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Caffeine Eye Drops Protect Against UV-B Cataract

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Abstract

The purpose of this study was to investigate if topically applied caffeine protects against in vivo ultraviolet radiation cataract and if so, to estimate the protection factor.

Three experiments were carried out. First, two groups of Sprague-Dawley rats were pre-treated with a single application of either placebo or caffeine eye drops in both eyes. All animals were then unilaterally exposed in vivo to 8 kJ/m² UV-B radiation for 15 min. One week later, the lens GSH levels were measured and the degree of cataract was quantified by measurement of in vitro lens light scattering. In the second experiment, placebo and caffeine pre-treated rats were divided in five UV-B radiation dose groups, receiving 0.0, 2.6, 3.7, 4.5 or 5.2 kJ/m² UV-B radiation in one eye. Lens light scattering was determined after one week. In the third experiment, placebo and caffeine pre-treated rats were UV-B-exposed and the presence of activated caspase-3 was visualized by immunohistochemistry.

There was significantly less UV-B radiation cataract in the caffeine group than in the placebo group (95% confidence interval for mean difference in lens light scattering between the groups = 0.10 ± 0.05 tEDC), and the protection factor for caffeine was 1.23. There was no difference in GSH levels between the placebo- and the caffeine group. There was more caspase-3 staining in UV-B-exposed lenses from the placebo group than in UV-B-exposed lenses from the caffeine group.

Topically applied caffeine protects against ultraviolet radiation cataract, reducing lens sensitivity 1.23 times.

Keywords

caffeine; cataract; ultraviolet radiation; protection factor

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Introduction

Caffeine, an alkaloid, is present at relatively high concentrations in many beverages like coffee, tea, cola and in chocolate-based food products (Ashton, 1987; Curatolo and Robertson, 1983). Caffeine is an effective scavenger of reactive oxygen species, particularly hydroxyl radicals, singlet oxygen and to some extent peroxy radicals (Devasagayam et al., 1996; Shi et al., 1991; Stadler et al., 1996). It was shown that the antioxidant effect of caffeine was similar to that of glutathione and significantly higher than that of ascorbic acid (Devasagayam et al., 1996). In the human body, caffeine is demethylated by the hepatic cytochrome P450 1A2 (CYP 1A2) to the main metabolites 1-methylxanthine and 1-methyluric acid which also have been shown to have significant antioxidant activity (Lee, 2000). Additionally, caffeine is very stable in topical eye drop preparation and not susceptible to auto-oxidative degeneration and photodynamic degradation. Topical application allows high concentration of caffeine to be used.

Varma showed the in vitro protective effect of caffeine against UVR-induced cataract and established that caffeine levels in the lens peaks 60 min after a single topical application in rat (Varma SD, 2008; Varma SD, 2010b, 2011).

UVR is a major contributor to the pathogenesis of cataract (McCarty and Taylor, 2002; Taylor et al., 1988). In the aqueous humor and the lens, UVR catalyses the generation of reactive oxygen species (ROS), singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals. Oxidative stress in the lens leads to caspase-3 activation (Talebizadeh, 2012) followed by apoptosis (Michael et al., 1998) in the lens epithelial cells, and further to a disturbance of the transmittance of the lens (Wang et al., 2010). The concentration of reduced glutathione (GSH) in the lens is very high and GSH is a major antioxidant in the lens (Giblin, 2000; Lou, 2003; Spector et al., 1985; Varma SD, 2008). GSH is essential in the protection against exogenous and endogenous ROS (Lou, 2003). Varma showed in vitro that caffeine protects the lens against UVR-induced cataract by ROS scavenging (Varma SD, 2008; Varma SD, 2010a).

