesses an aliphatic short chain that can bind to serum albumin[53] and thus, binding to similar proteins in the cornea, aqueous humour or lens may occur.

In the present study, the relative quantification method was used. The spectral data were first imported into the software for analysis of complex mixtures (AMIX, MestReC) and then reduced by dividing the spectra in 'buckets'. Each bucket contained absolute signal intensities from given shift range and thus, the peak areas were obtained by summation of the buckets. After correction for sample weights, the peak areas were compared relatively between different groups.

3.4 Statistical analysis

3.4.1 Principal component analysis

A typical experimental ophthalmologic study can generate several ocular samples that can be analyzed by NMR spectroscopy, and hence, several NMR spectra. Examining each spectrum individually can be a daunting exercise even for the trained spectroscopist. Among many statistical tools that have been developed or borrowed from other fields for assessing large number of NMR spectra in a relatively rapid fashion, principle component analysis (PCA) was chosen as an appropriate approach in the present study. PCA is a well-known and effective method of data compression and transforms the original data (intensity values in spectrum) into set of 'scores' for each sample, measured with respect to the principal component axes ('loadings'). The principal component (PC) scores replace the original variables, and are ordered, with successive PCs accounting for decreasing amounts of variance, and orthogonal, with no correlation between the scores on different axis. Thus, the first principal component explains the greatest variability in the data, the second principal component is independent on the

first component and second best explains the variability of the data and so on. Due to these properties, a small number of PCs can replace the many original variables without much loss of information. The results of this procedure are usually represented in the form of a two- or three-dimensional score plots, where each point represents all the data contained in one spectrum. Sample points that cluster together have more similar spectra (and hence more similar biochemical make-up) than samples that cluster apart. PCA plots are extremely powerful for rapid identification of inherent clusters in the data (which may be suggestive of a common effect or mechanism), assessment of dose-related and time-related changes, and the identification of individual outliers. However, the score plot itself adds little to biomarker identification and says nothing about the alterations in the metabolic profile on a molecular basis. The PCA data can be examined in more detail by examining the loadings to find out which variable relationships are responsible for the loadings. Thus, another important graphic presentation, the loading profile, displays the importance of each metabolite for the variation described by the PCs.

3.4.2 Quantitative statistical analysis

In the present study, relative quantification method was performed using absolute peak integrals normalised by sample weight. Percentage alterations in the metabolite concentrations in the ocular samples (cornea, aqueous humour, lens) of UVR-B exposed albino rabbits were calculated relative to the levels in the control group. Mean values of the relative differences were expressed with 95% confidence intervals, calculated according to Fowler et al.[38] Spectral data from the ocular samples were further analysed by Independent sample t-test and One-way ANOVA followed by Bonferroni multiple comparison test, in order to assign metabolites, significantly differing among the experimental groups ($P < 0.05$).

4 Results and discussion

4.1 Experimental animals

The human eye is in many respects different from the rabbit eye, however, compared to the other common laboratory species, rabbit represents one of the best experimental models. The anatomy of the anterior segment of the rabbit eye shows in many respects similarity to the humans. Furthermore, analogous to man, rabbit is a diurnal animal and thus, corneal and aqueous humour concentrations of ascorbate, the main protective agent against UVR, are similar. The main drawback of the design of the present study is the use of albino animals. The uveal levels of melanin are lower than in pigmented eyes and thus, a more profound UVR damage of the iritic tissue in the albino eyes might have increased the amount of the penetrating UVR and resulted in higher alterations in the composition of the aqueous humour. For the comparison studies, pigmented animals would be a better choice, however, only albino species were available in the collaborating laboratory.

4.2 Exposure to UVR

The wavelength (312 nm) and the ocular dose (3.12 J/cm^2 and 1.04 J/cm^2) of the UVR used in the present experiments were chosen in relation to available irradiation equipment in the collaborative laboratory in the Czech Republic. Figure 11 shows that approximately 33% of the overall UVR at 312 nm is transmitted by cornea, passes through the aqueous humour and reaches the lens, where the residual radiation is absorbed. Thus, all the structures of the anterior segment of the eye are influenced by the UVR exposure and the comparison of the effect of a single and repeated UV irradiation of the same overall dose can be evaluated in all the anterior compartments of the eye at the same time. As described by Pitts,[81] the UVR-B dose used in the present experiment for the repeated exposure (1.04 J/cm^2) corresponds approximately to the threshold corneal radiant exposure. Additionally, the dose used for single UVB irradiation (3.12 J/cm^2) was previously reported to be capable to induce permanent lenticular opacities. Regarding the irradiation scheme, both eyes of each rabbit were exposed to UVR and both eyes from the untreated

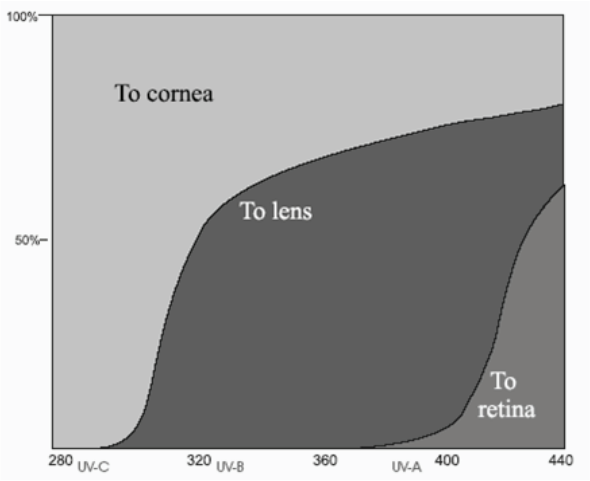

Figure 11 Transmittance of near UV to the lens and retina (adapted from Charman).[15]

rabbits were used as controls. Individual control animals were used to assure that control samples were not affected in any way by the alterations in the fellow eye, as described in former investigations.[29,70]

4.3 The NMR spectroscopy

In the previous studies,[45,64-68,92] extraction procedures were necessary to use prior to the NMR spectroscopy. These procedures required relatively large amounts of biological tissue and were time consuming because of the need for multiple extraction methods to study both lipophobic and lipophilic phases. Furthermore, the extraction of tissue samples is principally destructive in nature and might have an influence on the chemical composition with particular concern regarding the analysis of antioxidants (GSH), osmolytes (taurine, hypo-taurine and myoinositol) and membrane constituents (phosphocholine). However, the employment of the HR-MAS ^{1}H NMR spectroscopy in the present study enabled analysis of intact ocular tissues with no need for prior extraction procedures. Moreover, as shown in Figure 12, the resolution quality of the HR-MAS ^{1}H NMR spectra of the corneal and lenticular samples was comparable with results from the previous NMR studies, based on the extraction procedures.[64-66,92]

Unfortunately, although a high-resolution quality was achieved in the HR-MAS ^{1}H NMR spectra of rabbit lens and cornea, the spectral baselines were to a large degree influenced by signals from macromolecules as proteins and lipids. The HR ^{1}H NMR spectra of rabbit aqueous humour were also significantly altered following a single UVR dose of 3.12 J/cm^2, probably due

to a substantial increase in the protein levels resulting from an inflammatory process (Figure 13). However, as shown in Figure 13, applying the CPMG pulse sequence, resonances of proteins and other macromolecules were attenuated and the spectra could be subsequently assigned and quantified.

