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IN VIVO EXPOSURE TO ULTRAVIOLET RADIATION IN THE RAT LENS

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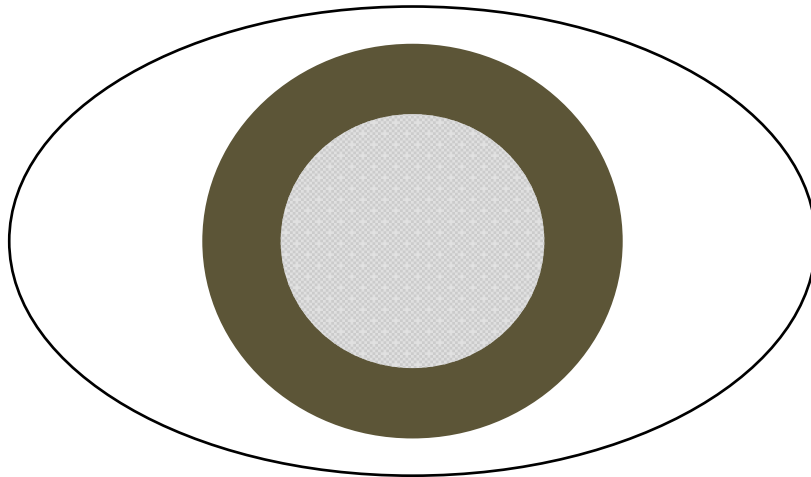
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Populärvetenskaplig sammanfattning: Start av starr

Grå starr, eller katarakt, är den vanligaste orsaken till blindhet i världen. Det finns flera riskfaktorer för att utveckla sjukdomen, men den främsta påverkbara riskfaktorn är exponering av ögat för solens ultravioletta strålar (UV-ljus). I följande studie har råttor exponerats för en lägre stråldos jämfört med i de flesta tidigare studier, vilket speglar de verkliga förhållandena på jorden bättre än höge doser.

Grå starr innebär att ögats lins grumlas. Linsen ska vara helt genomskinlig för att den ska kunna utföra sin normala funktion, vilken är att tillsammans med hornhinnan släppa in och bryta ljus så att ljuset kan träffa ögats näthinna. Näthinnan i sin tur omvandlar ljuset till elektriska signaler som sen skickas till hjärnan för tolkning – och tolkningen gör att vi kan se. När linsen grumlas, som vid katarakt, kommer ljuset att spridas så att fel andel ljus träffar den avsedda platsen på näthinnan, vilket gör att kontrasten i avbildningen av synfältet blir sämre. Resultatet blir att man ser dåligt. Försämringen kommer smygande och utan behandling leder sjukdomen till blindhet.

Grå starr är vanligt förekommande, särskilt hos äldre, men orsakerna till sjukdomen är inte helt klarlagda. Vi vet att linsen kan grumlas av mycket solljus, men exempelvis rökning och kortison ökar också risken för att drabbas. Den enda behandlingen är att i en operation byta ut den biologiska linsen mot en plastlins. Kataraktkirurgi innebär en stor kostnad för hälso- och sjukvården. För bättre prevention och behandling är det angeläget att bättre klarlägga UV-strålningens effekt på ögats lins.

Genom den här studien har en metod för mätning av intensiteten i UV-strålningen förbättrats och en metod för objektiv mätning av linsgrumling har förfinats. Tack vare dessa metoder har råttögon kunnat exponeras för en väldefinierad, förhållandevist liten, strålmängd (1 kJ/m^2), jämförbar med solstrålningens intensitet som träffar jordens yta. Linsgrumling har kunnat mätas och uttryckas i en internationell standard för grumlighet. Resultatet var inte konklusivt, men tydde på att även en liten stråldos kanske kan leda till en lättare kataraktutveckling. Detta har ökat vår förståelse för UV-strålningens effekter på ögats lins och är ett steg på vägen mot att i framtiden hitta en medicinsk kataraktbehandling.

Abstract

Purpose: To elucidate if exposure to subthreshold dose of UVR-B leads to measurable cataract in the rat lens within 24 hours after exposure.

Methods: The accuracy of three pipettes was measured using distilled water and a balance. A photodiode was calibrated against a thermopile for international standard of irradiance measurement. The measuring of forward light scattering with a light dissemination meter was standardized using a dilution series of Diazepam and a photodiode. Rat eyes were then unilaterally exposed for 300 nm UVR-B (1 kJ/m^2) and the lenses were extracted and measured for forward light scattering 1, 8, 16 and 24 hours post-exposure.

Results: The pipettes had an average mass close to that of the measured volumes, but due to its large average variance the 0.5-10 µl pipette had to be excluded from the rest of the experiments. The calibration factor for the photodiode was 0.0101 W/(nA*m^2). Plotting the Diazepam concentrations against the current gave a second order polynomial. The animal experiment showed a trend in increasing light scattering in the exposed lens compared to the contralateral unexposed lens (mean difference 0.026 tEDC) the longer the post-exposure time, but the result was not statistically significant.

Conclusions: This study indicates that subthreshold UVR-B exposure at 1 kJ/m^2 does not induce forward light scattering increase in rat lenses.

1. Background

1.1 Cataract

Opacification of the normally translucent lens give the mainly age-related disease *cataract*. The opacification stops the normal passage of the light through the eye by scattering the light, which in turn causes symptoms like reduced clarity, glare, monocular diplopia, progressive myopia, and eventually blindness (1).There are three main types of cataracts: infantile/juvenile, congenital and senile (or age-related). There is also traumatic cataract (typically anterior subcapsular cataract), that occurs after an injury. Senile cataract is the most common, and is clinically, depending on the morphology, further divided into subcapsular, cortical and nuclear sclerosis cataract. It can also present as a combination of the three (1).

Subcapsular cataract can be idiopathic or traumatic. It consists of abnormally positioned epithelial cells that enlarge to form bladder cells. Opacities in the lens cortex is called cortical cataract and is caused by biochemical changes in the lens fibre cells. Changes in the lens nucleus leads to nuclear cataract (2).

The pathogenesis of cataract is as of yet unclear, but some suggestions will be discussed below. Genetic mutations of, among others, lens proteins are associated with cataract (3), but there are also several other risk factors, including smoking, diabetes mellitus, alcohol, ionising radiation, hypertension and, most importantly, ultraviolet radiation (UVR). UVR has been associated with all three types of senile cataract (4), but especially with cortical cataract (5,6).

1.2 The Lens

The lens is biconvex and almost transparent, with a diameter of 6-6.5 mm at birth to 9-9.5 diameter at age 65 years (2). It is partly situated in the hyaloid fossa of the vitreous body and supported in the front by the cornea and the iris. It is connected to the ciliary body by the zonular fibres, and their contractions and relaxation give rise to accommodation through the lens. There are no blood or lymph vessels in the lens. The transparency is dependent on the order of cells, organelles and proteins (7).

Histologically, a thick, almost impermeable lens capsule surrounds the lens. Inside the capsule, there is a layer of cuboidal epithelial cells at the anterior surface, with new cells being laid down externally to the older ones (2). The epithelial layer can be divided into four zones. The central layer, consisting only of epithelial cells in resting phase G_0, is the largest. Towards the periphery, the pregerminative zone is found. It consists of cells that can go through mitosis. Next, there is the germinative zone

that produces secondary fibre cells. Furthest to the side is the transitional zone. It consists of epithelial cells that have started to elongate into fibre cells (7). Because of the thickness of the lens capsule, the epithelial cells cannot shed, and the lens became inwardly compacted with age. This is often accompanied by accumulating of yellow pigmentation, which together with the compaction leads to decreasing vision (2).