Caffeine is relatively well tolerated physiologically and the US Food and Drug Administration classify caffeine as a sufficiently safe compound for human consumption. The LD-50 of 10 to 15 g of a single dose is equivalent to 80-100 cups of coffee a day. There has been a concern that caffeine increases the intraocular pressure (Peczon and Grant, 1964). However, in a recent study in which caffeine was topically administered in humans with primary open angle glaucoma, no change of intraocular pressure was found (Chandra et al., 2011).

There is a need for quantification of the in vivo effect of anti-cataract agents that allow comparison of effect. Recently, we introduced the concept of the protection factor, which is the ratio of $MTD_{2.3:16}$ with protection, and $MTD_{2.3:16}$ without protection (Kronschläger et al., 2012).

The WHO estimated that due to demographic change, there would be a need of increase in cataract operations from 20 million cataract operations annually in 2004 to 32 million cataract surgeries annually in 2020 (Organization, 2004). The need for increase in cataract operations implies an enormous burden for existing health care systems. A delay of cataract onset of only 10 years could reduce the need for cataract surgery with as much as half (Kupfer, 1985).

Varma et al. recently showed in a galactosemic rat model that caffeine eye drops in vivo protects from cataract, probably due to the antioxidant properties of caffeine (Varma SD, 2010b).

In this study, we aimed to test if the protective effect of caffeine eye drops also holds for oxidative stress in the lens induced by in vivo UV-B radiation exposure. Here, we aimed to estimate the protection factor for caffeine eye drops against in vivo UV-B-induced cataract. We further wanted to qualitatively elucidate if caffeine impacts on the oxidative stress induced activation of caspase-3.

Materials and Methods

Three experiments were performed. The first experiment determined the potential protective effect of caffeine eye drops in UV-B-induced cataract and the effect on lens GSH concentration. The second experiment determined the protection factor for caffeine. The third experiment was designed to measure the impact of UVR on caspase-3 activation.

Experimental Animal

Six-week-old Sprague-Dawley rats were used adhering to the ARVO Statement for the use of animals in Ophthalmic and Vision Research. Ethical approval was obtained from the Uppsala Ethical Committee on Animal Experiments (protocol number: C29/10).

Eye drop Preparation

The caffeine eye drops were prepared by adding 0.9 % of hydroxypropylmethyl cellulose (Sigma-Aldrich; H7509) to a 72 mM aqueous solution of caffeine (Varma SD, 2010b). Placebo eye drops were prepared with the same vehicle but without caffeine.

Devices

The radiation from a high-pressure mercury arc lamp (HBO 200W, Osram, Munich, Germany) was collimated, passed through a water filter and then a double monochromator set at 300 nm (Söderberg et al., 1990). The resulting spectral output was measured with a spectrometer (PC 2000, Ocean Optics, USA) calibrated to deuterium tungsten halogen source with known spectrum.

The output centered around the mercury lines at 298 and 302 nm with an approximate half-maximum of 10 nm (Figure 1).

Irradiance was measured with a thermopile (No 7104, Oriel, USA). The thermopile had been calibrated to a standard traceable to the National Institute of Standards, USA.

Light scattering

The amount of cataract was quantified as forward lens light scattering, measured with a light dissemination meter (Söderberg et al., 1990). The instrument was calibrated with a standard lipid emulsion of diazepam (Stesolid Novum, Alpharma AB, Stockholm, Sweden) and the primary unit of intensity of forward light scattering was transformed to Equivalent Diazepam Concentration (tEDC) (Söderberg et al., 1990).

Experimental procedure

At 60 minutes preceding the exposure, the animal was anesthetized with a mixture of 94 mg/kg ketamine and 14 mg/kg xylazine, injected intraperitoneally. At 55 minutes preceding the exposure, eye drops were instilled in both eyes in the anesthetized animal. Then at 10 minutes before exposure, tropicamide was instilled in both eyes for pupil dilation. 10 minutes later, one eye of each animal was exposed to 8 kJ/m² UV-B radiation during 15 minutes (Ayala et al., 2000). The field of exposure covered only the cornea and the eyelids. The contralateral unexposed eye was shielded during exposure. Lubricating ointment was

applied to both eyes after the exposure. One week after exposure, the animal was sacrificed with carbon dioxide asphyxiation followed by cervical dislocation. The eyes were enucleated and the lenses were extracted using a microscope. Remnants of the ciliary body were removed from the lens equator, maintaining the lens in balanced salt solution (Alcon, Sweden). Immediately thereafter, forward lens light scattering was measured. Finally, cataract morphology was visualized by dark field illumination photography.