Generally, it was possible to identify more than 20 different metabolites in the spectra from the anterior segment of the rabbit eye (Figure 14). Assignment of peaks was based on comparison with previously reported data.[39,40,45,64,65,90,91,100,101,112-114] However, compared to some other analytical techniques (HPLC), the sensitivity of the NMR procedure is considerably lower. Additionally, in some regions of the spectra, high level of peak overlapping was observed and thus, two-dimensional ^{1}H-NMR techniques, homonuclear correlated spectroscopy (^{1}H-^{1}H-COSY) and J-resolved spectroscopy (JRES), were necessary to apply in order to assign particular signals in the spectra (Figure 15).

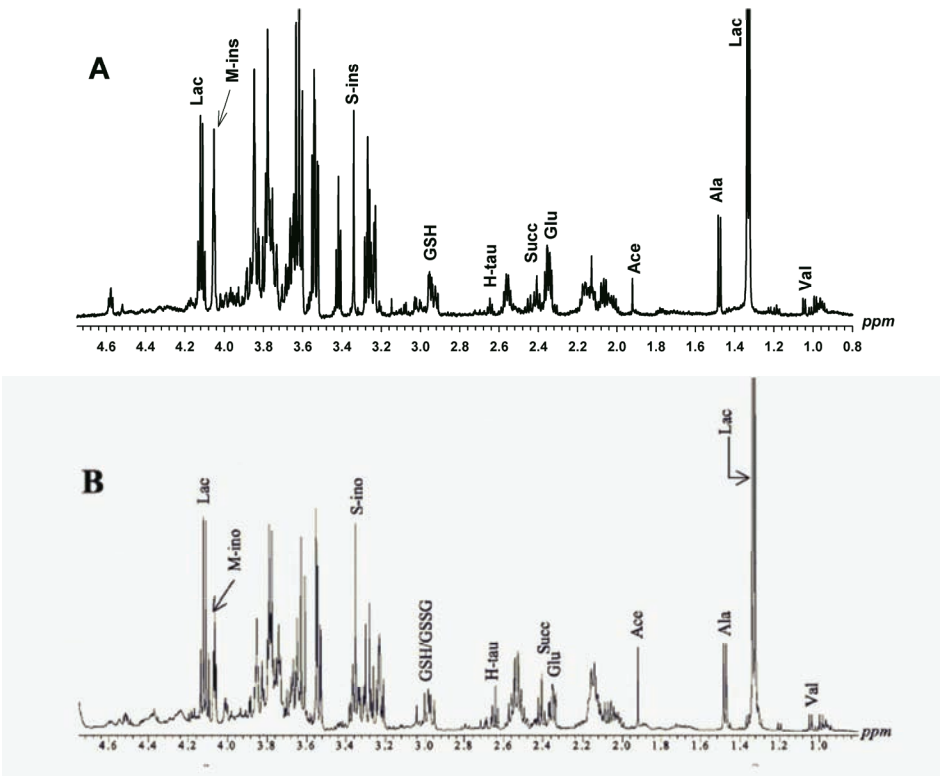

Figure 12 Comparison of the resolution quality of HR- HR-MAS ^{1}H NMR spectra (**A**) and HR ^{1}H NMR spectra (**B**) (adapted from Risa).[92] Ala, alanine; Ace, acetate; Glu, glutamate; GSH/GSSG, glutathione reduced/oxidized form; Lac, lactate; Mal, malate; M-ins, myoinositol; S-ins, scylloinositol; H-tau, hypo-taurine; Val, valine.

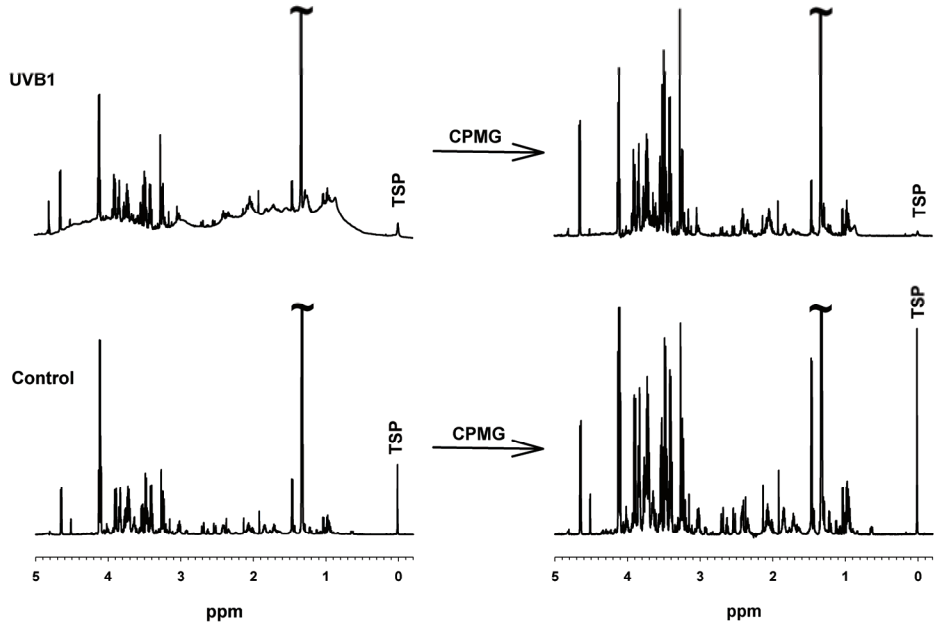

Figure 13 Attenuation of the resonances of macromolecular compounds and enhancement of the signals of low-molecular weight metabolites in rabbit aqueous humour after applying of Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse sequence in the ^{1}H NMR spectroscopy.

In the present study, relative quantification method was used. Following the CPMG spin echo pulse sequence, the resonances of proteins and other macromolecules were successfully attenuated and thus, complicated baseline correction procedures were not necessary to apply. The main drawback of this approach, compared to absolute quantification method, is the form of the obtained results, where the absolute concentrations of particular compounds are missing. In order to obtain absolute concentrations, a reliable standard of known concentration which does not alter the properties of the sample under investigation, or binds to various substances, has to be included. Unfortunately, the most commonly used internal standard in the biomedical NMR studies, TSP, did not prove its reliability in former studies.[53] As mentioned previously,[91] the volume of standard TSP solution added to each sample (cornea, lens) in the HR-MAS ^{1}H NMR spectroscopy may vary because of variations in the individual volume of the sample. Furthermore, TSP possesses an aliphatic short chain that can bind to serum albumin[53] and thus, binding to similar proteins in the cornea, the aqueous humour or the lens

may occur. The latter theory was confirmed in the present experiments. As shown in Figure 13, exposure to a single UVR dose of 3.12 J/cm^2 resulted in a significant increase in protein content accompanied by a concomitant reduction in the TSP signal intensity. Thus, binding of TSP to aqueous humour proteins resulting in its diminished spectral intensity was proved. Recently,[79,80] a new method has been developed, the ERETIC (Electronic Reference To Access In Vivo Concentrations), which can determine absolute concentrations by a electronically generated NMR signal. Thus, application of this novel method should be included in the future ophthalmic HR-MAS ^{1}H NMR experiments and improve the present quantification methods.