1.3 Ultraviolet radiation and its ocular transmittance

The sun's radiation can be divided into different groups depending on the wavelength of the light. Firstly, infrared radiation, or heat radiation, has a wavelength between 760 nm - 1 mm and is invisible to the human eye. Infrared radiation represents 43% of solar radiation on Earth after passing through the atmosphere. Secondly, representing 44% of Earth's solar radiation, there is visible light, with wavelengths between 400-760 nm, giving rise to different colours. Solar emission of UVR has a wavelength between 100-400 nm. Solar radiation on Earth consists of 13% UVR. (7,8). UVR can further be divided into UVR-A, UVR-B and UVR-C, as established by the CIE (Commission Internationale de l'éclairage, International Commission on Illumination) in the 1930s (9). Table 1 shows their different properties. When the sky is clear and the sun is in zenith, in central Europe the total solar irradiance is 1000 W/m^2 (10). Under these conditions, the irradiance provided by the 300 nm-radiation is 0.005 W/m^2 (8).

Table 1: The transmittance and health effect of different types of UVR (7).

UVR type	Wavelength (nm)	Transmittance	Health effect
UVR-A	315-400	Transmits through atmosphere and glass.	Skin cancer
UVR-B	280-315	Blocked by glass. 90% attenuated by the atmosphere.	Skin cancer Sunburn Cataract
UVR-C	100-280	Attenuated by the atmosphere, does not reach Earth's surface.	Mutagenic

In the eye, the light is transmitted to the retina through the cornea, aqueous humor, crystalline lens and vitreous body. The cornea constitutes the main protection from the UVR. In the human eye the cornea attenuates 92 % at 300 nm and 18 % at 400 nm (11). In rat eyes the percentage of attenuation in these wavelengths is 64 % and 20 % (12). Essentially all UVR is absorbed by the ocular media in the human eye, apart from wavelengths between 320 nm and 400 nm. These wavelengths reaches the retina (11). The UVR absorption in human lenses is a gradient with the lowest absorption in the anterior parts, and the highest absorption in the posterior parts, especially for wavelengths lower

than 310 nm (13). Only the radiation energy that is absorbed by the tissue may cause damage, and in the lens this radiation consists of wavelengths between 290-340 nm (14). Aromatic amino acids, kynurenine and advanced glycation end products (AGEs) are the major UV-absorbing molecules in the eye. The AGEs may crosslink and contribute to cataract development and accumulate if young human lenses are exposed to UVR-A (15).

1.4 Cataract and UVR

Epidemiological studies (4–6,12,16–19) have linked exposure to UVR, and development of cataract together. For example, Sasakia et al. 2003 (6) reported a higher prevalence of cataract in lower latitude areas because of their high UVR-levels, though this study did not take into account the different ethical backgrounds of the participants. They also concluded that the location of cortical cataract is also highest in the nasal quadrant of the eye, which is unprotected from the sun compared to the other quadrants. This has also been found by other studies (20). A study from 2015 (21) found higher burden of disease in regions with high UVR-B levels than in those with lower UVR-B levels.

There is epidemiological evidence that UVR-B is the main part of the solar radiation leading to cataract development (20). However, according to a study by Dillon et al. 1999 (12), many earlier studies concluded that it is mainly UVR-B that leads to cataract development on the basis that spectacles-wearers have a decreased risk of disease. Glasses do classically not admit transmission of UVR-B, but do admit transmission of UVR-A. The study, on the other hand, showed that plastic reading glasses reduces the UVR-A transmission as well, thereby concluding that UVR-A may have a role in cataract development, too. Another study showed that wearing plastic spectacles, sunglasses or a hat protects against cataract (16), but, as shown by Rosenthal et al. 1986 (22) the protection offered by prescription eyewear depends on a large number of factors such as the size of the spectacles, the material and the way of wearing them.

Experimental studies on rabbit and rat eyes show that the lens is the most sensitive to irradiation of wavelengths around 300 nm (23,24), which is in the UVR-B-spectrum. Other studies (7) have also suggested a linkage between UVR-B exposure and cataract. Conversely, UVR-A have experimentally been seen to produce nuclear cataract in guinea pig lenses (25).

Many *in vivo* experiments use extremely high levels of UVR, far beyond what would occur in humans (20). However, as shown in studies by Galichanin (7), repeated subthreshold exposures of UVR-B in rat lenses accumulate and cause lens opacities. The author also concludes that higher UVR-B doses cause cataract quicker and to a higher degree. Appendix 1 summarizes the experimental rat studies linking UVR and cataract together.

1.4.1 Photochemical reactions and toxicology

The absorption of a photon makes the absorbing molecule undergo electron changes by moving an electron to a higher orbital energy level. This can result in either a direct phototoxic reaction in the absorbing molecule, or in an indirect photosensitized reaction on adjacent ones. UVR causes these types of reactions in DNA, RNA, proteins and lipid fatty acids. In DNA, this induces mutations, DNA-protein cross-linking and DNA strand-breaks (7).

UVR causes toxicity that can be either acute or chronic (7). Acute toxicity is often the result of an exposure for high doses and gives an immediate effect. The dose-response can be described as either binary (the response is either an effect, or no effect occurs) or continuous. In the latter case a threshold can be defined as the dose in which a significant response can be seen.

A normally distributed response with a continuous dose-response gives a sigmoidal curve when plotted into a second order polynomial regression curve, omitting the first order (26). In UVR-B induced cataract the response can be measured as forward light scattering (7).

In the steepest part of the sigmoidal curve, the UVR-B induces a linear increase in light scattering. The slope, or the regression coefficient, can indicate the level of interaction between the toxicant (in this case the UVR-B) and the response (the light scattering, or degree of opacity). Previously, the UVR dose-response upon the lens has been binary, defined as "cataract" or "no cataract", giving a threshold dose for transient cataract of 1.5 kJ/m2 and 5 kJ/m2 for permanent cataract (24). However, it has been experimentally shown (27) that exposure for higher radiation doses leads to continuously increasing opacities in the lens and as such Söderberg et al (28,29) developed a method for estimation of the threshold dose, maximum acceptable dose (MAD). MAD, or MTD (maximum tolerable dose, as it was later called), is the dose which after exposure has a 16% probability of inducing more light scattering in exposed lenses compared to 2.3% of the normal, unexposed, population (30). Table 2 shows the MTD value for different species.

Table 2: The MTD value for different species (7).

Species	MTD-value (kJ/m^2)
Albino rat	3.65
Pigmented rat	4.2
Pigmented mouse	2.9
Pigmented guinea pig	69

1.4.2 Pathological mechanisms by UVR

UVR exposure induces swelling and rupture of epithelial cells and swelling and fusion of fiber cells (31). It has been suggested that UVR causes oxidative stress in the lens, thus increasing reactive oxygen species, ROS, which in turn damages the DNA and causes cross-linking of proteins (20). The oxidation of lens proteins leads to aggregation, which progressively forms opacities and finally cataract (32–34). It has been suggested that antioxidants, such as certain dietary micronutrients (34), may protect the lens from these kinds of damages (33). There are several natural antioxidants in the lens, among others thioredoxin reductase and glutathione. Blocking of these antioxidants leads to a higher degree of UVR-A-induced damage in the epithelial cells (35).

Human epithelial cells, rat lenses and rabbit lenses have been used to determine the action spectra from UVR for lens or cell damage (15). It has been shown that one of the mechanisms involved in both human and animal cataract is apoptosis of the lens epithelium. This is regardless of what triggered the disease (36). UVR-B has been showed to induce apoptosis of the epithelium *in vitro* (37) in a time-related manner, followed by opacification (38). The same thing has been showed *in vivo* by Michael et al (39), with the apoptosis rate peaking 24 hours after threshold exposure UVR-B. The authors also showed that DNA-damage occurs immediately after exposure, but cannot be seen between 1-6 hours afterwards, thereby concluding that the lens epithelial cells undergo reparation during this time. The cells that fail to do so will be cleared by apoptosis (39).

Caspase-3, an important apoptosis executioner, increases after UVR-B exposure (40), as do the expression of p53, an apoptosis initiator (7). Galichanin (41) has shown that at a subthreshold dose of 1 kJ/m^2, UVR-B induces apoptosis in epithelial cells, but not in lens fiber cells.

