Prevention of Experimental Cataract Induced by UVR

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Abstract

Cataract is the leading cause of blindness in the world and is defined by opacification of the normally transparent lens of the eye. The major avoidable cause of cataract is ultraviolet radiation (UVR), but no current strategies have been developed to prevent the onset of cataract. Apoptosis and internal and external antioxidant systems that inhibit apoptosis have been shown to play a significant role in cataractogenesis.

The main purposes of this thesis were to study the time evolution of apoptosis, to develop the concept of a protection factor (PF), and to investigate the effect of thioltransferase (Grx1) and topical caffeine in UVR cataract development. Further, to elucidate pharmacokinetics and influence on iris diameter of topical caffeine.

Sprague Dawley rats were exposed to UVR and TUNEL staining of the lens sections was analysed. Grx1+/− and Grx1−/− mice were exposed to 5 sub-doses of UVR. Based on the difference of light scattering between Grx1+/− and Grx1−/− mice, the concept of the PF was developed. Topical caffeine and a placebo were applied to the eyes of separate groups of Sprague Dawley rats that were exposed to sub-doses of UVR and protective effect was evaluated. Penetration of topical caffeine in Sprague Dawley rats to lens and blood was analysed by high performance liquid chromatography. Pupil diameter was measured in groups of unilaterally and bilaterally caffeine-treated ketamine/xylazine anesthetized Sprague Dawley rats.

TUNEL-labeling peaked between 5 and 120 hours after UVR exposure. The PF of Grx1 was 1.3. Moreover, topically administered caffeine protected against UVR-induced cataract development with a PF of 1.23. Topical caffeine peaked at 30 min in the lens, increased up to 120 min in the blood and antagonized ketamine/xylazine-induced mydriasis.

In conclusion, UVR induces apoptosis, which is evidenced by the peak of TUNEL-labeling at 24 hours after UVR exposure. The PF of Grx1 is an objective relative measure of protective properties that allows the comparison of different antioxidant systems and administered antioxidant substances. Grx1 and caffeine are protective against UVR-induced cataract. Topically administered caffeine penetrates to the lens and inhibits UVR-induced apoptosis. Additionally, a miotic effect of caffeine is described for the first time.

Keywords: cataract, ultraviolet radiation, apoptosis, thioltransferase, caffeine

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What really makes science grow is new ideas, including false ideas.

SIR KARL POPPER 1963
(Austrian philosopher)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their corresponding Roman numerals.


Reprints were made with permission from the respective publishers.
Not included manuscripts and papers in this thesis:

Kronschläger M, Yu Z, Talebizadeh N, Meyer LM, Söderberg PG. Delayed apoptosis in lens epithelial cells after in vivo exposure to UVR in the 300 nm wavelength region. submitted


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Background

Epidemiology
Cataract is the leading cause of blindness today (Mariotti, 2010). In fact, according to the latest World Health Organization (WHO) assessment, cataract is responsible for 51% of world blindness, which represents about 20 million people (WHO, 2010). Although cataracts can be surgically removed, barriers that prevent access to surgery are a problem in many countries. In industrialized countries, cataract extraction is one of the most frequently performed surgical procedures and accounts for 10% of the annual health care budget, i.e., $4.1 billion in the United States alone (Busbee, 2003; National-Eye-Institute, 2009). Though an estimated 7 million patients undergo operations for cataracts each year, 16 million are left without this beneficial operation (Thylefors, 1998). Furthermore, the overall need for cataract surgery will dramatically rise in the near future due to the growing aging population, thereby creating a big financial burden for national and international health care systems and a huge demand for cataract surgeons. In 2003, calculations indicated that the number of cataract surgeries taking place would double over the next 20 years (McCarty, 2003). Moreover, no prophylactic treatments are currently available to prevent the onset of cataract. Yet estimates indicate that a delay of only 10 years in the onset of cataract could reduce the need for cataract surgery by as much as 50% (McCarty, 2002).

Ultraviolet radiation (UVR) is the most avoidable risk factor contributing to age-related cataract development. The WHO estimates that 20% of cataracts in the world are directly attributable to excessive solar irradiation, particularly within the ultraviolet frequencies (WHO, 1999). Hollows demonstrated that Australian Aborigines living in areas with high UV irradiation have a higher incidence of senile cataract than Aborigines living in low UV irradiation areas (Hollows, 1981). Moreover, epidemiological studies have specifically linked UVR radiation to cataract development, but the reason for age-related nuclear cataract development is not known (Truscott, 2000). The Chesapeake Bay study of 838 watermen found a significant association between the dose of solar UVR and the cortical opacity (Taylor, 1988). Moreover, the relationship between cortical cataract development and UVR was confirmed for the male population in the Beaver Dam Eye Study (Cruickshanks, 1992). Furthermore, through comparisons between the Reykjavik Eye Study; the Vitamin E, Cataract, and Age-related Maculopathy (VECAT)
study in Melbourne, Australia; and the Singapore-Japan Cooperative Cataract Study, Sasaki showed that cortical cataract occurs at a higher prevalence in subjects living in low latitude regions, such as the population in Singapore (Sasaki, 2003) (Figure 1).

The predominant localization of lens opacification in these studies was the lower nasal quadrant. This supports the theory of Coroneo demonstrating that light coming from the temporal edge of the cornea focuses on the nasal side of the limbus (Coroneo, 1991). Additional experimental studies are needed to further understand cataractogenesis caused by UVR exposure and to test the efficacy of anti-cataractogenic agents.

**UVR and Apoptosis**

In vivo, the lens demonstrates maximum sensitivity to the 300 nm waveband region. (Merriam, 2000; Pitts, 1977). UVR causes photooxidative stress, including the formation of various reactive oxygen species (ROS) (e.g., singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals) in the aqueous humour and other intraocular compartments in the lens (Varma, 1984). As a consequence, lens proteins undergo photochemical reactions. Furthermore, UVR can combine two pyrimidine molecules of DNA to form a ring product, a pyrimidine dimer. If UVR exposure reaches a critical point, the cell is unable to repair the damage, and apoptosis is induced (Michael, 1998b).