Immunohistochemistry

At 7 hours post-exposure, the animal was sacrificed and the eyes removed. The eyes were fixated in 4 % freshly prepared paraformaldehyde in phosphate-buffered saline for 20 minutes at room temperature. Afterwards, the eye was washed in phosphate buffered saline for 30 minutes and thereafter immersed in 30 % sucrose for dehydration overnight at 4°C. The dehydrated eye was then positioned in a cryomold, embedded with Optimal Cutting Temperature compound (Sigma, USA) and then put in a freezer at -70°C. The frozen eye was cryosectioned in 10 µm thick mid-sagittal sections. From each eye, sequential sections were cut and at least 5 sections were discarded between sections collected for analysis. Selected sections were placed on a microscope slide stored at -70°C for subsequent histological staining in one batch. For staining of activated caspase-3, we used a 1:10 diluted rabbit polyclonal primary antibody (ab 2302, Abcam) and a 1:300 diluted fluorescein tagged donkey polyclonal secondary anti-rabbit IgG antibody (ab6798, Abcam).

Finally, all stained sections were mounted with Vectashield® HardSet™ mounting medium with DAPI (Vector Labs, USA). After approximately 20 minutes, the sections were examined and photographed with a fluorescence microscope (Universal Microscope Axioplan 2 Imaging, Carl Zeiss, Germany).

GSH measurement

The frozen lens was homogenized in 500 µl of ice-cold mammalian protein extraction reagent (Pierce, USA) on ice, using a glass homogenizer (Duell, Kontes Glass Co., Vineland, USA). 120 µl homogenate was transferred to a test tube and 120 µl of TCA 10 % was added for protein precipitation. The vial was then centrifuged at 15 000 g for 15 min at 4°C. The supernatant was transferred to a new test tube.

For normalization of GSH, the lens protein concentration was measured with the bicinchoninic acid (BCA) method (Reagent Kit 23225 23227; Pierce Inc., Rockford, USA).

The assay of total free non-protein-SH was adapted from the protocol (Uptima/Interchim) based on Seldak and Lindsey (Sedlak and Lindsay, 1968). 50 µl supernatant aliquots were transferred to a 96 well plate and mixed with 200 µl of Tris-ETDA buffer (30 mM Tris, 3 mM ETDA, pH 8.9; Sigma AB, Sweden) and 20 µl of 16.8 mM DTNB (D8130, Sigma AB, Sweden). The mixture was incubated at room temperature for 5 min for oxidation of all available-SH groups. The reduced DTNB was quantified spectrophotometrically at 412 nm. The sample readings were compared to an external GSH standard curve.

Experimental design

In the first experiment, 40 animals were randomly divided into a caffeine and a placebo subgroup with 20 animals each. In each subgroup the eye drops were administered to both eyes. Each animal was unilaterally exposed to UV-B radiation. The intensity of forward light-scattering was measured 3 times in each lens. For biochemical analysis of GSH, lenses from 10 animals in each eye drop group were put in a freezer at -70°C immediately after the lens light scattering measurement. The protein and GSH assays were made in triplicate. During lens preparation, two lenses from the placebo group were lost.