All of this disturbs the regular order of cells, proteins and organelles and, since that is what the lens transparency depends on, opacities develop. The light no longer has a clear passage through the lens and light scattering occurs.

1.4.3 Repair mechanisms

Studies have shown that close-to-threshold and above-threshold UVR-B exposure leads to cataract, but the changes in epithelium and in fiber cells are largely reversible (14,38). Signs of reparations can be seen within 7 days of exposure, by way of apoptosis, DNA reparations and epithelial proliferation (14). Regenerative repair in the epithelium occurs during 2 weeks post-exposure (38). The damage to the fiber cells are, however, irreversible (38), or at the very least not complete, with a remaining disarray (14).

1.5 Global problems

45% of global blindness is caused by cataract, making the disease the world's most common cause of blindness and the second most common cause of moderate to severe vision impairment (number one being undercorrected refractive errors). In 2020, for people 50 years and older, there was 15.2 million cases worldwide (42). There exists no method of prevention, delaying or reversing of the development of the disease, and the only treatment is surgery, which is associated with complications (3). This makes cataract a huge burden on the global health care, especially with an ageing population (16). Furthermore, surgery is not easily available in all areas. For example, Zhu et al. (21) found a higher disease burden in more rural areas compared to urbanized, supposedly due to higher availability of healthcare in the urban areas. WHO (43) states that the proportion of visual impairment caused by cataract is higher in low- and middle-income countries than in high-income countries. For the individual patient, cataracts has an enormous personal impact with lower quality of life, depression, anxiety, social isolation, risk for falls and fractures, and also lower work participation (43).

To delay the debut of the disease 10 years could reduce the need of an operation with 50 % (44), and cataract prevention could reduce health care costs with 5-6 billion USD (34). Further studies are needed in order to develop delaying treatment, and this in turn requires more experiments to assess the lens's sensitivity for UVR-exposure. This in turn requires an instrument of measuring the intensity of UVR on the eye and an instrument for quantitative measurement of lens opacification.

1.6 Aims

It has previously been shown (27) that a single exposure to UVR-B of less than 3 kJ/m^2 does not induce significant light scattering in the lens one week after exposure. However, a later study (40) has shown that exposure to a total dose of 1 kJ/m^2 induces a transient upregulation of apoptosis marker caspase-3, peaking at 8 hours and 16 hours after exposure. Therefore, the aim of this study was to elucidate if exposure to 1 kJ/m^2 UVR-B induces quantitatively measurable cataract changes in the lens within 24 hours after exposure and if these changes are dependent on the time after exposure.

2. Material and Methods

2.1 Experiment animals and ethical approval

Because of previous experience in the research group, 6 weeks old female albino Sprague-Dawley rats were used in this experiment. Ethical approval was obtained from Uppsala Animal Experiment Ethics Committee, Dnr 5.8.18-07627/2021. This work is part of the study "Effekter av optisk strålning med och utan tillförsel av antioxidanter i ögonen av råttor". That study was given a medium degree of difficulty because the animals were expected to go through several short periods of slight suffering.

All animals were kept and treated in an approved animal facility according to Swedish laws and regulations and the Declaration of Helsinki. All personal that have interacted with the animals have fulfilled a course in animal ethics and practices. In order to fulfil the 3 R:s (replace, refine, reduce) of animal experiments, the personal have practiced removing lenses from unused eyes from other experiments (refine and reduce), and only one eye from each animal was exposed for UVR, making it possible to use the other as control (reduce). Reflexes were checked to control the depth of anesthesia (refine) and soft tissue were placed around the sleeping animals to keep the body temperature at acceptable levels (refine). Because of the complex interaction between the lens and the other tissues of the eye, it was deemed that the animals of this experiment could not be replaced by cultured cells (replace). A "mixed box" was used to sacrifice the animals, with a mix of medical gas and carbon dioxide first putting the rat to sleep and then sacrificing her (refine).

2.2 Calibration of micropipette

Three micropipettes were measured – 0.5-10 µl, 10-100 µl and 100-1000 µl. At set intervals (0.5/5.5/10 µl, 10/55/100 µl, 100/550/1000 µl) distilled water at room-temperature was drawn up and the mass was recorded using a Mettler AT261 DeltaRange® FACT scale. The process was repeated 10 times for each volume. To allow for time variance, everything was repeated on three successive days.

The average mass, the standard deviation, the coefficient of variance (CV%), the confidence interval and the average coefficient of variance (average CV%) were calculated. The average CV% was then plotted against each day to show the variance over time.

2.3 UVR source

The UVR source (Figure 1) was originally designed by Söderberg (31). The UVR radiation is generated by inducing excitation, corresponding to photon energy, in the outer electrons of Mercury atoms. This is achieved using a high-pressure Mercury lamp (HBO 200W, Osram, Germany). A spherical reflector placed behind the lamp collects the radiation from backwards direction (7).

The emitted radiation from the Mercury lamp is collimated[1] by a condensor lens and passed through a water filter in order to decrease the infrared radiation. Another condensor is placed after the water filter to collect the light. The light is passed through a shutter controlled by an electric watch in order to control the exposure time for UVR on the eye.

Since the light spectrum emitted from the Mercury lamp is broad, it must be narrowed down to a more specific range of wavelengths. This can be achieved using either an interference filter[2] (as originally used by Söderberg) or a monochromator (as used in later experiments).
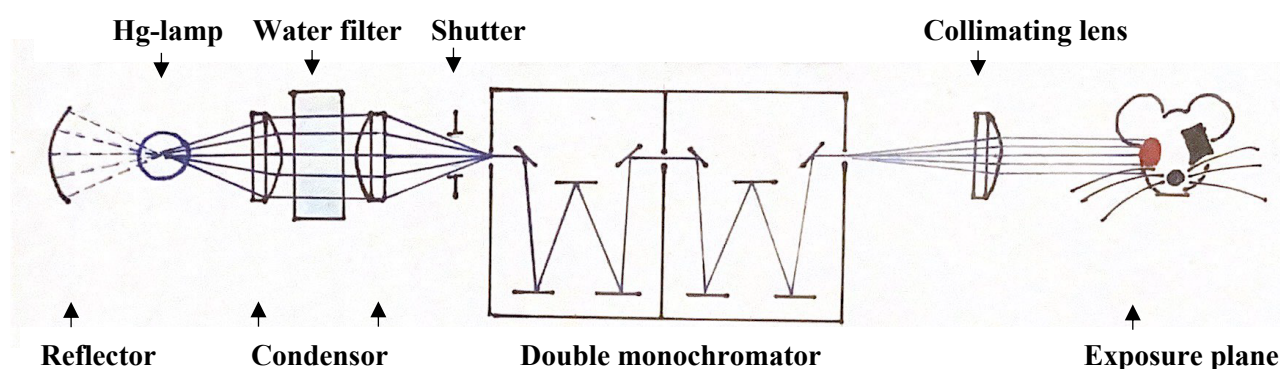
Figure 1: Schematic drawing of the UVR source. Note that the photodiode and the thermopile were placed in the exposure plane. Made after a drawing originally published in an article by Ralph Michael. Development and Repair of Cataract Induced by Ultraviolet Radiation. *Ophtalmic Research* 2000; February (suppl): 7.

A monochromator is a device that separates light of several wavelengths (polychromatic light) into a range of individual wavelengths (monochromatic light) and then selects a narrow band of these wavelengths. It consists of an entrance slit, mirrors, a dispersive element[3] and an exit slit. By rotating the dispersive element the resulting wavelength of monochromatic light changes (46).

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[1] made parallel

[2] An *interference filter* is a filter that only transmits a specific range of wavelength. This is achieved using constructive and destructive interference, which is the reinforcement or the removal of wavelength (45).

[3] Something that separates light into individual wavelengths. In a monochromator it may consist of either a prism, or a reflective grating.