To fully understand how apoptosis relates to cataract development, we must distinguish between apoptosis and necrosis. Apoptosis is a physiologi-
cal process that is not harmful to neighbouring cells. However, the same stimulus, e.g., UVR, may cause apoptosis at a low exposure and cause necrosis at a high exposure. Necrosis, however, causes damage to all neighbouring cells. Cardinal signs of necrosis are cell swelling, a disrupted cell membrane, and released cytoplasm, whereas cell shrinkage and convolution, an intact cell membrane, and apoptotic bodies with retained cytoplasm are signs of apoptosis (Kerr, 1972). Michael et al. observed that UVR doses just above the threshold dose cause apoptosis in the lens, but necrosis was not detected at these doses (Michael, 1998b).

There are three different routes for induction of apoptosis: the extrinsic pathway, the intrinsic pathway, and the cytotoxic pathway. The extrinsic pathway involves transmembrane receptor-mediated interactions. To date, the following death ligand-receptor systems have been identified: FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4, and Apo2L/DR5 (Elmore, 2007). The intrinsic pathway has many triggers, including UVR, toxins, and hypoxia. In the cytotoxic pathway, apoptosis is mediated by perforin and granzyme. All three pathways merge into the executive pathway, which is characterized by the activation of execution caspases. Caspase-3 is considered the most important execution caspase, and Talebizadeh recently described the evolution of caspase-3 expression in the lens after UVR exposure (Talebizadeh, 2014). Execution caspases trigger cytoplasmic endonucleases. Endonuclease activation finally results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, and expression of ligands for phagocytic cell receptors. Apoptosis ends with the uptake of apoptotic bodies by phagocytic cells.

Glutathione (GSH) and cytosolic thioltransferase glutaredoxin 1 (Grx1)

To prevent damage from ROS, the lens has a strong defense system including antioxidants like GSH, Vitamin C, Vitamin E, and carotenoids and an enzymatic defense system consisting of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-dependent Grx1. A failure in these defense systems leads to accumulation of ROS, deactivation of sulfhydryl-dependant enzyme systems, a change in lens colour due to the formation of chromophores, and a disruption of membrane structures (Chylack, 2004). Furthermore, proteins are aggregated by protein-protein disulfide bridges and protein-thiol mixed disulfides (PSSG) (Harding, 1996). PSSGs are thought to trigger high molecular weight aggregation and hence cataract development by changing the refractive index (Wu, 1998) (Figure 2).
Reduced GSH is the major antioxidant in the lens (Giblin, 2000; Spector, 1995). The concentration of GSH in the lens is very high in order to protect the lens from exogenous and endogenous ROS and to keep proteins in the lens in a reduced state (Lou, 2003). In vitro and in vivo experiments demonstrated that after exposure to UVB irradiation, GSH concentration in the lens was significantly reduced (Hightower, 1992; Wang, 2010).

The GSH-dependent Grx1 regenerates PSSG in proteins damaged by oxidation by restoring free SH groups for proper enzymatic or protein function. In essence, it is acting as a dethiolating repair enzyme, preventing early oxidative damage to lens proteins (Lou, 2003). Under oxidative stress, when other anti-oxidative enzyme systems, such as GR and GPX, are already inactivated by ROS damage, the Grx1 gene is overexpressed 2-3 times in lens epithelial cells (Raghavachari, 2001). The up-regulated expression of Grx1 under sustained oxidative stress emphasises its role in cell protection, since Grx1 repairs the oxidatively damaged key enzymes and proteins via dethiolation (Xing, 2002). Recently, mitochondrial thioltransferase glutaredoxin 2 (Grx2), an isoform of Grx1, was also found to protect against oxidative stress (Wu, 2010; Wu, 2011). Furthermore, a dysfunction of Grx1 was found in the pathogenesis of diabetic retinopathy, Alzheimer’s disease, and obstructive lung disease (Akterin, 2006; Chung, 2010; Shelton, 2007).
Maximum Tolerable Dose (MTD)

The threshold dose refers to the dose of an agent (UVB in our studies) above which significant response occurs and below which significant response does not occur (Finney, 1971). A dose-response function represents the relationship between the dose of an agent and the corresponding response. Herein, the response is the difference in forward light scattering between UVB-exposed and non-exposed lenses, a measure of the degree of cataract development. The International Commission for Non-Ionizing Radiation Protection (ICNIRP, 2004) published its latest safety limits for the avoidance of UVR-induced cataract and based these safety standards on the assumption that the dose-response function is binary. The response is categorised only as “cataract” or “no cataract” with no graduation (Pitts, 1977). However, Michael et al. demonstrated in a study on albino rats that UVR-induced lens light scattering follows a continuous dose-response function (Michael, 1998a). Consequently, Söderberg et al. developed the Maximum Acceptable Dose (MAD) concept, which is based on determining a limit for the intensity of light scattering that corresponds to the selected tolerance for normality, i.e., 1 - α. A high probability (α) results in a large risk of misclassification, whereas a low probability results in a small risk of incorrect classification. For prevention of toxic effects, only the lower dose region of the sigmoid dose-response function for UVR-induced light scattering is of interest in this study. This lower part of the dose-response function can be approximated to a second order polynomial. The limit for the intensity of light scattering is then projected onto this dose-response function, and the UVR dose that induces on average an equivalent amount of light scattering can be calculated. The big disadvantage of the MAD is that a large sample size is needed to find the adequate tolerance limit for normality. Yet, samples are limited in experimental research, and appropriate sample size is certainly an ethical question. Therefore, Söderberg et al. (Söderberg, 2002) developed the Maximum Tolerable Dose (MTD) strategy.