In the second experiment, 40 animals were subdivided into five dose subgroups of four rats. All the animals were unilaterally exposed to UV-B radiation. The dose subgroups were selected in order to optimize the regression by splitting the total dose interval squared into 5 dose levels, setting the increment squared dose constant (Equation 1).

$$H_{g:th} = \sqrt{(g-1) \frac{[E(MTD_{2.3:16})]^2}{2}} \quad \text{Calculation of subdoses} \quad \text{Eq.1}$$

Here, H_g , is the subgroup dose, for the g :th subgroup ($g=1\dots5$) and $E(MTD_{2.3:16})$, is the expected $MTD_{2.3:16}$. The expected $MTD_{2.3:16}$ was set to 3.7 kJ/m^2 based on a previous study (Söderberg et al., 2002). Thus, the subgroup doses were 0, 2.6, 3.7, 4.5 and 5.2 kJ/m^2 . The intensity of forward light-scattering was measured 3 times in each lens. For the estimation of $MTD_{2.3:16}$, it was planned to include the light scattering measurements of the lenses from the animals that were exposed to 8 kJ/m^2 in experiment 1.

In the third experiment, both eyes were topically treated with caffeine eye drops in 3 animals, and in another 3 animals both eyes were treated with placebo eye drops. The animals were unilaterally exposed to UV-B radiation during 15 minutes and the contralateral eye was kept as control. The UV-B radiation dose in the third experiment was 8 kJ/m^2 . For each lens, three sections were processed for immunohistochemistry.

For light scattering measurements, the paired difference between the UV-B-exposed and the contralateral unexposed lens in each animal was used as primary data.

Statistical parameters

The significance level and confidence coefficients were set at 0.05 and 0.95 respectively, considering sample size.

Results

Experiment 1

Macroscopic morphology after 8 kJ/m^2 UV-B radiation—All UV-B-exposed lenses developed opacities while all unexposed lenses were clear. Typical photographs in darkfield illumination are shown in Figure 2. 8 kJ/m^2 UV-B-induced more cataract in the placebo group than in the caffeine group (Figure 2).

Lens light scattering after 8 kJ/m^2 UV-B radiation—The light scattering was significantly higher in the UV-B-exposed lens than in the contralateral unexposed lens, in both the caffeine ($CI(0.95) = 0.10 \pm 0.05 \text{ tEDC}$, d.f. = 17), and the placebo group ($CI(0.95) = 0.20 \pm 0.02 \text{ tEDC}$, d.f. = 17) (Figure 3).

The difference between the caffeine and placebo group was statistically significant as indicated by a 95 % confidence interval for the mean of the difference between the caffeine and the placebo group ($CI(0.95) = 0.103 \pm 0.05 \text{ tEDC}$, d.f. = 26). Since the variances for differences were unequal, the degree of freedom was reduced according to approximate t-testing for independent groups.

Caffeine impact on GSH concentration after 8 kJ/m^2 UV-B radiation— 8 kJ/m^2 UV-B radiation did not change the lens GSH concentration in neither the caffeine ($CI(0.95) = 0.14 \pm 0.37 \text{ nmol/mg protein}$, d.f. = 9) nor the placebo group ($CI(0.95) = 0.10 \pm 0.50 \text{ nmol/mg protein}$, d.f. = 7) (Figure 4), and there was no significant difference between the

two eye drop groups 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).

Experiment 2 (five different UV-B radiation doses, protection factor)

Macroscopic morphology—Subcapsular cataract developed in all UV-B-exposed lenses. Caffeine eye drop treatment reduced the lens opacification in the lower UV-B radiation dose groups, compared to placebo eye drops. The placebo group exhibited increasing lens opacification with increasing UV-B radiation dose (Figure 5).

Light scattering with increasing UVR dose—The MTD_{2.3; 16} for the caffeine group was 5.7 kJ/m² (CI(0.95) = [4.9; 6.8] kJ/m², d.f. = 37) and 4.6 kJ/m² (CI(0.95) = [4.0; 5.2] kJ/m², d.f. = 37) for the placebo group (Figure 6).

A higher MTD means higher tolerance to UV-B radiation. The protection factor was calculated to 1.23.