The polychromatic light, in this case from the Mercury lamp, is let into the monochromator by the entrance slit. It hits a concave mirror that reflects and collimates the light and sends it at a specific angle towards a reflection grating[4]. Light of different wavelengths is reflected at different diffraction angles and thus hits the next concave mirror at different points. This concave mirror focuses the light and depending on the wavelength it will be focused on different points. The wavelength that is focused on the exit slit will be able to leave the monochromator (46).

The problem with monochromators with reflection grating is that stray light in the monochromator limit the efficiency of wavelength selection, thus they do not block unwanted wavelengths very well. As such, the exit light will be contaminated with unwanted wavelengths. By using two different serially connected monochromators, this problem can be solved (31,46).

In order to achieve a high degree of flexibility in wavelength-selection and a high grade of spectral purity, two serially coupled monochromators (Oriel 2x 77250: LOT-Oriel) were used in this paper. Wavelengths around 300 nm (UVR-B), *UVR-300 nm*, were selected due to it being the most damaging wavelength region (31).

A projection lens placed after the monochromators collimates the emerging radiation and homogenizes it before it hits the exposure plane. A spectrometer (PC 2000; Ocean Optics, Dunedin, FL) is used to record the spectral irradiance of the emerging radiation (7,40) (Figure 2).
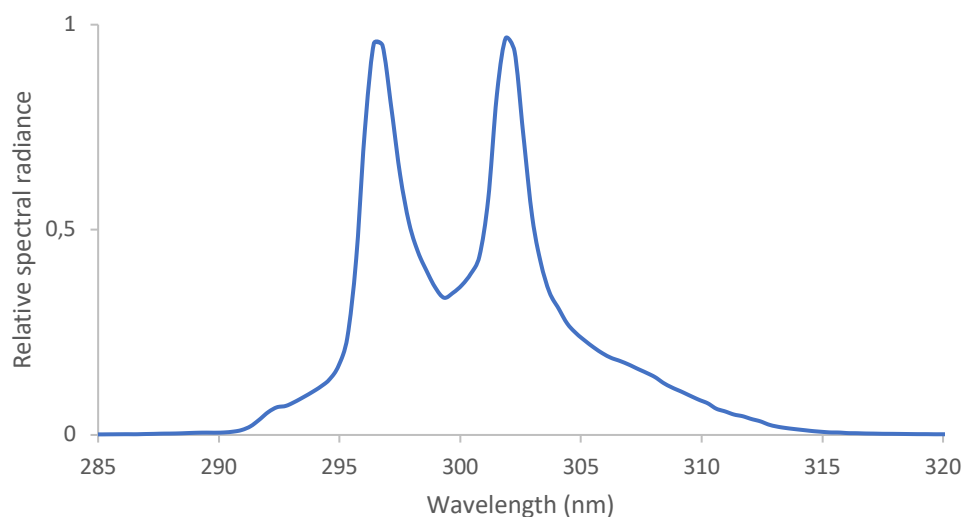

Figure 2: The relative spectral radiance of the chosen UVR-B.

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[4] Grating (sv. gitter) is an optical device that splits and diffracts light. It can either be reflective or transmitting. Parallell light will hit the groves at an incident angle α and leave the grating at a diffraction angle β (47).

2.3.1 Calibration of photodiode

The science of measuring light is called the *radiometry* (48). Irradiance, incident light intensity or electromagnetic radiation per time and area, is measured in watts[5] per square meter (W/m^2) (49). It can be measured by radiometers based upon a thermopile or photodiodes, phototubes or photomultipliers (31). The radiometers used in this experiment were based upon a thermopile and a photodiode. Both of them were covered by a diffuser to decrease angular dependence, thus softening the incoming light (50).

A thermopile works by converting temperature, in this case caused by UVR-B absorption at the exposure plan of the UVR source (7), into electrical voltage. The voltage can then be measured using a multimeter (31). The advantage of a thermopile is that it is insensitive to wavelength. It is, however, slow, and it is sensitive to both over-exposure of light and temperature disturbances during measurement (31).

A photodiode converts light energy into voltage or current (51). The generated current is proportional to the irradiance and is strong enough to be detected by a multimeter (Keithley 159A, Keithley instruments, USA). It is dependent on the wavelength but has a more rapid response than thermopiles (31). Since a thermopile-based radiometer is unsensitive to wavelength, it can be used to calibrate the photodiode-based radiometer to different wavelengths (31).

In order to measure the irradiance of the UVR-source, the photodiode was calibrated to the thermopile. To do this, the UVR-source was turned on and the incoming light was recorded with the spectrometer (reference line set at 300 nm). The band width was adjusted by turning the grit inside the monochromator. When the band width of the exiting light was around 300 nm, a thermopile connected to the multimeter was placed at 10, 20, 30 and lastly 40 cm from the monochromator output. The voltage was recorded at each point. Afterwards, the same was done with a photodiode, and the current was recorded. The process was repeated three times.

The voltage from the thermopile as a function of the current from the photodiode was plotted. The calibration factor for the photodiode could be calculated using the rate constant, k, from the resulting linear equation and the known calibration factor for the thermopile (44,2353 $mV^{-1}*W*m^{-2}$). The result was used to calculate the current needed for the exposure of rat lenses.

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[5] Watt is the derived SI-unit of power (sv. effekt) and is defined as joule per second (J/s), or energy per unit of time.

2.4 Light dissemination meter

Light scattering may be used for quantitative measurement of lens opacities (e.g., cataract), as forward light scattering. The light dissemination meter was originally designed by Söderberg (31) and uses dark-field illumination. It consists of a lens and shutter, a photodiode, a cold light source reflected to a circular reflector and a transparent disc upon which the dissected rat lens is placed (Figure 3).
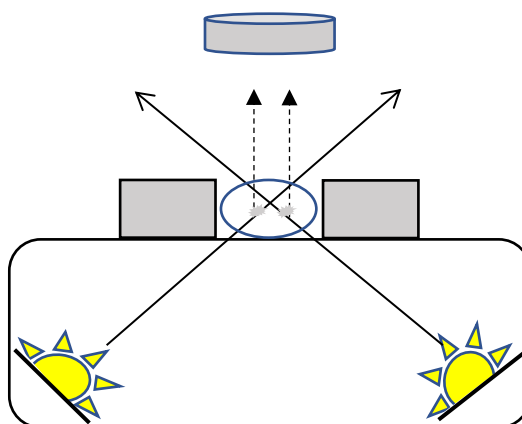

Figure 3: Schematic drawing of the light dissemination meter.

Cold light is directed at a 45° angle towards the lens. If the lens is transparent, the light cannot reach the optics at this angle, but if there are any opacities in the lens it will scatter the light. The light that scatters in the forward direction will be collected by the optics and projected onto the photodiode. The photodiode in turn will produce a current proportional to the light incident.

In order to standardize the measurement, the readings are calibrated to an opacity standard using a commercially available and standardized lipid emulsion of Diazepam (Stesolid Novum, Dumex-Alpharma, Denmark). To do this, 3 tubes of stock solution with desired concentration 8 mg/ml, using Diazepam 5 mg/ml and balanced salt solution (BSS, Sterile Irrigating Solution, Alcon, TX, USA), was prepared using the micropipettes described above. The real concentration was calculated using the same balance as in the pipetting experiment. Each stock was dilatated into a series of 7 dilutions (desired concentration 0, 0.25, 0.5, 1, 2, 4, 8 mg/ml) and once again the real concentration was measured.