The concept of MTD is based on the definition of two standards, the tolerance limit for normality and the criterion for significant toxicity. The tolerance limit is set by calculating the difference in lens light scattering in unexposed animals at two standard deviations above zero. In a normally distributed population, the tolerance limit then is 2.3%. The criterion for significant toxicity is set at the point where the difference of lens light scattering between the exposed and contralateral unexposed lens corresponds to one standard deviation above the dose response function, resulting in a probability of 16%. Finally, the MTD is the dose that meets the criteria for the tolerance limit for normality and the criteria for significant toxicity and is denoted as $MTD_{2.3:16}$. The $MTD_{2.3:16}$ can be expressed as Equation 1 and summarized as follows: an animal unilaterally exposed to the $MTD_{2.3:16}$ of UVR has a 16% probability of showing a difference in the forward light scattering of...
the exposed lens as compared to that of the unexposed lens, and this difference is greater than what is found in 97.7% of unexposed animals.

\[ 2\sigma = k(MTD_{2.3:16})^2 + \sigma \quad or \quad MTD_{2.3:16} = \sqrt{\frac{\sigma}{k}} \]

Eq. 1

Table 1 presents the \( MTD_{2.3:16} \) values for different experimental animals.

Table 1: MTD\(_{2.3:16}\) values for experimental animals

<table>
<thead>
<tr>
<th>( MTD_{2.3:16} )</th>
<th>Animal</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9 kJ/m(^2)</td>
<td>Pigmented mouse</td>
<td>(Meyer, 2008)</td>
</tr>
<tr>
<td>3.7 kJ/m(^2)</td>
<td>Albino rat</td>
<td>(Söderberg, 2002)</td>
</tr>
<tr>
<td>4.2 kJ/m(^2)</td>
<td>Pigmented rat</td>
<td>(Kakar, 2003)</td>
</tr>
<tr>
<td>69 kJ/m(^2)</td>
<td>Pigmented guinea pig</td>
<td>(Mody, 2010)</td>
</tr>
</tbody>
</table>
Protection factor (PF) and sunscreens

Irradiation of sun causes erythema in the unprotected skin depending of skin pigmentation. Sunscreen allows prolonged sun exposure before erythema occurs. The protection factor (PF) of a sunscreen is calculated as the threshold dose of UVR for erythema with the sunscreen, $H_{\text{Th: sunscreen}}$, compared with the threshold dose for erythema without the sunscreen, $H_{\text{Th: No sunscreen}}$, (Equation 2).

$$PF = \frac{H_{\text{Th: Sunscreen}}}{H_{\text{Th: No sunscreen}}}$$  

In other words, sunscreen allows a PF-fold longer exposure to sun before erythema develops than without. An analogous concept could be introduced for UVR-induced cataract, using MTD as the threshold dose.

Caffeine

Caffeine was first found in 1819 by a German chemist named Friedrich Ferdinand Runge. Caffeine was independently isolated in 1821 by three French chemists: Pierre Jean Robiquet, Pierre-Joseph Pelletier, and Joseph Bienaime Caventou. Despite this long history, almost one hundred years passed before Nobel Prize Winner Hermann Emil Fischer decoded the structure of caffeine and synthesised it (Figure 3).

![Chemical structure of caffeine](image)

Figure 3: Chemical structure of caffeine.

Today caffeine is consumed by 90% of people around the world (the Food and Drug Administration (FDA), 2007) in the form of cola or other soft drinks, tea, coffee, and/or chocolates. The FDA recommends a limit of 100
to 200 mg (i.e., one to two 5-ounce cups of coffee) per day and considers 600 mg (i.e., four to seven 5-ounce cups of coffee) per day excessive.

Caffeine stimulates the central nervous system, thereby increasing blood pressure, decreasing the heart rate, reducing cerebral flow, relaxing bronchial and vascular smooth muscles, and promoting diuresis and intestinal motility. Despite its stimulating effects, a relationship between coffee and hypertension was not found in a study with 155,000 nurses (Cromie, 2005). Moreover, in a Harvard study with 128,000 people, an association between coronary heart disease and coffee was not established except when it was consumed while smoking cigarettes and consuming excessive amounts of alcohol (Lopez-Garcia, 2006).

Caffeine offers some beneficial effects for certain health issues. It was found to reduce the incidence of Alzheimer’s and Parkinson’s disease (Benedetti, 2000; Lindsay, 2002; Maia, 2002; Ritchie, 2007; Ross, 2000). In addition, coffee lowers the risk of type 2 diabetes (Iso, 2006; van Dam, 2006), decreases the risk of impaired liver function and cirrhosis (Cadden et al., 2007; Klatsky et al., 2006), and decreases the risk of certain cancers (Ganmaa, 2008; Inoue, 2005; Song, 2008; Tavani, 2003).

Interestingly, caffeine has a high antioxidant potential (Shi, 1991). It scavenges reactive oxygen species (ROS) (Leon-Carmona, 2011). This could be quite important in the lens. The main source for exogenous ROS that stress the lens is ultraviolet light (Lou, 2003). In in vitro experiments, Varma showed that caffeine protects the lens from oxidative damage and reduces UVR-induced cataract development (Varma, 2008; Varma, 2010a; Varma, 2010b). Furthermore, Varma demonstrated in vivo that topically administered caffeine prevents cataract development in a galactosemic rat model (Varma, 2010c) and that intraperitoneally injected caffeine prevents cataract in a selenite rat model (Varma, 2010). Varma also showed that caffeine preserves GSH concentration levels in the lens (Varma, 2010a) and protects cells from apoptosis (Varma, 2011). On the other hand, little is known about topically administered caffeine and its pharmacokinetics. Varma described a peak caffeine concentration in aqueous humour and in the lens at 60 min after topical application (Varma, 2010c). No data is available on systemic absorption of caffeine from topical eye drops. Caffeine is metabolized by cytochrome P-450 1A2 (Cyp 1A2) in the liver. Two main metabolites are 1-methylxanthine and 1-methyl uric acid. These metabolites must be taken into account when considering the biological activity of caffeine. It has been shown that they have significant antioxidant activity (Lee, 2000). On a cellular level, at micromolar concentrations, i.e., a concentration achieved after a cup of coffee, caffeine blocks the effects of adenosine on A2A and A1 receptors. At millimolar concentrations, caffeine inhibits phosphodiesterase (which catalyzes cAMP) and therefore increases cAMP levels; it also blocks GABA<sub>A</sub> receptors and mobilizes intracellular calcium depots. Recently,
Varma described the suppression of toxic microRNAs and consequent gene silencing by oral caffeine intake (Varma, 2013).