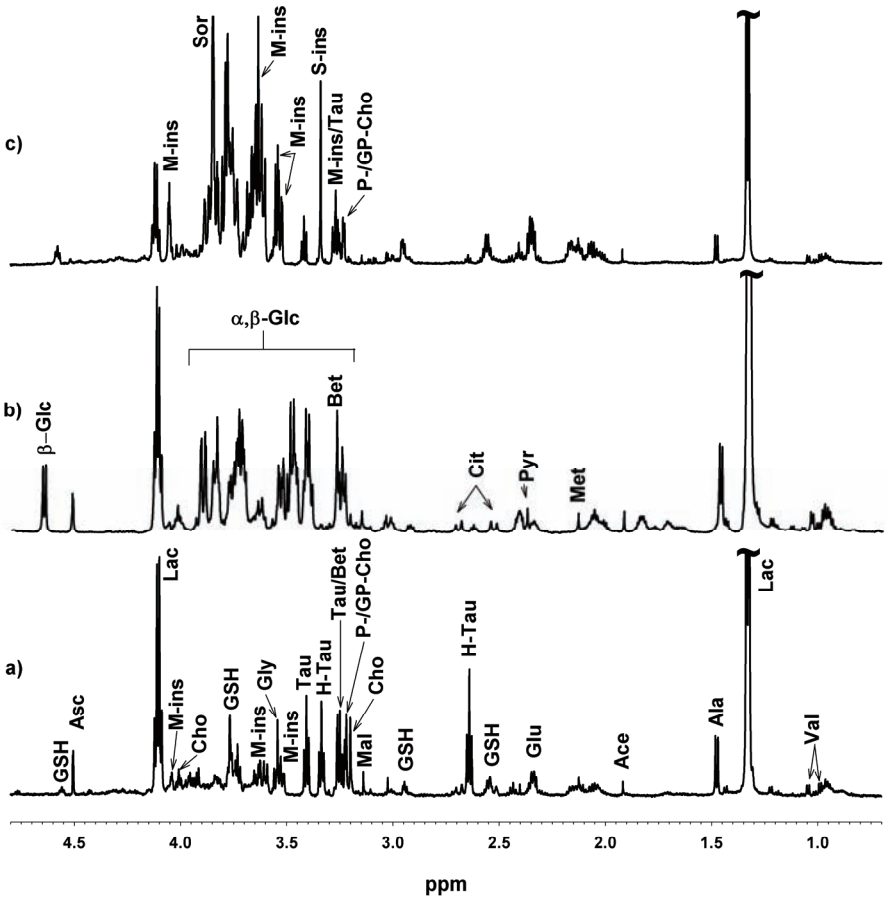

Figure 14 Representative metabolic profile of 600 MHz HR-MAS ^{1}H NMR and HR ^{1}H NMR spectra of control rabbit eye: **(A)** cornea, **(B)** aqueous humour, **(C)** lens. The ppm values are assigned using TSP as reference substance at 0 ppm. Ala, alanine; Ace, acetate; Asc, ascorbate; Bet, betaine; Cho, choline; Cit, citrate; Glu, glutamate; Gly, glycine; GPcho, glycerophosphocholine; GSH, reduced glutathione; H-tau, hypo-taurine; Lac, lactate; Mal, malate; Met, methionine; M-ins, myoinositol; Pcho, phosphocholine; Pyr, pyruvate; Sor, sorbitol; S-ins, scylloinositol; Tau, taurine; Val, valine; α,β-Glc, α,β-glucose.

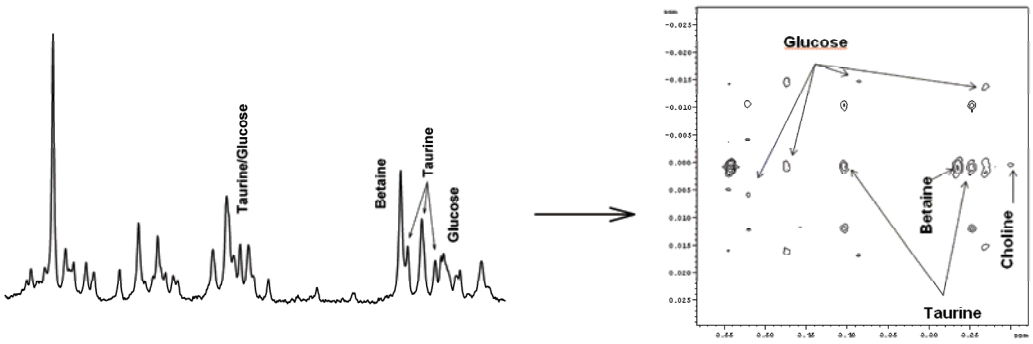

Figure 15 Utilization of J-resolved spectroscopy (JRES) in assignment of complicated coupling patterns in the ^{1}H NMR spectra.

4.4 Statistical analysis

In the present study, application of NMR spectroscopy in connection with advanced statistical methods (PCA, Independent sample t-test, one-way ANOVA and Bonferroni multiple comparison test) was favourably used to extract special grouping patterns among the tissue samples and to evaluate relative percentage changes in particular metabolite concentrations. Principal component analysis was used prior to the quantitative analysis, and was invaluable in giving an overall view of the damaging effect of UVR on the anterior segment of the eye. The score plot showed the characteristic grouping patterns among the experimental samples and additionally helped to assign the outlying samples that were subtracted from the subsequent quantitative statistical analysis. Moreover, the alterations in the metabolic profiles of examined tissues, standing behind the grouping pattern found in the score plot, were showed in the loading profile format. The metabolites dominating in the process of UVR damage could be thus assigned and subjected for further quantitative analysis. The main drawback of the quantitative statistical analysis was the small number of signals that could be investigated. In the present experiments, several metabolites overlapped each other in some regions of the spectra, and for some other compounds, the signal to noise ratio was too low to extract valuable information. Moreover, the results obtained from the quantitative statistical analysis and PCA did not always show a high degree of similarity. Especially the alterations in the

lenticular sorbitol and scylloinositol levels, or aqueous humour glucose concentrations revealed a substantial variation between the two statistical approaches. The reason standing behind this phenomenon might be the small number of experimental animals used in the present study. In spite of the small sample size, the effect of the variations among the individual experimental animals is minimized by the operations used in the PCA, however, it might affect the quantitative statistical analysis results and increase the experimental error. Thus, in the present study, the PCA results were of primary interest, quantitative statistical analysis giving supplementary information about the magnitude of the observed alterations.