All 21 dilutions were then individually measured 3 times for light scattering in the light dissemination meter. The current, or intensity of light scattering (I, measured in 10^{-10} A), was recorded using a photodiode connected to a multimeter. The cuvette in which the dilutions was

measured was carefully cleaned with tap water and distilled water and dried between each reading. The measured currents (I) and concentrations (c) were then fitted into the following second order polynomial equation, omitting the first order (Eq. 1). k and ε are the constants of the equation.

$$I = k_1 c^1 + k_2 c^2 + \varepsilon \qquad\qquad \text{Eq. 1}$$

The current was plotted against the concentrations. The concentrations were then log transformed in order to make the data normal distributed. The result was used to express light scattering in the rat lenses in log transformed Equivalent Diazepam Concentration (tEDC). The tEDC value for a healthy rat lens and an opaque is around 0.1 and 1, respectively (52).

2.5 In vivo experiment

Eight Sprague-Dawley rats, described in section 2.1, were anesthetized with 94 mg/kg ketamine (Ketalar, 50 mg/ml, Pfizer) and 14 mg/kg xylazine (Rompun vet., 20 mg/ml, Bayer AB, Germany), 15 minutes before UVR-B exposure. Both eyes were instilled with Tropicamide 10 mg/ml (Mydriacyl, Alcon, Belgium) to produce mydriasis. One eye on each animal was covered with a piece of tape and the animal was placed so that it could be unilaterally exposed to 1 kJ/m^2 of UVR-300 nm for a total of 15 minutes.

Two animals were sacrificed at 1 hour, 8 hours, 16 hours and 24 hours post-exposure, using carbon dioxide asphyxiation, followed by cervical dislocation. The lenses were extracted from the enucleated eyes and the remnants of the ciliary body were removed under microscope. The extracted lenses were kept in BSS.

The light scattering of each lens, including the non-exposed control lenses, were measured 3 times using the light dissemination meter. The results were converted into log transformed EDC (tEDC), using the solution for a second order polynomial equation (Eq. 2).

$$c = -\frac{k_2}{2k_1} \pm \sqrt{\left(\frac{k_2}{2k_1}\right)^2 + \frac{I}{k_2}} \qquad\qquad \text{Eq. 2}$$

The difference in tEDC between the UVR-B exposed and the contralateral unexposed lens were then calculated. Linear regression was used to analyze the result. All the result from each rat was then pooled together and the mean tEDC, standard deviation and the confidence interval for mean difference between the exposed and unexposed lenses for the whole group were then calculated.

2.6 Design of the animal experiment

The animal experiment was designed for regression analysis assuming a straight-line response of forward light scattering as a function of post-exposure time. In each animal one eye was exposed to UVR and the contralateral eye was not exposed. Altogether, two animals per group, were sacrificed at each of 1 h, 8 h, 16 h or and 24 h post-exposure. The intensity of forward light scattering in a lens was the average of 3 measurements.

2.7 Statistical parameters

The significance level was set at 0.05 and the confidence coefficient 0.95 considering the limited sample size.

3. Results

In order to answer the aim of the study, a micropipette needed to be measured for accuracy, the irradiance of an UVR-source needed to be measured and an instrument for quantitative measurement of cataract needed to be calibrated. In this section, the results of this will be presented.

3.1 Micropipette

Table 3 shows the average mass, standard deviation, coefficient of variation (CV%) and confidence interval (CI(0.95)) for each measured volume of the three pipettes over the course of three days. The average mass was always close to that of the wanted volume and for 10-100 µl pipette and the 100-1000 µl pipette the CV% was less than 2%. It was however found that the low volume pipette had considerably lower accuracy than the higher volume pipettes (Figure 4). The scale setting on the 0.5-10 µl pipette at 5.5 µl seemed to over estimate the real volume considering the confidence interval for the mean mass (Table 3). The variation of the average CV% for each pipette over the days can be seen in Figure 4.
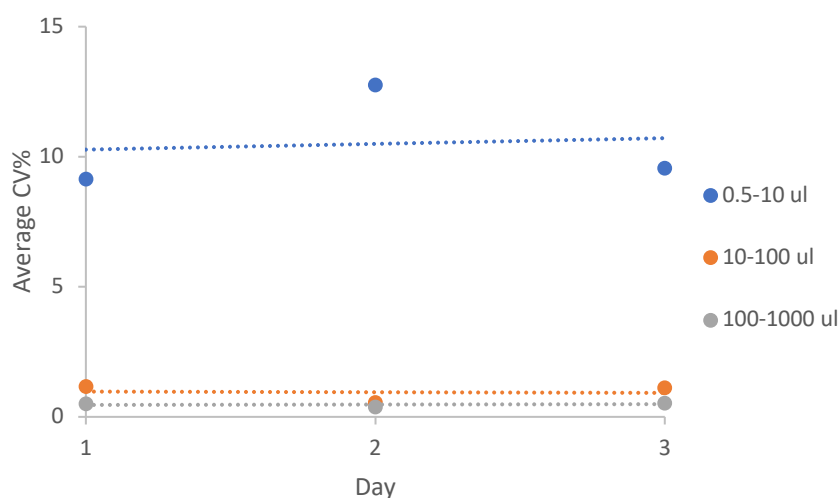

Figure 4: Change of average variation coefficient (Average CV%) for each pipette and each day.

Table 3: The average mass, standard deviation, coefficient of variation (CV(%)) and confidence interval (CI(0.95)) for each measured volume for the three pipettes.

Pipette	0.5-10 ul			10-100 ul			100-1000 ul		
Volume (ul)	0.5	5.5	10	10	55	100	100	550	1000
Day 1									
Average mass (mg)	0.46	5.39	9.96	10.91	55.61	100.87	100.65	557.03	1009.56
Standard deviation (mg)	0.09	0.14	0.48	0.25	0.47	0.38	1.00	1.79	0.18
CV(%)	19.98	2.60	4.83	2.28	0.85	0.37	1.00	0.32	0.18
CI(0.95) (mg)	0.46	5.39	9.96	10.91	55.61	100.87	100.65	557.03	1009.56
	+/-0.07	+/-0.10	+/-0.34	+/-0.18	+/-0.34	+/-0.27	+/-0.72	+/-1.28	+/-1.28
Day 2									
Average mass (mg)	0.49	5.35	9.80	10.97	55.92	101.42	100.99	553.81	1007.86
Standard deviation (mg)	0.14	0.37	0.30	0.13	0.12	0.21	0.54	1.60	3.43
CV(%)	28.42	6.83	3.04	1.21	0.22	0.21	0.54	0.29	0.34
CI(0.95) (mg)	0.49	5.35	9.80	10.97	55.92	101.41	100.99	553.81	1007.86
	+/-0.10	+/-0.26	+/-0.21	+/-0.09	+/-0.09	+/-0.15	+/-0.39	+/-1.15	+/-2.45
Day 3									
Average mass (mg)	0.52	5.37	9.85	10.73	55.45	100.53	98.93	550.37	1003.85
Standard deviation (mg)	0.11	0.28	0.21	0.27	0.26	0.36	0.79	2.44	3.66
CV(%)	21.36	5.23	2.15	2.52	0.46	0.36	0.79	0.44	0.36
CI(0.95) (mg)	0.52	5.37	9.85	10.73	55.45	100.53	98.93	550.37	1003.85
	+/-0.08	+/-0.20	+/-0.15	+/-0.19	+/-0.18	+/-0.26	+/-0.56	+/-1.75	+/-2.62

3.2 Calibration of photodiode

Within the dynamic range of irradiances used, both the thermopile, T, and the photodiode, P, are expected to provide a straight-line response to irradiance, E (W/m^2), through origo. The response is a voltage (μV) for the thermopile (Eq. 3) and a current (10^{-10} A) for the photodiode (Eq. 4) with the proportionality constants for the thermopile k_T (μV/(W/m^2)) and for the photodiode k_P (10^{-10} A/(W/m^2)).

$$U = k_T \cdot E \qquad\qquad \text{Eq. 3}$$
$$I = k_P \cdot E \qquad\qquad \text{Eq. 4}$$

Dividing Eq. 3 with Eq. 4 provides Eq. 5

$$\frac{U}{I} = \frac{k_T}{k_P}, \text{ or}$$

$$U = \frac{k_T}{k_P} \cdot I \qquad\qquad \text{Eq. 5}$$

Thus, a response on the thermopile (μV) is proportional to a response in the photodiode (10^{-10} A), with the proportionality constant $\frac{k_T}{k_P}$ (μV/10^{-10} A), below called *the k-value*.