Considering the impact of UVR on the lens and the shielding of the lens by the iris, the impact on iris diameter becomes important. Up to date, there are no reports of topically administered caffeine and its influence on pupil diameter. Overdoses of caffeine have either induced miosis or mydriasis (Brooks, 2011; Jorens, 1991; Rowland, 1976; Yew, 2013).

Ketamine/Xylazine and pupil diameter

Ketamine in combination with xylazine is a commonly used anesthetic in animal experiments. It has been widely used in UVR exposure experiments and is considered a safe and reliable drug mixture for anesthesia. Its effect on pupil diameter is mydriasis. The mechanism of action for ketamine-induced mydriasis is inhibition of the cholinergic system both centrally (by causing a prominent sympathetic tonus) and peripherally (by directly blocking muscarinic acetylcholine receptors in the iris). Xylazine induces mydriasis in two ways: by inhibiting central parasympathetic tonus and by stimulating alpha-2 adrenergic receptors in the iris. This knowledge will be essential in studying caffeine and pupil diameter in anesthetized rats.
Aims

Paper I
To quantitatively elucidate the evolution of TUNEL-labeling in the rat lens after in vivo exposure to just above the threshold dose of UVR for up to 120 hours.

Paper II
To determine the protection factor (PF) for Grx1 with regard to UVR-induced cataract development by comparing the in vivo UVR lens toxicity between double knockout Grx1$^{-/-}$ and Grx1$^{+/+}$ mice.

Paper III
To investigate the possible protection that topically applied caffeine affords against in vivo UVR cataract development and, if topically applied caffeine proves to be protective in this case, estimate the PF.

Paper IV
To study the penetration of topical caffeine in the lens and blood and to evaluate the safety of locally and systemically absorbed caffeine.

Paper V
To examine the influence of topically administered caffeine on iris diameter in ketamine/xylazine anesthetized rats.
Materials and Methods

UVR Source (Paper I, Paper II and Paper III)
In all UVR exposure studies, the same UVR source was used. UVR from a high-pressure mercury lamp (HBO lamp, Osram) was collimated, passed first through a water filter to absorb infrared radiation, and then passed through a double monochromator (2x77250, Oriel). The monochromator allowed for the selection of a specific waveband of radiation, i.e., 300 nm in this study, and the double alignment minimized stray light. The emerging radiation was finally projected as a field covering the cornea of the exposed eye (Figure 4).

![Diagram of the UVR source](image)

Figure 4: Schematic diagram of the UVR source adapted from Ralph Michael (Michael, 2000)

The spectral distribution of the output beam was measured with a fibre-optic spectrometer (Ocean Optics PC 2000, Ocean Optics, Dunedin, Florida, USA). The full spectrum is shown in Figure 5.
Irradiance of UVB on the corneal plane was controlled with a thermopile detector (7104, Oriel, USA), calibrated to a National Institute of Standards and Technology (NIST) traceable source. The thermopile senses the temperature gradient produced by the UVB absorption and converts the thermal energy of the UVB to a voltage.

Measuring forward light scattering (Paper II and Paper III)

Experimentally induced cataract was quantified as forward lens light scattering. The intensity of the forward light scattering in the lens was measured with a light dissemination meter (Söderberg, 1990) (Figure 6).

Figure 5: Relative spectral irradiance of the UVR source

Figure 6: The principle of measurement for the determination of the intensity of forward light scattering in the lens
This instrument uses the principle of dark field illumination. The lens is transilluminated at 45 degrees against the horizontal plane. If the lens under measurement is crystal clear, the illuminating light goes through the lens without being collected onto the photo detector. If light scattering in the lens is measured, a fraction of the light scattered is collected and projected onto the photodiode. The scattering standard was an emulsion of diazepam (Stesolid Novum, Dumex-Alphapharma, Denmark). Light scattering was therefore expressed as the transformed equivalent diazepam concentration (tEDC) (Söderberg, 1990).

The protection factor (Paper II and Paper III)

The concept of the PF was adapted from the protection provided by sunscreens against UVR-induced erythema. For sunscreens, the protection factor is calculated as the quotient of the threshold dose for erythema with sunscreen and the threshold dose for erythema without sunscreen. In UVR-induced cataracts, the threshold dose is the MTD_{2.3:16}; thus, the protection factor is determined by the quotient of the MTD_{2.3:16} with protection and the MTD_{2.3:16} without protection (Equation 2).

\[
P_F = \frac{\text{MTD}_{2.3:16} \text{ (with protection)}}{\text{MTD}_{2.3:16} \text{ (without protection)}}
\]

Eq. 2

Thus, a higher PF indicates that longer exposure to UVR is possible before the development of a cataract is observed.

Immunohistochemistry (Paper I and Paper III)

Cryosectioned lenses were analysed using immunohistochemistry. To detect apoptosis, we chose TUNEL-labeling and caspase-3 staining. For TUNEL-labeling, the In Situ Cell Death Detection Kit-Fluorescein (No. 684 795, Boehringer Mannheim) was used according to the manufacturer’s protocol. For the staining of activated caspase-3, we used 1:10 diluted rabbit polyclonal primary antibodies (ab 2302, Abcam) and 1:300 diluted fluorescein tagged donkey polyclonal secondary anti-rabbit IgG antibodies (ab 6798, Abcam). The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and examined with a fluorescence microscope (Universal Microscope Axioplan 2 Imaging, Carl Zeiss, Germany).
GSH Measurement (Paper III)

Whole lenses were homogenized using a lysis buffer and centrifuged. The supernatant was further processed following an adapted protocol (Uptima/Interchim) based on that of Sedlak (Sedlak, 1968). The GSH concentration was then indirectly estimated using colorimetric and spectrophotometric analyses; i.e., the change in the absorption of an agent oxidizing GSH was recorded. Lens protein levels were measured with the bicinecinonic acid (BCA) method (Reagent Kit 23225 23227; Pierce Inc., Rockford, USA). All GSH measurements were then normalized to the lens protein concentration.