4.5 Alterations in the biochemical profile of the anterior segment of the rabbit eye

The changes found in the metabolic profile of the anterior segment of the rabbit eye, following a single and repeated UV irradiation of the same overall dose, are summarised in Figure 16 and 17. It has to be remembered that the results of the present study display the average composition of the particular structures in the anterior segment of the eye. Thus, regarding the cornea, our data reflect alterations in all three tissue layers (epithelium, stroma and endothelium) at the same time. When considering the lens, a small meridional sector containing the whole radial profile with both cortical and nuclear parts of the lens was cut and analyzed. Previously, significant changes in the metabolite content were found both among the separate corneal layers and the particular lenticular compartments.[55,113] Moreover, in the rat lens, even the response to UVR-induced damage revealed substantial spatial variations.[113] Thus, in the future studies on the comparison of the effects of single and repeated UVR exposures, this phenomenon has to be considered and the particular corneal and lenticular compartments should be analyzed separately.
In the present study, the PCA results revealed that cornea was the most vulnerable tissue towards the UVR-induced damage (Figure 16A). The significant shift between the UV irradiated and control corneal samples along the PC1 axis resulted from alterations in contents of several metabolites that are shown in the loading spectra of the first principal component. In the UVR exposed samples, the concentrations of glucose, pyruvate, betaine and

glycine were increased, whilst the contents of taurine, hypo-taurine, choline, acetate, ascorbate, GSH and myoinositol were reduced. There was no apparent grouping pattern between the UVB1 and UVB2 samples except for a slight shift along the PC2 axis. The relative percentage alterations in particular metabolites were subsequently revealed by the quantitative statistics (Figure 17A) and the slight shift along the PC2 axis in the scores of UVR exposed samples was explained as a result of partially stronger effect of the single UVB irradiation.

As shown in Figure 16B,C, the principal component analysis revealed a similar grouping pattern of aqueous humour and lenticular samples following the UVR-B exposure. The samples were shifted mainly along the axis of the first principal component in the score plot representation. Control samples were moved towards low PC1 scores, while the UVB1 group was equally distributed along the zero value and UVB2 samples had the highest PC1 values. Stronger impact of repeated UVR-B exposure on the rabbit aqueous humour and lens was thus evident. However, the lenticular and aqueous humour biochemical profiles are substantially different. In order to explain the observed alterations in the particular metabolites following the UVR exposure of both ocular structures, loading profile of the first principal component was investigated. In the aqueous humour, the highest PC1 scores of UVB2 samples were explained as a result of increased glucose concentration and reduction in ascorbate, GSH, betaine, citrate and alanine content (Figure16B). The similar grouping pattern of the lenticular tissue was interpreted as caused by elevation in sorbitol and glutamate levels and decrease in the concentration of myoinositol, GSH, taurine, glycerophosphocholine, phosphocholine, acetate and alanine (Figure 16C). The percentage alterations in the metabolites both from the aqueous humour and lens confirmed that the effect of repeated UVR exposure is stronger compared to a single irradiation and are shown in Figure 17B,C.

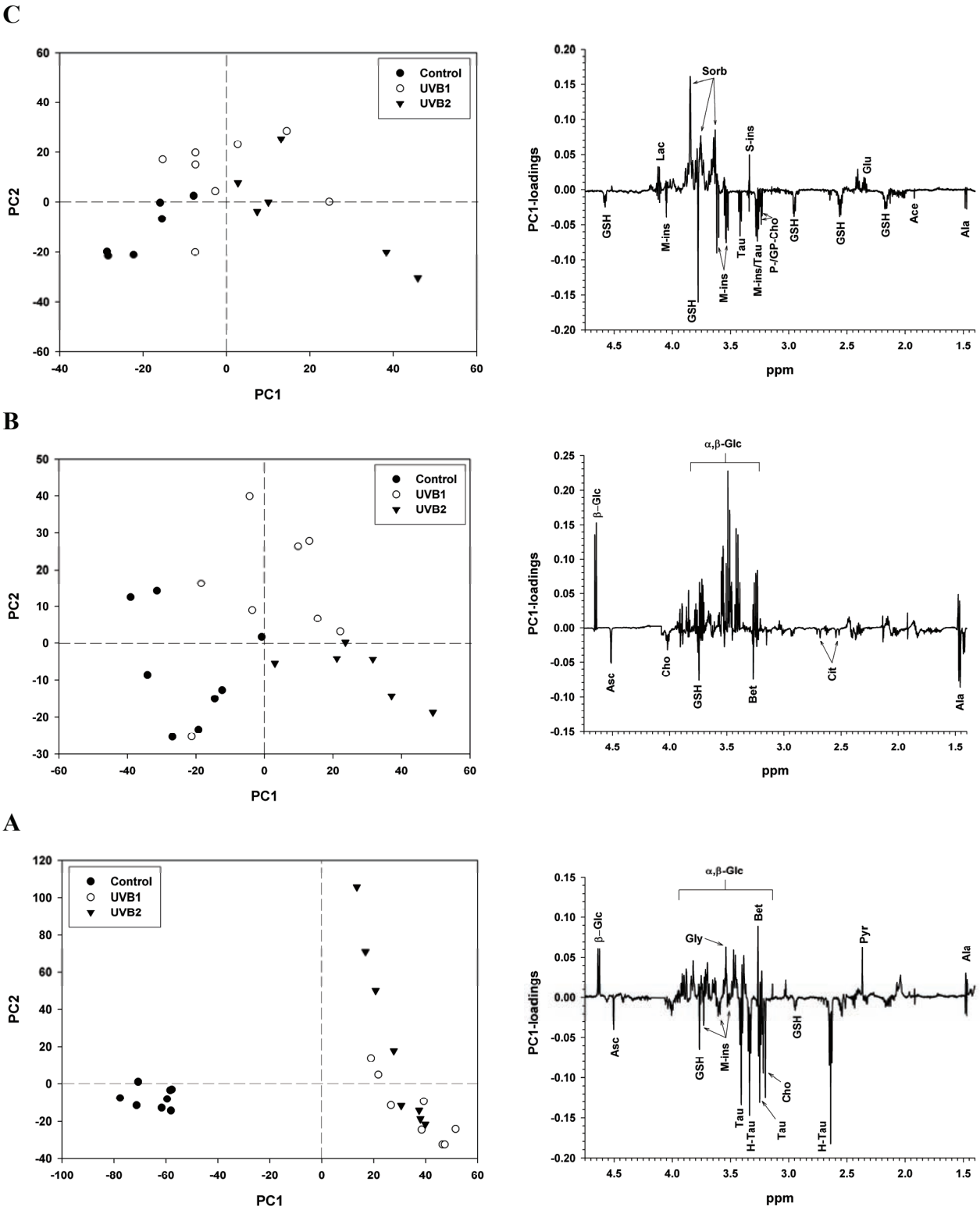

Figure 16 Principal component analysis results interpreted in the form of score plot (right) and loading spectra (left) representation: (**A**) cornea, (**B**) aqueous humour, (**C**) lens. Abbreviations as in Figure 14.