Plotting the current from the photodiode against the voltage (Figure 5) from the thermopile resulted in a k-value of 0.227 μV/10^{-10} A with a coefficient of determination (R^2) of 99.66%. This gave the calibration factor for the photodiode of 0.0101 W/(10^{-10}A*m^2). The p-value was < 0.05, and the CI(95) was 0.219-0.236.
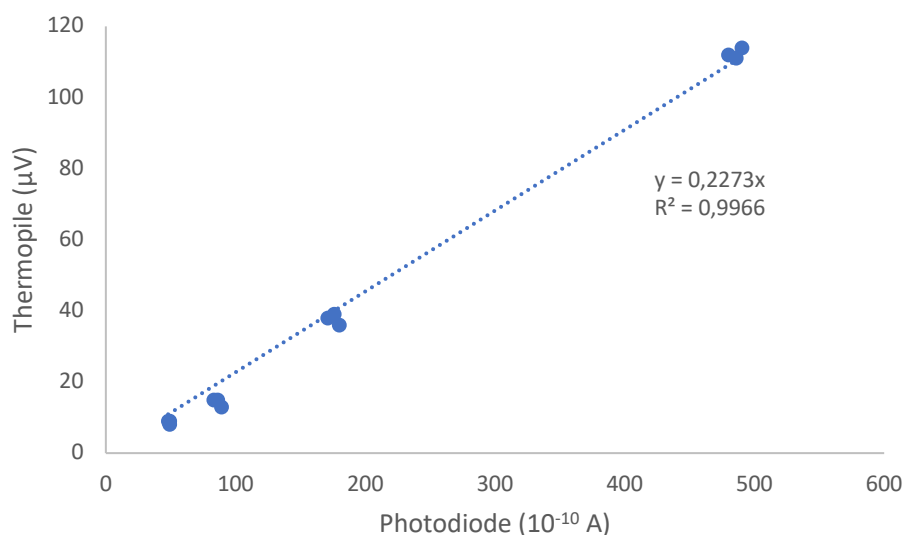
Figure 5: Current from photodiode plotted against voltage from thermopile resulted in the equation. Dotted line is the best fit linear regression omitting the 0th order term.

In order to calculate what current was needed to deliver a total dose of 1 kJ/m^2 of radiation to the rat lenses, the calibration factor of the photodiode was used. At an explorer time of 15 minutes, the power density was 1.1111 J/m^2/s (1000 J/m^2 / 900 s). The current was calculated by dividing the power density with the calibration factor for the photodiode ((1.1111 J/m^2/s)/(0.0101 W/(10^{-10}A *m^2))), which gave a current of about 111*10^{-10}A.

3.3 Calibration of the light dissemination meter

Figure 6 shows the concentrations of Diazepam plotted against the current. It follows the line of a second order polynomial equation, without the first order. The k_1 and k_2- values for each stock solution was calculated and the mean of the values was $k_1 = 52.9$ and $k_2 = -2.54$.
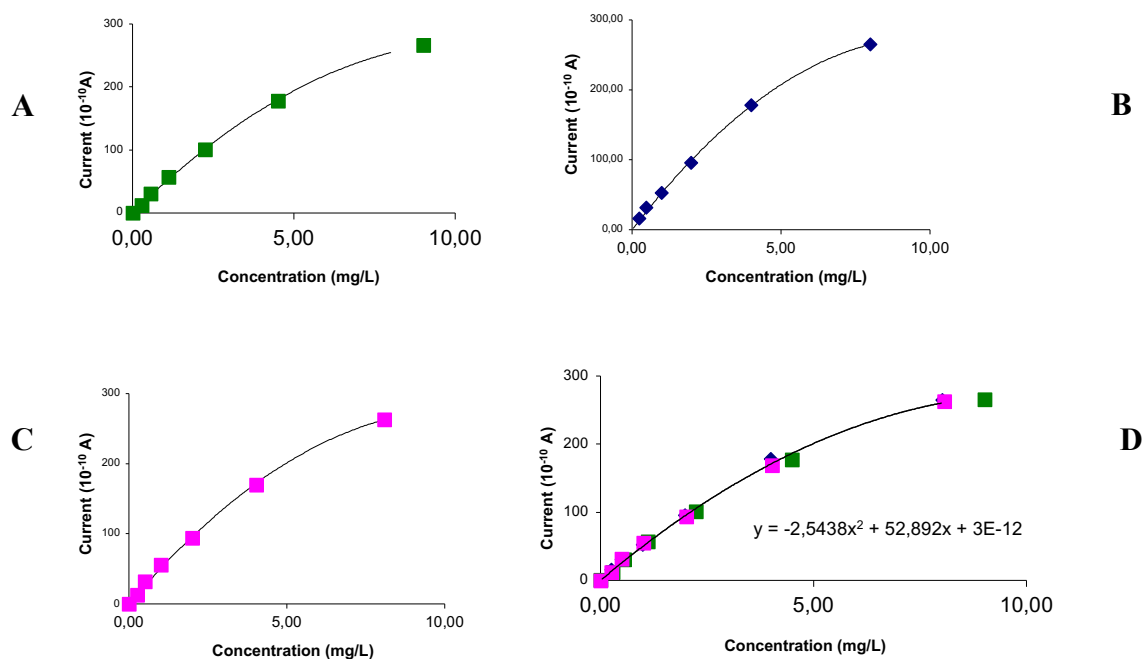
Figure 6: The recorded current plotted against the different concentrations of Diazepam. A = stock 1, B = stock 2, C = stock 3, D = all of the above plotted in the same diagram.

3.4 Light scattering in the exposed and in the unexposed lenses

The lenses of in total seven animals were examined. One rat, in the 1-hour-post-exposure group, died of the anesthesia. Table 4 shows the light scattering, expressed as tEDC, for each lens. The difference in light scattering between the lenses of each rat is shown in Figure 7.
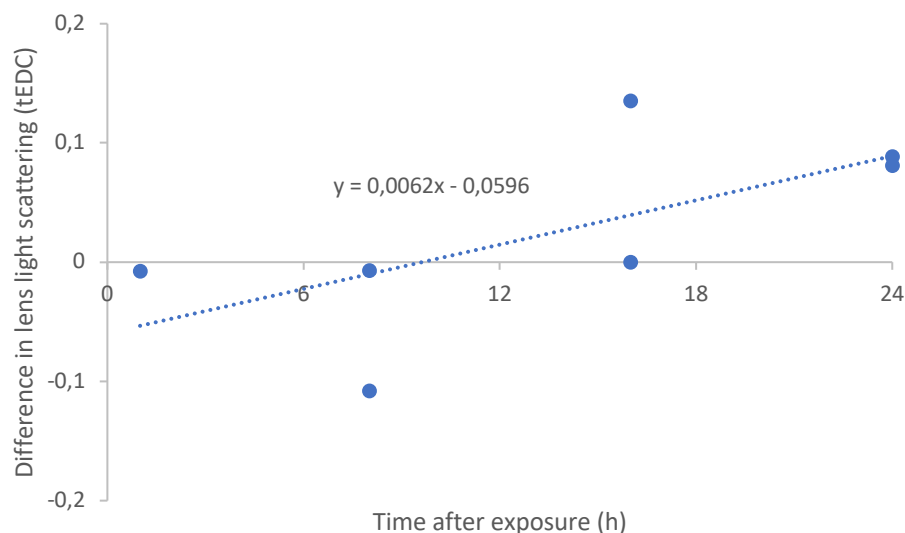
Figure 7: Paired difference in lens light scattering (exposed-unexposed), expressed as tEDC, for each individual rat. Dotted line is least square fitting to a 1st order polynomial.