Macrophotography (Paper II, Paper III and Paper IV)

All morphological changes in the lenses after UVR exposure were documented via macrophotography with dark field illumination against a dark background. In dark field illumination, the light is incident on the lens examined at an angle to the optical axis of the lens examined. Minimal changes in the local refractive index can be visualized by this method.

Caffeine eye drop preparation (Paper III, Paper IV and Paper V)

Caffeine eye drops were introduced by Varma (Varma, 2010c). In our studies, we adopted his preparation by adding 0.9% hydroxypropylmethyl cellulose (Sigma-Aldrich, H7509) to 0.72, 3.34, 15.51 and 72 mM aqueous solutions of caffeine.

HPLC (Paper IV)

HPLC has been tested to be a reliable and simple method to analyse caffeine in tissue and serum (Biaggioni, 1988). Before analysis by HPLC, lens homogenates and blood samples were centrifuged at 12000 g at 4° C for 10 min. The supernatant of lens and blood samples was then used for further analysis. HPLC was performed by using the following equipment: Reversed-type column, 100 x 3 mm, 5 μm (packing material: ChromSpher C18); HPLC pump (Jasco-PU-980 HPLC Pump); degasser (Jasco DG-980-50 Degasser); and a Jasco UV-975 UV/VIS Detector at 273 nm. The mobile phase used in this assay was composed of 0.05 M acetic acid, 0.05 M ammonium acetate and 9% acetonitrile in MilliQ water. The flow of the mobile phase was 0.5 ml/min. The standards for the calibration curve were prepared from different dilutions of 80 μM caffeine in MilliQ water.
Pupil diameter (Paper V)

Pupil diameter was measured with a ruler in the slit lamp microscope.

Experimental design

In Paper I, the evolution of TUNEL staining in the rat lens after unilateral 8 kJ/m² UVR exposure was investigated. Sixteen Sprague Dawley rats were divided into 4 latency groups (i.e., exposure for 1, 5, 24, and 120 hours) with each group containing 4 rats. TUNEL staining was conducted in each lens, and the ratio between the TUNEL-positive epithelial cells in exposed and unexposed lenses was calculated. Furthermore, the ratio between TUNEL-positive epithelial cells in the upper and lower hemiquadrant was calculated.

In Paper II, the concept of the PF was developed by comparing Grx1+/+ with Grx1−/− mice. Twenty Grx1+/+ and 20 Grx1−/− mice were each subdivided into five exposure groups. The exposure doses were calculated using Equation 3, resulting in sub-threshold doses of 0.0, 2.1, 2.9, 3.6, and 4.1 kJ/m².

\[ H_g : \theta_h = \sqrt{(g-1) \left( \frac{E(MTD_{2,3:16})}{2} \right)^2} \]  

Eq. 3 Calculation of subdoses

After unilateral UVR exposure, forward light scattering was measured.

Paper III examined the protective effect of topically applied caffeine in three experiments. In the first experiment, 40 Sprague Dawley rats were randomly assigned to a caffeine group and a placebo group. All animals were unilaterally exposed to UVR at a dose of 8 kJ/m², which is just above the threshold dose. Light scattering was measured, and the lens GSH concentration of the first ten animals from each group was analysed. In the second experiment, the PF was calculated following the design of Paper II and the sub-dose calculation using Equation 3. The data from the 8 kJ/m² exposure were included in the PF calculation. Finally, in the third experiment, three Sprague Dawley rats were unilaterally exposed to 8 kJ/m² of UVR. Seven hours after exposure, the eyes from the rats were cryosectioned and analysed for active caspase-3 expression in the lenses.

In Paper IV, we looked into the pharmacokinetics of caffeine. Three experiments were conducted. First, four groups of three animals were treated with 72mM of topically applied caffeine and sacrificed at 30, 60, 90 or 120 minutes (depending on the group), to study local toxicity. Thereafter, four groups of ten rats received caffeine eye drops bilaterally. Caffeine concentrations in lens and blood supernatant were analysed at 30, 60, 90 and 120 min (depending on the group) after caffeine administration. Finally, four groups of ten rats received eye drops having different concentrations of caf-
feine, i.e., 0.72, 3.34, 15.51 and 72 mM. Caffeine concentration in lens and blood supernatant was analysed at 30 min after topical caffeine administration.

Paper V uncovered the influence of topical caffeine on pupil diameter in ketamine/xylazine anesthetized rats. Two experiments were carried out. In the first experiment, ten rats received caffeine eye drops in one eye and placebo eye drops in the contralateral eye. In the second experiment, ten rats received caffeine eye drops bilaterally and ten rats received placebo eye drops bilaterally. Pupil diameter was measured at 10, 20, 40 and 60 min after caffeine administration. In the second experiment, three rats of the caffeine group also received 1% tropicamide 60 min after caffeine application. Pupil diameter was measured 5 min after tropicamide application.

Experimental procedure
In the first three papers, the animals were exposed to UVB in vivo. The unexposed eye was shielded during exposure and used as a control. Before exposure, the animals were anesthetized. The rats were anesthetized via intraperitoneal injections of a mixture of 94 mg/kg ketamine and 14 mg/kg xylazine, and the mice were anesthetized via subcutaneous injections of a mixture of 40 mg/kg ketamine and 5 mg/kg xylazine. Additionally, 1% tropicamide was administered by eye drop in both eyes to induce mydriasis. For the experiment detailed in Paper III, caffeine or placebo drops were instilled in both eyes 55 minutes prior to UVR exposure.