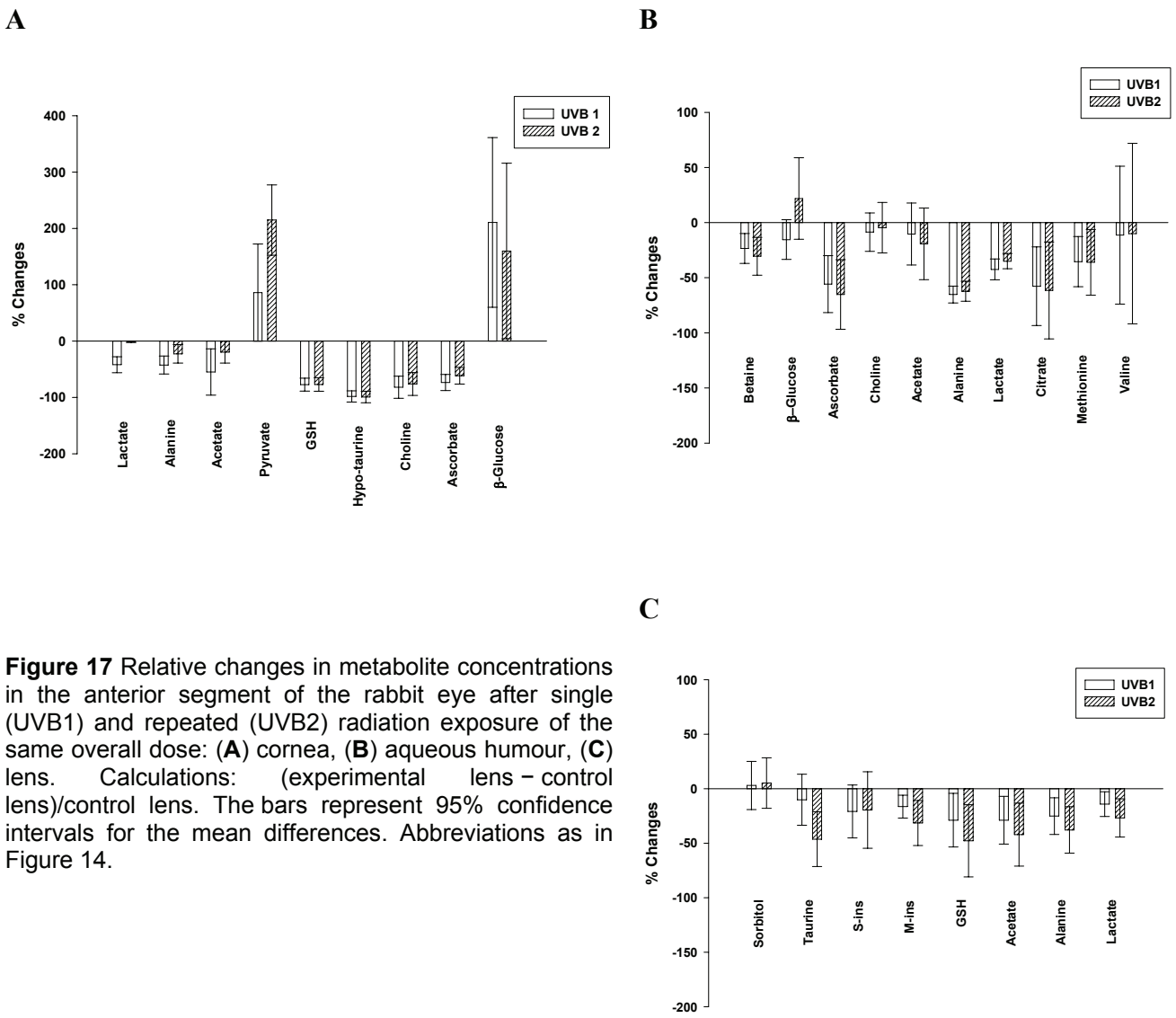

Figure 17 Relative changes in metabolite concentrations in the anterior segment of the rabbit eye after single (UVB1) and repeated (UVB2) radiation exposure of the same overall dose: (**A**) cornea, (**B**) aqueous humour, (**C**) lens. Calculations: (experimental lens − control lens)/control lens. The bars represent 95% confidence intervals for the mean differences. Abbreviations as in Figure 14.

4.5.1 Antioxidants

The well-known effect of UVR acting on the ocular tissues is the generation of reactive oxygen species (hydrogen peroxide, singlet oxygen and free radicals such as superoxide anions and hydroxyl radicals). Reactive oxygen species (ROS) are a danger for biological systems and might cause cellular damage by reacting with lipids, proteins and DNA[14]. In the corneal epithelium, the activities of the specific enzymes, scavenging ROS (superoxide dismutase, glutathione peroxidase and catalase), were reported to decrease dramatically following the repeated UVR-B exposure.[12,13] Similar action may occur also in other ocular structures and thus, the eye has to rely on the low-molecular

weight antioxidants, ascorbate and GSH as the main ocular defence systems against the enhanced oxidative stress.

In the present study, high ascorbate levels were found in the cornea and the aqueous humour, however the sensitivity of the NMR technique was not sufficient to investigate the low content of lenticular ascorbate. Glutathione concentration was quantified in the lens and cornea. The low spectral intensity of the GSH signal in the aqueous humour did not enable its quantification, but the alterations in GSH content were apparent in the loading spectra (Figure 16B).

In accordance with previous investigations,[100,114] significant reduction in ascorbate (74% - UVB1, 62% - UVB2) and GSH (77% - UVB1, UVB2) content were found in the cornea in the present experiments. Concentrations of both of these metabolites are interrelated and further linked to the pentose phosphate pathway (production of NADPH). The process of neutralization of reactive oxygen species by ascorbate and the employment of GSH and NADPH is well described by Rose et al[93] and is shown in Figure 2. But severe decrease in the levels of both antioxidants suggests impairment of this process. This would be in agreement with a previous study of Tsubai,[122] where a large dose of UVR-C resulted in pentose phosphate pathway damage and disturbance in homeostasis of redox balances such as GSH/GSSG in the porcine corneas.

Formation of the oxidized form of ascorbate, dehydroascorbate (DHA), as well as the oxidized form of GSH (GSSG), was not observed in the NMR spectra in our experiments. It seems the effect of radiation was so intense, that DHA was further hydrolyzed to diketogulonic acid with a consequent degradation to oxalate and CO_2. Damage of the pentose phosphate pathway could also result in decrease of NADPH-dependent reduction of GSSG and subsequently lead to reversible conjugation of GSSG with protein sulfhydryls resulting in the formation of protein-glutathione mixed disulfides (PrSSGs). This mechanism is necessary for the inhibition of disulfide-linked light scattering protein aggregate formation and have been already described in previous studies.[130] Both carboxyl groups of oxalate and PrSSGs are not detectable in the CPMG ^{1}H-NMR experiments and thus, could not be analyzed in our present study.

Similar processes might be responsible also for the significant decrease in the ascorbate content in the aqueous humour (55% - UVB1, 65% - UVB2) and GSH concentration in the lens (29% - UVB1, 48% - UVB2), as found in the present experiments. Moreover, it has been recently proposed that modification of the lens proteins is associated with the covalent binding of the UV filter compounds to amino acid residues of proteins[118,119] and that the linkage may proceed photochemically.[30] Thus, the levels of lenticular glutathione might be also reduced by the formation of the UV filter adducts, a mechanism which is crucial for the protection of the lens proteins from modification by UV filters.[110]

4.5.2 Compounds related to sugar metabolism

When the antioxidative system is not sufficient for buffering the reactive oxygen species, the ocular tissues are exposed to a substantial oxidative stress and the biochemical status of cells might be severely affected. As described previously,[55,56] UVR exposure may lead to inhibition of corneal carbohydrate metabolism and result in elevation of glucose concentration. This is in agreement with the results observed in our present study, where a significant increase in glucose concentration (210% - UVB1, 160% - UVB2) was observed in the cornea. Moreover, a concomitant reduction in corneal lactate content (42% - UVB1, 1% - UVB2) further supports this theory.