The k-value is 0.0062, with a CI(95) ± 0.0081. The whole group had a mean difference in light scattering between exposed and unexposed lens of 0.026, with a CI(95) ± 0.075.

Table 4: The light scattering for the exposed (Exp.) and unexposed (Unexp.) lenses of each rat.

Time to sacrifice (h)	Exp. lens light scattering (tEDC)	Unexp. lens light scattering (tEDC)
1	0.196	0.203
8	0.173	0.281
	0.228	0.235
16	0.301	0.166
	0.212	0.212
24	0.247	0.166
	0.183	0.094

4. Discussion

In this study the accuracy of three pipettes was measured, a photodiode was calibrated against a thermopile for international standard of irradiance measurement and an objective and international way of quantifying light scattering was established. The results above were used in the following experiment to expose rat eyes for UVR-B with a dose of 1 kJ/m^2 and measure the forward light scattering in the lenses.

Regression analysis of the result from the animal experiment showed a slight increase in light scattering the longer the time from exposure. However, the R^2-value was low, the confidence interval for the rate constant, k, overlapped 0, indicating that there is no forward light scattering increase with time after exposure. The mean difference between exposed lens and unexposed lens was 0.026 tEDC, but the confidence interval was very large and overlapped 0. It is therefore not possible to conclude that this subthreshold exposure for UVR leads to increased light scattering in the rat lens within 24 hours after exposure.

Though the result is not statistically verified, the experiment indicates that time after exposure might matter for the light scattering. Previous experiments have shown that apoptosis in the rat lens occurs before the opacification (38), and that apoptosis occurs within 24 hours after subthreshold exposure (40). Active apoptosis marker caspase-3 peaks at 16 hours after exposure, but decrease at 24 hours, possibly representing the last stages in apoptosis and death of cells (40). The last post-exposure time in this experiment was 24 hours, which is when most apoptosis is occurring (39). The difference in light scattering between exposed and un-exposed lenses were the highest 24 hours after exposure but given that apoptosis occurs before opacification of the lens and that apoptosis markers is at their highest levels 8-16 hours after subthreshold UVR-B exposure, light scattering might have kept increasing if the experiment had had a longer duration. Further studies are needed to investigate this.

The experimental design of this study has not, as far as the author knows, been done before. From earlier experiments it is known that higher UVR-B dose leads to light scattering within 24 hours (39), but it has also been seen that there is a reparation process that leads to a smaller light scattering a week after exposure (14). Exposure for 1 kJ/m^2 has been done before, but the animals were either sacrificed a week after exposure, giving time for repair processes to heal the cataract, or light scattering was not recorded (40). The result from this experiment is in line with previous experimental studies that have shown that subthreshold doses of UVR-B do not lead to significant light scattering one week after exposure (27) and that reparation processes occur during this time (14,38). The slight increase in light scattering that has been seen in this experiment could very well

repair within a week if the animal had been kept alive, and thus no light scattering would have been seen one week post-exposure.

This study is largely focused on the prework of the animal experiment. No experiment is better than its materials and methods and by refining these, the result of the animal experiment is also refined. For example, even though the smallest of the pipettes (0.5-10 µl) had a mean mass close to that of the wanted volume, the variance between each use was very large. As such, it was excluded from use in developing the standardized light scattering measurement, because it could not be trusted to measure correctly, which in turn could have made the whole animal experiment invalid. Ethically, refining the methods before animal experiments means that fewer animals are needed to get the same results, which fill the requirements Refine and Reduce in the 3 R:s of animal experiment ethics. Since the rats each have two eyes, the individual rat also works as its control, thereby reducing the number of needed animals.

One of the advantages with this method is that the research group has experience with it. To expose one eye and keep the other as control and to measure cataract quantitatively as forward light scattering has been done before and is known to work. The UVR source is flexible regarding wave lengths, but it is not very precise. As seen in figure 2, it generates two wave lengths peaks around 300 nm, but it is not precisely 300 nm. In order to improve the method, an industrial laser with a well-defined wavelength could have been used. Another source of error is the balance used for accuracy measurement of the pipettes and for measuring the concentration of Diazepam. Errors here would make the standardized scale of opacification measuring invalid, and thus the entire result of the animal experiment. This problem could possibly have been avoided by recalibration of the scale or by cross-measuring the mass on another scale. Other improvements of the method would be to have more animals and longer post-exposure interval times.

Only very few animals were used in this experiment, which might have had an impact on the statistics. Increasing the number of animals would have reduced the random error. However, as always with animal experiments, it must be discussed if the results are applicable to human life. For example, the human cornea absorbs more UVR-B than the rat cornea (11,12), thereby protecting the human lens from the radiation to a higher degree than the rat lens. As seen in table 2, the maximum tolerable dose UVR-B before developing cataract has been calculated for rats, around 4 kJ/m^2 (7), but it is reasonable to assume that this dose would be much higher for humans. Furthermore, these kind of experiments have oftentimes used albino rats, and they are more sensitive to UVR than pigmented rats (53). This also makes the corresponding effect of the radiation less than what it

would have been on the average human. Nevertheless, subthreshold doses of UVR-B do lead to increased apoptosis in the lens (41) and the repeated doses may accumulate to cause cataract (26,54). *In vivo* experiments are also more applicable on humans than *in vitro* experiments.

In the experimental situation, 1 kJ/m^2 of UVR-B is a low dosage but compared to the solar radiation on Earth it is high. At 300 nm, the irradiance is 0.005 W/m^2/nm on a clear day with the sun in zenith (8). A total dose of 1 kJ/m^2 under 15 minutes, corresponds to 1.111 W/m^2, which is 220 times higher than the solar radiance with 300 nm. According to Michael et al 1998 (27) an outdoor worker can be exposed to up to 3 kJ/m^2 of 300 nm UVR-B during a period of 75 hours. So even though it takes longer than 15 minutes to be exposed to 1 kJ/m^2 UVR-B in the real life, certain groups of people may still reach comparable subthreshold doses quickly. This experiment does not conclusively show that a single subthreshold dose UVR-B causes significant cataract, but as previously discussed, repeated subthreshold doses accumulate. The eyes need to be protected against UVR, even at low doses. Outdoor workers are at great risk, and though sunglasses are the most used sun protection in this group (55) it is mostly used for glare protection. Since UVR-B is not blocked by clouds (56), this would mean that these people go unprotected on cloudy days. Additionally, the shape and way of wearing the spectacles matter for the protection offered by them, as previously discussed. Ideally, wrap-around glasses should be used (57), but this is beyond the scope of this article.

Conclusion

In this *in vivo* experiment rats were exposed for a single subthreshold dose of UVR-B and the difference in opacification between the exposed and unexposed lenses were measured as light scattering. The dosage was closer to what can be seen on Earth than what has been used in many previous experiments and the post-exposure time was also shorter. In conclusion, single exposure for 1 kJ/m^2 UVR-B does not lead to a statistically significant increase in forward light scattering. Hence, it does not give rise to cataract in the rat lens within 24 hours after exposure. Larger studies with more animals and longer post-exposure times are needed in order to explore the link between subthreshold UVR-B exposure and cataract further, and to get closer to the development of a medical treatment for cataract.