After the animals were sacrificed, the eyes were enucleated, and, for the experiments in Papers II and III, the lens was extracted from each eye. For the study detailed in Paper I and the third experiment in Paper III, the whole eye globe was processed for cryosectioning and immunohistochemical analysis. Each extracted lens was placed in a balanced salt solution (BSS), and remnants of the ciliary body were removed using microsurgical instruments. Forward light scattering was quantitatively measured three times for each lens, followed by dark field illumination photography. In the first experiment of Paper III, the lenses were analysed for GSH concentration.

In Papers IV and V, rats were anesthetized with a mixture of 94 mg/kg ketamine and 14 mg/kg xylazine, as in Paper I and III. Caffeine or placebo drops were applied when the rats showed no more reflexes. In Paper IV, immediately after sacrifice of the rat, the lenses were taken and homogenized for further analysis. Blood was taken directly from the heart and centrifuged in vials to extract the blood homogenate. Caffeine eye drop concentration used in Papers III and V was 72 mM, respectively.

Statistical analysis
For all the papers, the significance level was set to 0.05, and the confidence coefficients were set at 0.95.
Results and Discussion

As previously indicated, cataract is the most common cause of blindness in the world (Mariotti, 2010). Increasing age is associated with increasing risk for the development of cataract (McCarty, 1999). As previously implied, the burden of the increasing aging population demands the development of prophylactic treatments. UVR has been identified as the major avoidable risk factor (WHO, 1999). In the present thesis, UVR-induced apoptosis of lens epithelial cells is elucidated as the primary event in cataract development, the concept of the PF is developed, topically applied caffeine as a prophylactic treatment against the development of UVR-induced cataract is demonstrated, and the pharmacokinetics and influence on pupil diameter of topically applied caffeine are determined.

Evolution of TUNEL-labeling in the rat lens after in vivo exposure to just above threshold dose UVB (Paper I)

TUNEL-labeled nuclei were observed in both exposed and unexposed lenses, though to a greater extent in exposed lenses. A two-sided paired sample Wilcoxon test was used to evaluate the difference in the fraction of TUNEL-stained cells from exposed versus contralateral unexposed lenses. The results indicated a significant difference of 9% (test statistics: \( T_- = 2, T_+ = 76 \); significance limit: \( T_{0.05;16} = 29 \)). In exposed lenses, the most TUNEL-labeling was observed at the anterior pole of the lens, whereas fewer TUNEL-labeled nuclei were observed in the equatorial region. The relative difference in TUNEL-labeling for the exposed lens minus that of the contralateral unexposed lens transiently increased and peaked at 24 hours after exposure (Figure 7).
Figure 7: Evolution of TUNEL-labeling after in vivo exposure to 5 kJ/m² UVR-300 nm in the rat lens. Intervals are maximum and minimum difference of the ratio of stained cells between exposed and contralateral unexposed lenses.

Post-exposure intervals were compared using orthogonal double sided Mann-Whitney tests according to the following strategy: 1 hour versus 5 hours; 1 hour and 5 hours versus 120 hours; and 1 hour, 5 hours, and 120 hours versus 24 hours. Significant differences (p < 0.05) were found for the comparisons of 1 hour and 5 hours versus 120 hours and for 1 hour, 5 hours, and 120 hours versus 24 hours. No significant difference in TUNEL-labeling was observed between the upper and lower midsagittal hemiquadrant.

Our finding of a transient peak in TUNEL-labeling between 5 and 120 hours after UVR exposure confirms the study results of Michael (Michael, 1998b). In fact, Michael et al. qualitatively described a peak in TUNEL-labeling at 24 hours after UVR exposure. Furthermore, our findings supports a previous study showing an increase in TUNEL-labeling up to 24 hours after exposure to far broadband UVR (Sun, 2001). The coverage of the equatorial lens by the iris despite mydriasis during UVR exposure might result in fewer TUNEL-labeled cells in the equatorial region. The homogeneous distribution of TUNEL-labeling between the upper and lower midsagittal hemiquadrant regions demonstrates that no favoured location for apoptosis is associated with equivalent UVR exposure.
Protective effect of the thioltransferase gene on in vivo UVR-300 nm-induced cataract (Paper II)

The exposed lenses showed increased development of subcapsular and cortical cataract with increasing UVR dose, and this increase was most prominent in mice missing the Grx1 gene. The estimated MTD$_{2.3:16}$ was 3.0 kJ/m$^2$ (CI(0.95) = [2.3;4.0], d.f. = 17) and 3.8 kJ/m$^2$ (CI(0.95) = [2.8;6.4], d.f. = 18) for Grx1$^{+/}$ and Grx1$^{+/+}$ mice, respectively, resulting in a PF of 1.3 (Figure 8).

Figure 8: Light scattering difference between exposed and contralateral unexposed lenses at 48 h after exposure as a function of in vivo UVR-300 nm dose. Grey dots correspond to lenses from glutaredoxin double knockout animals (Grx1$^{-/-}$) and dark dots correspond to lenses from the same strain of animals with the glutaredoxin gene intact Grx1$^{+/+}$. The solid lines represent the least squares fit for differences between lenses originating from Grx1$^{-/-}$ animals (grey curve) and Grx1$^{+/+}$ animals (dark curve).

In other words, the thioltransferase gene therefore allows a 1.3 times longer UVR exposure time before damage occurs. This result supports previous findings indicating that Grx1 is involved in resistance to antioxidant stress (Löfgren, 2008; Lou, 1995; Xing, 2002); moreover, these results also support findings that the missing thioltransferase gene increases lens susceptibility to UVB (Meyer, 2009). Several external and internal antioxidant systems in the lens have been described. The development of the PF concept provides a unique tool for the comparison of antioxidant systems.
Caffeine eye drops protect against UV-B cataract (Paper III)

When we designed the study, we again chose the Sprague Dawley rat as our experimental animal because of the extensive knowledge about UVR-induced cataract in the Sprague Dawley rat (Galichanin, 2010; Mody, 2008; Söderberg, 1990; Söderberg, 2012; Söderberg, 2002; Talebizadeh, 2012; Wang, 2010). Varma recently demonstrated caffeine penetration to aqueous humour and lens (Varma, 2010c). Caffeine eye drops that contain 72 mM caffeine result in a peak concentration of 6 mM in the lens 60 min after application (Varma, 2010c). We therefore instilled caffeine drops 55 minutes before UVR exposure. In the first experiment, the placebo group developed more cataract than the caffeine group (Figure 8).