Elevated glucose levels in the corneal tissue may decrease the diffusion rates of the nutritional glucose from aqueous humour and consequently be responsible for increased aqueous humour glucose concentration observed in the present study (Figure 16B). Moreover, enhanced protein content in the UVB irradiated aqueous humour samples (Figure 13) indicates inflammatory response in the iris and ciliary body resulting in the breakage of the blood-aqueous barrier,[78] a process which might be also responsible for the elevation in glucose levels in the aqueous humour.[112]

In our experiments, the NMR spectroscopy did not reveal glucose in detectable amount in the rabbit lens. However, sorbitol, a product of polyol pathway was found in high concentrations and UVR exposure further increased its content. As previously described in the diabetic lens,[50] the toxic

levels of glucose enter lens cells and activate aldose reductase. This enzyme converts glucose to sorbitol which is not able to escape from the cell and which can generate high intracellular osmotic pressure sufficient to burst lens cells. A significantly high glucose levels in the UVR-B exposed aqueous humour samples found in our study thus may simulate the diabetic model and would explain the elevation in sorbitol concentration following the UVB irradiation. Another plausible mechanism standing behind the elevated sorbitol levels and a concomitant reduction in lactate concentration in the lens might be the inhibition of the glycolysis. In the normal lens, a small portion of sorbitol is metabolised to fructose which is converted to pyruvate by glycolytic pathway and might be subsequently reduced to lactate.[128] Thus, a mechanism similar to the previously described inhibition of the glycolytic pathway in the cornea[55,56] might be probably responsible also for the observed alterations in lenticular sorbitol contents in the present study.

4.5.3 Osmolytes

UVR exposure is known to cause water imbalance and osmotic stress in the corneal and lenticular tissues.[47,62] The main osmolytes in the anterior segment of the eye are taurine, hypo-taurine and myoinositol. In agreement with previous studies,[90,91,100,101,113,114] a general decrease in osmolytes in the UVR exposed cornea and lens was found in the present experiments. The UV-induced corneal swelling observed in the prior studies[18,23,35,87] and the enhanced osmotic pressure caused by elevated lenticular levels of sorbitol, as found in our present experiments, would be logically accompanied by a concomitant release of the osmolytes to aqueous humour in order to reduce the cellular osmotic pressure. However, a subsequent elevation in the concentrations of these compounds would be expected in the aqueous humour, a phenomenon which was not observed in our study. A reasonable explanation of this observation might be the UVR-induced disturbance of the osmotic function which was previously reported in the lens epithelium.[11]

Myoinositol may further function as a cellular signal transducer and has a significant role in growth and differentiation.[111] However, the observed reduction in its corneal and lenticular concentration in the present study (Figure 16A,C and 17C) seems to be caused by the inhibition of the

myoinositol biosynthesis (Figure 18). According to prior hypotheses,[86] myoinositol is synthesized in the cornea from glucose and is consequently transported to the aqueous humour and the lens, where it is accumulated by an active transport mechanism. As previously reported,[55,56] UVR is capable of impairment of the carbohydrate metabolism in the cornea and thus, it might possibly impair also the myoinositol biosynthesis. The significant elevation in the concentration of corneal glucose, found in our present experiments (Figure 17A), further confirms this theory.

Considering the hypo-taurine and taurine contents, their antioxidative properties[52] have to be taken into account. UVR has been formerly reported to induce oxidation of hypo-taurine to taurine[85] and might be thus responsible for a nearly 100% reduction in the corneal levels of hypo-taurine observed in our study. Taurine has been proposed to enhance cell survival as a membrane stabiliser or as an antioxidant in the human corneal epithelial cells.[96] Moreover, taurine may protect the lens against the oxidative stress and consequent cataract formation.[28] Thus, the depletion of both taurine and hypo-taurine in the UVR-B exposed rabbit eyes was probably caused by an enhanced UV-induced oxidative stress in the anterior segment of the rabbit eye.

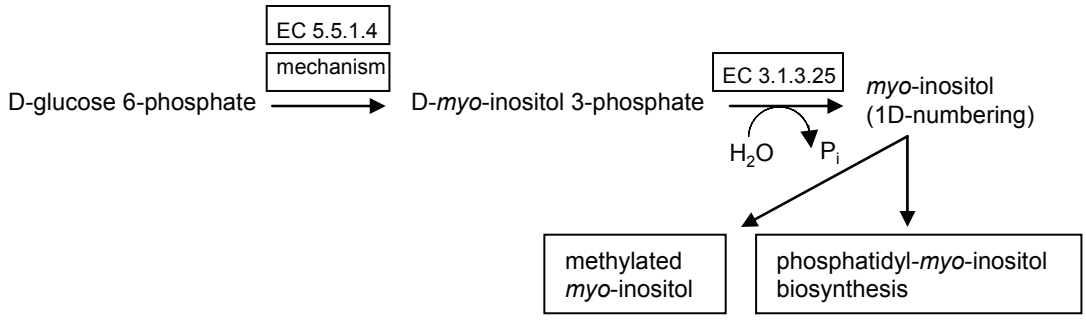

Figure 18 Myoinositol biosynthesis. EC 3.1.3.25, Inositol-phosphate phosphatase; EC 5.5.1.4, inositol-3-phosphate synthase (adapted from INTERNATIONAL UNION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY).[48]

4.5.4 Choline-containing compounds

UVR exposure of the eye has been found to cause apoptosis in the corneal tissue and necrosis among the cells of the lens epithelium.[129] This process would be a reasonable explanation for a significant reduction in the concentrations of membrane phospholipids precursors, choline, phosphocholine and glycerophosphocholine in the UVR exposed rabbit lens and cornea, as observed in the prior investigations of our research group.[90,91,100,101,113,114] In the present study, a significant decrease in choline concentration in the UVB irradiated cornea was accompanied by a concomitant elevation in betaine content. Betaine is formed by an oxidative transformation from choline, an intermediate reaction stage in the metabolism of choline containing compounds (Figure 19). Thus, the elevated betaine levels in the UVR-B exposed corneal tissue seem to be caused by the enhancement of the oxidative stress in the rabbit eye resulting in the rise of the rate of choline oxidation. Moreover, betaine can act as an osmolyte,[133] and the pathologically elevated level of its concentration, observed in the cornea in our present experiments, might be partly responsible for the process of previously described corneal swelling following the UVR ocular exposure.[18,23,35,87]