5. Acknowledgements

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Appendix 1: Earlier experimental studies

Title	Publication year	Species	Dosage (kJ/m2)	Time to sacrifice	Purpose	Conclusions	Other
Unscheduled DNA synthesis in lens epithelium after in vivo exposure to UV radiation in the 300 nm wavelength region (58)	1986				Development of an autoradiographic method for studing the pattern of DNA synthesis in the rat lens epithelium	Unscheduled DNA synthesis was induced	
Development of light dissemination in the rat lens after in vivo exposure to radiation in the 300-nm wavelength region (59)	1990		30		Investigating the development of light dissemination in UVR-B exposed lenses	Light dissemination reaches maximum 72 h after exposure. Contralteral non-exposed lens also increases its light scattering.	
Long-term development of lens opacies after exposure to ultraviolet radiation at 300 nm (60)	1996	Sprague Dawley	5 or 20	1, 4, 8, 16 and 32 weeks	Investigating the long-term development of lens opacies after short-term exposure.	Higher doses induce more light scattering.	Only abstact available
Location and severity of UVB irradiation damage in the rat lens (61)	1997	Brown Norway	0.65 every 6 days	Unknown	Investigating the location and severity of lens opacies and epithelial alterations following UVR-B exposure.	Lens epithelial cells and their associated fiber cells are the first target of UVR-B damage.	Only abstact available

Dose-response function for lens forward light scattering after in vivo exposure to ultraviolet radiation (27)	1998	Sprague Dawley	0.1, 0.4, 1.3, 3, 5, 8 or 14 kJ/m2 UVR	1 week	Determination of the dose-response function for UVR-induced opacities	"The intensity of forward light scattering in the rat lens increase exponentially with increased UVR dose between 0.1 and 14 kJ/m2." Doses of at least 3 kJ/m2 produced lens opacities visible to the naked eye.	
Apoptosis in the rat lens after in vivo threshold dose ultraviolet irradiation (39)	1998	Sprague Dawley	5	1, 6, 24 h, 1 week	Investigating DNA damage in rat lenses after close-to-thershold UVR-B exposure	Apoptosis peaks 24 hours after UVR-exposure, involving the entire epithelium, and dead cells are removed via phagocytosis.	
Repair in the rat lens after threshold ultraviolet radiation injury (14)	2000	Sprague Dawley	5	1, 7, and 56 days	Inversigating the lens damage and repair after in vivo close-to theshold UVR-B-exposure.	Close-to-threshold dosage of UVR-B causes cataract, but the changes are largely, though not completely, reversible.	
In vivo cataract after repeated exposure to ultraviolet radiation (54)	2000	Sprague Dawley	8, divided on two separate occasions.	1 week after last exposure	Investigating the effects of repeated close-to-threshold exposure for UVR-B on rat lenses.	The lens is most sensitive to second exposure after 3 days. 30 days between exposures seems to give time to physical repair of the lens.	Intervals between both exposures: 6 h, 1, 3, 9 and 30 days.
Lens Growth and Protein Density in the Rat Lens after In Vivo Exposure to Ultraviolet Radiation (62)	2001	Sprague Dawley	0.1-20	1, 4, 8, 16, or 32 weeks	Investigating the damage mechanism of UVR-B on the eye.	Lenses exhibit a dose-dependent growth-inhibition. Different doses leads to different water content in the lens.	

Metabolic changes in rat lens after in vivo exposure to ultraviolet irradiation: measurements by high resolution MAS 1H NMR spectroscopy (63)	2004	Sprague Dawley	2.5, 5.0, 7.5	1 week	Investigating metabolic changes in the lens after UVR-B exposure.	All groups showed significantely increased light scattering. All groups showed a significantely decrease in water-soluble metabolites, but these were not dose-dependent.	
Ultraviolet radiation-B-induced cataract in albino rats: maximum tolerable dose and ascorbate consumption (64)	2006	Sprague Dawley	0, 0.25, 3.5, 4.3 and 4.9	1 week	Determination of the maximum tolerable dose (MTD) for avoidance of UVR-B induced cataract and studying the UVR-B effect on lens ascorbate (vitamin C) content.	MTD was 3.1 kJ/m2 for 7-weeks-old Sprague Dawley rats. Lens ascorbate content decreased.	
Maximum tolerable dose for avoidance of cataract after repeated exposure to ultraviolet radiation in rats (30)	2007	Sprague Dawley	0-10	1 week after last exposure	Determination of the maximum tolerable dose (MTD) for avoidance of UVR-B induced cataract after repeated UVR-B exposure with different inter-exposure intervals.	Shorter inter-exposure time leads to greater damage. The accumulated MTD2.3:16 was 5.3, 5.1, 5.4, 5.8, and 6.0 kJ/m2 UVR-B for the 6 h, 1, 3, 9 and 30 day inter-exposure interval.	The inter-exposure intervals were 6 h, 1, 3, 9 and 30 days.
p53 expression and apoptosis in the lens after ultraviolet radiation exposure (52)	2007	Sprague Dawley	8	1 week	To compare the localization and expression of p53 and caspase-3 in healthy rat lenses and UVR-B exposed lenses.	p53 and caspase-3 is localized in the lens epithelium and their expression increases after UVR-B exposure.	

	Year	Strain	n	Time	Aim	Result
Evolution of damage in the lens after in vivo close to threshold exposure to UV-B radiation: cytomorphological study of apoptosis (38)	2010	Sprague Dawley	8	1, 7, 48 and 336h	Investigating the damage mechanism of cataract and the repairmechanism.	The cataract is mostly reversible, but not the damage to the cortical fibre cells. Apoptotic features starts showing in the epithelium after 1 hour.
Evolution of light scattering and redox balance in the rat lens after in vivo exposure to close-to-threshold dose ultraviolet radiation (65)	2010	Sprague Dawley	8	1, 3, 7 days	Investigating the cataract development and redox balans in the lens after close-to-threshold UVR-B exposure.	Light scattering increased the longer the time after exposure. The redox balance is altered in a time dependent manor.
Impact of iris pigment and pupil size in ultraviolet radiation cataract in rat (53)	2012	Brown Norway and Fischer-344	5	1 week	Investigating the impact of pigmentation and pupil size in the cataract development.	Pigmentet rats (Brown Norway) developed less cataract than albino rats (Fischer). For the albino rats the pupil size (miotic) plays an important role in the cataract development.

	Year	Strain	Dose	Time	Objective	Result	Notes
Kinetics of GADD45α, TP53 and CASP3 gene expression in the rat lens in vivo in response to exposure to double threshold dose of UV-B radiation (66)	2012	Sprague Dawley	8	1, 5, 24 and 120 h	Investigation of the evolution of the mRNA expression of apoptosis markers and genome stressors following UVR-B exposure.	"Double threshold dose of UVR, for short delay onset of cataract, in vivo causes a transient upregulation of the stress sensor GADD45α, a concurrent downregulation of TP53 and CASP3, followed by a constant upregulation of TP53 that precedes a constant upregulation of CASP3."	
Evolution of TUNEL-labeling in the rat lens after in vivo exposure to just above threshold dose UVB (67)	2013	Sprague Dawley	5	1, 5, 24 and 120 h	Analyzing the TUNEL-labeling (marker for end stage apoptosis) after UVR-B exposure in vivo.	TUNEL-labeling was induced, gradually increasing and peaking between 5 and 120 h.	
Caffeine eye drops protect against UV-B cataract (68)	2013	Sprague Dawley	8 (experiment 1), 0.0, 2.6, 3.7, 4.5 or 5.2 (experiment 2)	1 week	Investigating to which degree caffeine eye drops protects against UVR-induced cataract.	Caffeine applied topically in the eye protects against UVR-cataract with a protection factor of 1.23.	
Cataract after repeated daily in vivo exposure to ultraviolet radiation (54)	2014	Sprague Dawley	0.0, 3.18, 4.50, 5.51, 6.36 (cumulative doses)	1 week after last exposure	Verifying the dose additivity of subthershold dose of UVR exposure over a longer period of time.	Low doses of UVR-B accumulate to cause cataract, but the lens sensitivity for UVR-B decreases.	The rats were exposed on 1, 3, 10 or 30 days in total.

Exposure to subthreshold dose of UVR-B induces apoptosis in the lens epithelial cells and does not in the lens cortical fibre cells (41)	2017	Sprague Dawley	1	120 h	Investigating which part of the lens that goes through apoptosis after in vivo UVR-B exposure.	Apoptosis markers p53 and caspase-3 increases in the lens epithelium, but not in the lens fiber cells.