![Figure 9: Darkfield illumination photographs of 8 kJ/m² UVB-exposed and control lenses from caffeine- and placebo-treated rats.](image)

The difference in forward light scattering was significant as specified by a 95% confidence interval for the mean of the difference between the caffeine and the placebo group (CI(0.95) = 0.103 ± 0.05 tEDC, d.f. = 26). The GSH concentrations in the caffeine and the placebo groups after exposure to 8 kJ/m² of UVR and a latency time of one week were not significantly different as indicated by a 95% confidence interval for the mean of the difference (CI(0.95) = -0.04 ± 0.56 nmol/mg protein, d.f. = 16). Wang (Wang, 2010) has shown that GSH levels were restored one week after UVR exposure, which supports our findings. However, we observed a significant protective
effect from topically applied caffeine; therefore, we continued with the second experiment.

In the second experiment, the MTD$_{2.3:16}$ was 5.7 kJ/m$^2$ (CI(0.95) = [4.9; 6.8] kJ/m$^2$, d.f. = 37) and 4.6 kJ/m$^2$ (CI(0.95) = [4.0; 5.2] kJ/m$^2$, d.f. = 37) for the caffeine group and the placebo group, respectively (Figure 10).

![Figure 10: Lens light scattering at one week after exposure to various doses of UVB. Grey colour corresponds to lenses from the caffeine group and black colour corresponds to lenses from the placebo group. The solid lines represent the least squares fit for each group. Vertical lines are the MTD$_{2.3:16}$ of the two eye drop groups.]

The PF was calculated as 1.23. To our knowledge, this is the highest protection factor calculated for any administered antioxidant to date. Orally administered vitamin E has a protection factor of 1.14 (Söderberg, 2012), and orally administered vitamin C was found to have no protection (Mody, 2008). Only the presence of the Grx1 gene has a higher protection factor (i.e., 1.3) as compared to mice that were missing the gene.

Finally, in the third experiment, we observed much less active caspase-3 expression in the caffeine group. The post-exposure latency for active caspase-3 staining was set to 7 hours based on recent data on the evolution of caspase-3 staining after UVR exposure (Talebizadeh, 2012). These results suggest that caffeine prevents cataract development by inhibiting apoptosis, which agrees with previous findings (Varma, 2010c, 2011).

**Pharmacokinetics for topically applied caffeine in the rat (Paper IV)**

In the first experiment, no macroscopic changes to the eyelids, the conjunctiva, the cornea and the lens were observed.

In the second experiment, which explored caffeine concentration in lens and blood as a function of time, lens caffeine concentration peaked at 30 min after administration of 72 mM caffeine (Figure 11).
Figure 11: Lens caffeine concentration 30, 60, 90 and 120 minutes after 72 mM caffeine eye drop application. Bars are estimated CI(0.95) for the mean (d.f. = 9). Solid line: best fit regression, $C = 3.7e^{-0.008t}$.

In orthogonal testing, we found a significant difference for the interval 30 versus 60, 90 and 120 min (test statistic: 5.47; significant limit (0.05): 2.47). Caffeine concentrations ranged from 3.75 nmol/ mg protein at 30 min to 1.73 nmol/mg protein at 120 min. While caffeine concentrations peaked very early in the lens, in blood supernatant, caffeine concentration increased up to 120 min. Concentrations ranged from 40.54 µM at 30 min to 69.12 µM at 120 min. Similar variances of the groups were shown with Bartlett’s test and a regression line ($y = 0.64t$) with a 95% confidence interval = $Y_i \pm t_{0.05}(1), 35 s_{Y_i}$ was fitted (Figure 12).

Figure 12: Caffeine concentration in supernatant of blood 30, 60, 90 and 120 minutes after topical administration of 72 mM caffeine. Bars are estimated CI(0.95) for the mean (d.f. = 8). Solid lines: regression line (black: best fit, gray: CI(0.95), $C = 0.64t$).
The analysis of variance indicated a variation among the time points (F-statistic = 9.38, $F_{3;32,0.95} = 2.91$).

In the third experiment, application of eye drops containing different concentrations of caffeine, we found significant differences for all intervals analysed by orthogonal testing. Caffeine concentrations in the lens ranged from 0.14 nmol/mg protein for those administered 0.72 mM caffeine eye drops to 4.86 nmol/mg protein for those administered 72 mM caffeine eye drops (Figure 13).

![Figure 13: Lens caffeine concentration at 30 minutes after application of 0.72, 3.34, 15.51 and 72 mM caffeine eye drops. Bars are estimated CI(0.95) for the mean (d.f. = 9). Solid line: best fit regression, $C_L = 0.07C_T$.](image)

Similarly, significant differences were also found in caffeine concentrations in blood supernatant, with a concentration of caffeine from 1.65 µM to 43.6 µM depending on the eye drop concentration applied (Figure 14).
Varma reported a peak caffeine concentration at 60 min after eye drop application (Varma, 2010c) using $^{14}$C1-caffeine and radioactivity measurements. The time resolution of the peak in that paper is ambiguous because neither confidence intervals nor standard deviation were reported. Varma’s data may be consistent with the present data. Moreover, HPLC has been proven a valid method to measure caffeine in plasma and tissue (Biaggioni, 1988). In Paper III, we demonstrated that caffeine eye drops prevent UVR-induced cataract as assessed 60 min after caffeine administration. The caffeine concentration in the lens at 60 min is almost identical to that at 120 min. Based on our results, it can be speculated that it would be necessary to administer eye drops three times in a two hour interval in order to obtain protection against 6 hours’ exposure to the sun. Regarding the safety of systemically absorbed caffeine, blood levels in rats at 120 min after caffeine administration reached levels equal to five-fold lower than the blood caffeine concentration after consuming a cup of coffee when extrapolated to humans.