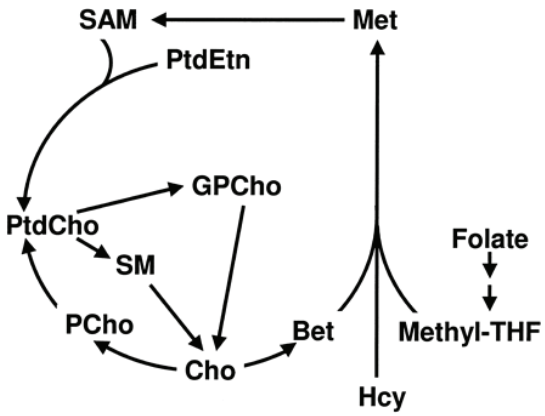

Figure 19 Metabolic pathways for choline and betaine. Phosphocholine (PCho), phosphatidylcholine (PtdCho), glycerophosphocholine (GPCho), and sphingomyelin (SM) are formed from choline (Cho) and can be hydrolyzed to form Cho. The formation of betaine (Bet) from Cho is irreversible. Betaine can donate a methyl group to homocysteine (Hcy) to form methionine (Met). Met is converted to S-adenosylmethionine (SAM), which is an important methyl donor. PtdCho can be formed from SAM and phosphatidylethanolamine (PtdEtn). Folate and Cho metabolism intersect because methyltetrahydrofolate (Methyl-THF), a product of folate metabolism, can also donate a methyl group for the formation of Met from Hcy (adapted from Ruiz-Cabello).[95]

4.5.5 Amino acids

The alterations in taurine and hypo-taurine levels in the UVR exposed rabbit eyes were already described together with osmolytes. Additionally, only the changes in alanine content showed statistically significant results in the present experiments. Former investigations of our research group on the damaging effect of UVR on the eye revealed substantial variations in the alanine concentration in the cornea,[100,114] the aqueous humour[100,112] and the lens.[90,91,101,113,114] In the cornea,[100,114] an UV-induced decrease in the alanine levels was found in the previous studies and is in agreement with our observations (55% - UVB1, 65% - UVB2). Regarding the aqueous humour metabolic profile, Tessem et al[112] revealed a substantial increase in alanine content after UVR exposure, while Sæther[100] observed an opposite tendency. Our results support the latter study with a significant reduction in alanine content (65% - UVB1, 62% - UVB2). When examining the whole lens, the UVB irradiation resulted in the elevation of alanine concentration.[90,91,101,113] However, as shown by Risa,[90] this increase was substantially time dependent and up to 24 hours post irradiation, the concentration of alanine was reduced. Since the experimental animals were killed 24 hours post last UVR exposure in our experiments, the results are in agreement with the findings made by Risa.[90]

Previously, it has been reported that some amino acids may function as both osmolytes[72] and antioxidants[52] in the lens. Thus, similarly to taurine and hypo-taurine, decrease in the alanine concentrations in the rabbit eye was probably caused mainly by an extensive UVR-induced oxidative stress in the anterior segment of the rabbit eye. Additionally, cellular repair mechanisms may accelerate protein biosynthesis in order to restore the ocular integrity, and thereby enhance the amino acid consumption in the cornea and the lens. Prior examinations of alanine metabolism in normal rabbit corneas[42,43] and bovine lenses[117] showed that alanine might be used as a metabolic substrate. It is largely deaminated and the pyruvate formed is consequently reduced to lactate. A significant decrease in corneal (42% - UVB1, 1% - UVB2) and lenticular (14% - UVB1, 26% - UVB2) lactate content found in the present study would be thus in accordance with a concomitant alanine reduction.

4.6 The effect of single versus repeated UVR exposure of the same overall dose

As already described, UVR exposure of the rabbit eye resulted in significant alterations in the metabolic profile of the anterior segment of the rabbit eye. Moreover, the PCA results revealed a substantial additivity of the repeated UV irradiations (Figure 16). Nevertheless, investigation of the PCA and quantitative statistical analysis results in detail show variations in the effects of single and repeated UVR exposures of the same overall doses on rabbit cornea, aqueous humour and lens. The corneal tissue seemed to be more vulnerable towards single irradiation, while the aqueous humour and the lens showed an opposite tendency. Cornea absorbs about 70% of the UVR at 312 nm wavelength[15] and thus, the effect of the UV irradiation on this tissue was expected to be the strongest. This was apparently confirmed by our experiments, where a substantial higher shift between the control and UVR exposed corneal samples along the PC1 axis was observed (Figure 16), compared to the aqueous humour and lenticular samples. The biomicroscopic studies has previously showed,[21] that when exceeding the interval of four hours between two subsequent threshold irradiations, the resultant effect of these exposures does not correspond to an effect of a single double-threshold dose. Furthermore, the longer is the interval the smaller is the cumulative effect. In our experiments, the interval between the repeated subsequent threshold UVR exposures was 48 hours. Despite of that, nearly the same effect of the repeated exposures was observed in relation to the single dose. As shown by Risa,[90] the observed metabolic changes are indirectly related to the light scattering mechanism in the lens and are delayed compared to the evolving lenticular opacity. Similar phenomenon seems to account also for the corneal tissue and the alterations in the microscopic signs following the UVR exposure may occur prior to the changes in the biochemical profile. Thus, the results of the present study might imply that the interval between two subsequent threshold UVR exposures, giving the same effect on the corneal metabolic profile as a single double-threshold exposure, is prolonged.

Only approximately 30% of the UVR at wavelength 312 nm penetrates the cornea,[15] 6-16% is absorbed by the aqueous humour[9] and the rest (14-24%)

is absorbed by the lens. Thus, compared to the corneal tissue, the aqueous humour and the lens are exposed to a significantly lower amount of UVR and the damage is expected to be considerably smaller. This was apparently confirmed by the present experiments (Figure 16). Additionally, when investigating the metabolic profiles of the aqueous humour and the lens, the results indicate synergistic additivity of the repeated UVR exposures. These observations might be possibly caused by two mechanisms, as suggested by Ayala et al:[4] photoproducts formation or cellular repair. Photoproducts are formed in response to the first UVR exposure and the sensitivity of the aqueous humour and the lens might be thus enhanced to a subsequent UVR exposure. When examining the effects of the cellular repair, cell cycle has to be considered. Damaged cells undergo mitosis as a repair mechanism after the first UVR exposure and during certain phases of the cell cycle, the cells might be probably more sensitive to a second UVR exposure.[4] Thus, the results of the present study imply that the additivity of the repeated UVR exposures needs to be considered and may play a key role in assessment of the cataract risks.

5 Conclusions

The application of the NMR spectroscopy in connection with effective statistical methods was found to be beneficial to evaluate the effect of a single and repeated UVR exposure of the same overall dose on the anterior segment of the rabbit eye. Moreover, the resolution quality of HR-MAS ^{1}H NMR spectra was comparable with HR ^{1}H NMR spectra and thus, no need for prior extraction procedures, which might influence the chemical composition of the samples, made this method superior. Additionally, CPMG spin echo pulse sequence was shown to have a crucial role in order to provide correct assignment and quantification of the NMR spectra. More than 20 different metabolites in the spectra from the anterior segment of the rabbit eye could be thus evaluated. Absolute quantification method was found to be less reliable procedure in the present study, because the aliphatic chain of the classic standard substance TSP seems to bind to the aqueous humour proteins.

Significant alterations following the UVR exposure were found in the metabolic profile of the cornea, aqueous humour and the lens. Especially, changes in antioxidants (ascorbate, GSH), compounds related to sugar metabolism (glucose, lactate), osmolytes (taurine, hypo-taurine, myoinositol), choline-containing compounds (choline, phosphocholine) and amino acids were observed (Figure 20). With respect to the high UVR absorption ability, cornea was found to be the most vulnerable tissue towards the effects of UVR. The main mechanism behind the UVR-induced damage seemed to be generation of reactive oxygen species, breakage of the blood-aqueous barrier and cellular death.

The comparison of the effects of single and repeated UVR exposures of the same overall doses on the metabolic profile of the anterior segment of rabbit eye revealed particularly the danger of synergistic additivity of the subsequent UV irradiations in the aqueous humour and the lens. The main factors responsible for this phenomenon seem to be the UVR-induced formation of photoproducts and the enhanced sensitivity of cells in certain phases of the cell cycle during the process of cellular repair.

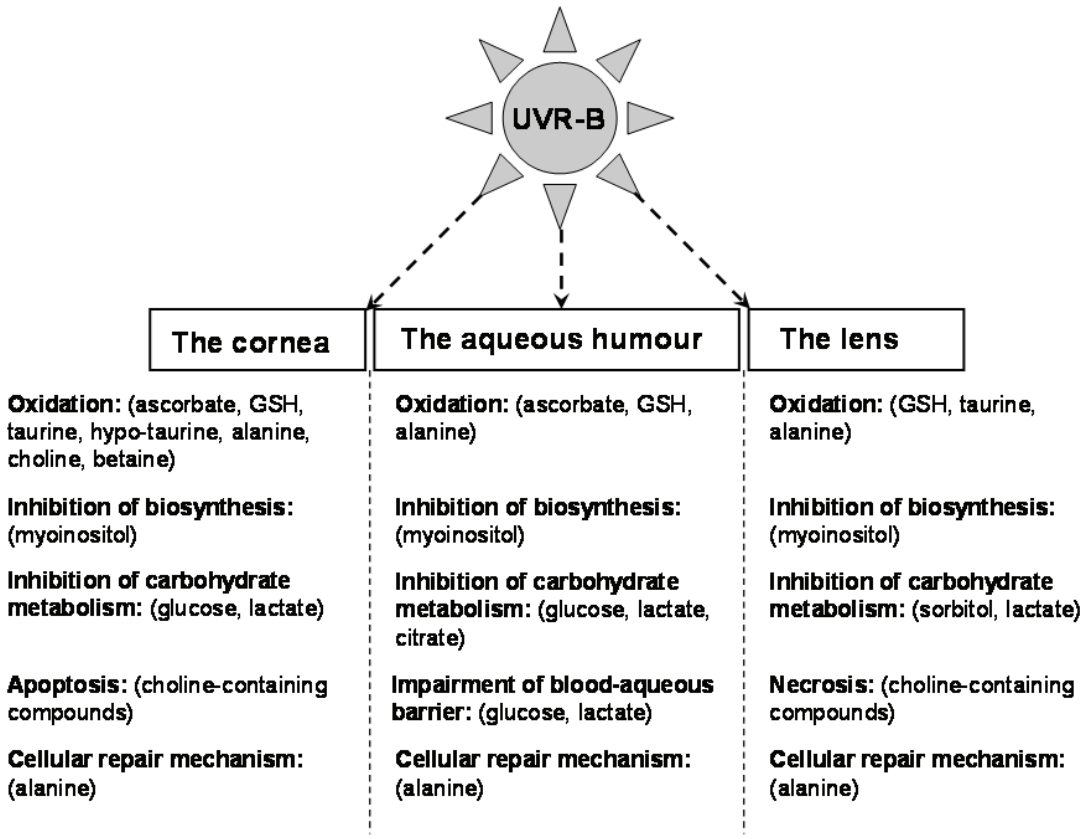

Figure 20 UVR-B damage mechanisms responsible for the alterations in the metabolic profile of the anterior segment of the rabbit eye. Based on the results of the present study.

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10. Torbjørn Iversen: SQUAMOUS CELL CARCINOMA OF THE VULVA.

1984

11. Tor-Erik Widerøe: ASPECTS OF CONTINUOUS AMBULATORY PERITONEAL DIALYSIS.
12. Anton Hole: ALTERATIONS OF MONOCYTE AND LYMPHOCYTE FUNCTIONS IN REALTION TO SURGERY UNDER EPIDURAL OR GENERAL ANAESTHESIA.
13. Terje Terjesen: FRACTURE HEALING AN STRESS-PROTECTION AFTER METAL PLATE FIXATION AND EXTERNAL FIXATION.
14. Carsten Saunte: CLUSTER HEADACHE SYNDROME.
15. Inggard Lereim: TRAFFIC ACCIDENTS AND THEIR CONSEQUENCES.
16. Bjørn Magne Eggen: STUDIES IN CYTOTOXICITY IN HUMAN ADHERENT MONONUCLEAR BLOOD CELLS.
17. Trond Haug: FACTORS REGULATING BEHAVIORAL EFFECTS OG DRUGS.

1985

18. Sven Erik Gisvold: RESUSCITATION AFTER COMPLETE GLOBAL BRAIN ISCHEMIA.
19. Terje Espevik: THE CYTOSKELETON OF HUMAN MONOCYTES.
20. Lars Bevanger: STUDIES OF THE Ibc (c) PROTEIN ANTIGENS OF GROUP B STREPTOCOCCI.
21. Ole-Jan Iversen: RETROVIRUS-LIKE PARTICLES IN THE PATHOGENESIS OF PSORIASIS.
22. Lasse Eriksen: EVALUATION AND TREATMENT OF ALCOHOL DEPENDENT BEHAVIOUR.
23. Per I. Lundmo: ANDROGEN METABOLISM IN THE PROSTATE.

1986

24. Dagfinn Berntzen: ANALYSIS AND MANAGEMENT OF EXPERIMENTAL AND CLINICAL PAIN.
25. Odd Arnold Kildahl-Andersen: PRODUCTION AND CHARACTERIZATION OF MONOCYTE-DERIVED CYTOTOXIN AND ITS ROLE IN MONOCYTE-MEDIATED CYTOTOXICITY.
26. Ola Dale: VOLATILE ANAESTHETICS.

1987

27. Per Martin Kleveland: STUDIES ON GASTRIN.
28. Audun N. Øksendal: THE CALCIUM PARADOX AND THE HEART.
29. Vilhjalmur R. Finsen: HIP FRACTURES

1988

30. Rigmor Austgulen: TUMOR NECROSIS FACTOR: A MONOCYTE-DERIVED REGULATOR OF CELLULAR GROWTH.
31. Tom-Harald Edna: HEAD INJURIES ADMITTED TO HOSPITAL.
32. Joseph D. Borsi: NEW ASPECTS OF THE CLINICAL PHARMACOKINETICS OF METHOTREXATE.

33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
41. Arne Kristian Sandvik: RAT GASTRIC HISTAMINE.
42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.

1989

43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.

1990

52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.

68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.

109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANSER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

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124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

1998

132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.

139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

1999

141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.

168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM

198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
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