**Topically applied caffeine induces miosis in the ketamine/xylazine anesthetized rat (Paper V)**

Caffeine eye drops induced miosis in ketamine/xylazine anesthetized rats. Miotic effect of caffeine drops peaked at 40 min both in the first and the second experiment (Figures 15 and 16).
Figure 15: Difference of pupil diameter between placebo-treated eye and contralateral caffeine-treated eye. Bars are 95% confidence intervals for the mean (d.f. = 9).

Figure 16: Time evolution of pupil diameter associated with topical administration of either placebo or caffeine after ketamine/xylazine-induced anesthesia. Bars are 95% CI for the mean (d.f. = 9). Solid line: Average of regressions obtained by fitting the data from each animal with non-linear regression to a model predicting exponential decline towards a minimum asymptote pupil diameter (dotted horizontal line).

We observed no major influence of systemically absorbed caffeine on pupil diameter of the contralateral eye (to which placebo was administered) in the first experiment. Since we know from the results of Paper IV that topically applied caffeine is systemically absorbed, we would have expected an earlier induction of miosis in the placebo-treated eye.

There are two known peripheral effects that could possibly play a role in the induction of miosis by topically applied caffeine. First, caffeine’s interaction with the adenosine A1 receptor could induce acetylcholine release (Carter, 1995). Second, caffeine could mobilize intracellular Ca^{++}-depots and
further stimulate the contraction of smooth muscle cells (Fredholm, 1999). Tropicamide immediately antagonizes the miotic effect of caffeine, which makes it less likely that tropicamide and caffeine competes for the same receptor and more likely that caffeine locally stimulates contraction in the iris sphincter or relaxation in the dilator.

Miosis of topically applied caffeine could have influenced UVR-induced cataract in the experiments described in Paper III. However in the experiments described in Paper III, we used tropicamide before UVR exposure. Therefore, the PF of caffeine is not influenced by a shielding effect from the iris.
Conclusions

- Near-threshold doses of UVR-300 nm induces TUNEL-labeling.
- TUNEL-labeling peaks around 24 hours after exposure to UVR-300 nm.
- Deficiency of the Grx1 gene makes the lens 30% (PF 1.3) more sensitive to early onset UVR-300 nm-induced cataract, indicating the important role of the Grx1 gene in the oxidation defense of the lens.
- The PF is an objective relative measure of protective properties.
- Topically administered caffeine protects against the early onset of cataract development induced by in vivo exposure to UVR-300 nm.
- Topically administered caffeine reduces the lens sensitivity to UVR-300 nm by 23% (PF1.23).
- Topically administered caffeine prevents UVR-300 nm-induced cataract development by inhibiting apoptosis.
- Among the administered substances we have tested so far, Vitamin C has no PF and Vitamine E has a PF of 1.14. Caffeine provides the highest PF.
- Topical caffeine penetrates to the lens and blood in rats.
- Caffeine quickly accumulates in the lens with a slow washout, whereas caffeine slowly accumulates in the blood.
- The accumulation of caffeine in lens and blood is directly proportional to its topically applied dose.
- Rat caffeine blood levels achieved are far below the equivalent threshold dose of FDA recommended daily dose for humans.
- No toxic effects are observed on eyelids, conjunctiva, cornea and lens.
- Topically administered caffeine antagonizes ketamine/xylazine-induced mydriasis.
- The miotic effect of caffeine is immediately antagonized by tropicamide.
- Systemically absorbed caffeine seems not to influence pupil size.
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Future perspective

Today’s solution for cataract is cataract surgery. Cataract surgery is the most common surgical procedure worldwide. It is a standard procedure with minimal risks. But there are still some risks like corneal decompensation, capsule rupture, infections, retinal detachment and macula oedema, each of which may result in reduced vision and blindness. In a time where vitrectomy is preferred over buckling in retinal detachment and where intravitreal injections with cortisone (e.g., Ozurdex©, Iluvien©, Retisert© and Triamcinolon) are popular treatments, cataract is an inevitable side effect. Cataract surgery may improve vision in those cases but will also take accommodation in young patients.

Delaying the onset of cataract is the ultimate goal of cataract research. Up to date, there is no effective treatment to delay cataract development. We have for the first time established a PF to compare different candidate agents (Table 2).

Table 2: PF of different candidate molecules

<table>
<thead>
<tr>
<th>Substance</th>
<th>Protection Factor</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>0</td>
<td>(Mody, 2008)</td>
</tr>
<tr>
<td>Vitamin E (α-tocopherol)</td>
<td>1.14</td>
<td>(Söderberg, 2012)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>1.23</td>
<td>(Kronschläger, 2013)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1.3</td>
<td>(Kronschläger, 2012)</td>
</tr>
<tr>
<td>Substance X</td>
<td>???</td>
<td></td>
</tr>
</tbody>
</table>

More molecules with different molecular structures will be tested to allow identification of lead candidates and to design a potent anticataract agent. Once a potent anticataract agent is designed, it would be feasible to use it from the age of 50, after vitrectomy or intravitreal injections with cortisone, or from birth if at risk to develop juvenile cataract. Cataract may increase intraocular pressure by angle closure. Combining anticataract agents with
glaucoma drops might have positive influence on intraocular pressure profiles. Moreover, cataract research is essentially research of lens epithelial cells, which cause secondary cataract after cataract surgery. Preventing secondary cataract will improve cataract surgery outcomes and even enable implantation of accommodative artificial lenses. In other words, cataract basic research and cataract surgery research enrich one another and help to tackle future challenges like the demographic change.
References

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