Experiment 3 (8 kJ/m² UV-B radiation and caspase-3)

Lens epithelial cells in lenses exposed to UV-B radiation exhibited extensive nuclear staining for activated caspase-3 (Figure 7).

Caffeine protected against activation of caspase-3 after exposure to UV-B radiation (Figure 7) while placebo did not.

Discussion

This study shows for the first time a protective effect of topical caffeine against in vivo UV-B radiation cataract.

The albino Sprague-Dawley rat was chosen considering the extensive data base on UV-B-induced cataract (Galichanin et al., 2010; Mody et al., 2008; Söderberg, 1990; Söderberg, 2012; Söderberg et al., 2002; Talebizadeh, 2012; Wang et al., 2010). The UV-B radiation dose was selected to be approximately twice the threshold dose (Söderberg et al., 2002). The lens light scattering was measured at 7 days after exposure since it has been shown that the light scattering increases up to approximately 1 week after exposure (Galichanin et al., 2010; Söderberg, 1990).

Considering that it was shown that a single topical application induces a peak of caffeine around 60 min later, we decided to apply the caffeine eye drops at 55 min before UV-B radiation exposure.

The immunohistochemistry for activated caspase-3 was limited to qualitative evaluation as a primary investigation of the interplay between the antioxidant caffeine and caspase-3 after exposure to UV-B radiation. The 7 hours latency time after exposure was chosen because we previously showed a peak of caspase-3 activation at 7 hours after UV-B radiation exposure (Talebizadeh, 2012).

The method used for free non-protein-SH is not specific to GSH but it has been shown that the major thiol in supernatants after protein precipitation of lens homogenates is GSH (Lou, 2003).

Varma demonstrated that the preservation of GSH levels is one of the key issues of topical caffeine (Varma SD, 2008). Our finding that caffeine protects from development of lens opacities (Figure 2, Figure 3, Figure 5, Figure 6) after exposure to oxidative stress from in

vivo UV-B radiation strongly supports that caffeine is a powerful antioxidant (Shi et al., 1991) and is in agreement with previous in vitro findings (Varma SD, 2008). Our finding that caffeine did not induce any significant difference in GSH content in the lens at 1 week after exposure (Figure 4) confirms the finding of Wang et al. indicating that GSH consumption in the lens, due to UV-B-induced oxidation, recovers within a week after exposure (Wang et al., 2010).

The estimated protection factor for caffeine of 1.23 (Figure 6), represents for administered substances, the highest protection against UV-B radiation and agrees with previous findings indicating that the antioxidant effect of caffeine is similar to that of glutathione and significantly higher than that of ascorbic acid (Devasagayam et al., 1996). 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 et al., 2008). Only Grx1 (thioltransferase), as shown when comparing Grx1^{-/-} mice with Grx1^{+/+} mice, has a higher protection factor of 1.3 (Kronschläger et al., 2012).

Caffeine absorbs very little in the 300 nm wavelength region (National Institute of Standards and Technology, 2011). It was shown that at 60 min. after topical administration of 72 mM caffeine, there is approximately 10 mM of caffeine in the aqueous humor (Varma SD, 2010b). Based on absorbance measurements, of caffeine at 300 nm and the corneal thickness and the aqueous humor depth of the rat, we estimated the transmittance of 72 mM caffeine in the cornea and the aqueous humor to approximately 90 %. Thus, it cannot be excluded that the effect of caffeine demonstrated is due to filtering of caffeine. However, caffeine is an effective scavenger of reactive oxygen species (Devasagayam et al., 1996; Shi et al., 1991; Stadler et al., 1996). Further, caffeine was shown to protect against oxidative stress cataract induced by galactose (Varma SD, 2010b).

Our control measurements of the absorbance of methylcellulose, at the concentrations used in the solvent showed a slight absorption at around 300-nm. However, since methylcellulose is a macromolecule it is highly improbable that it penetrates through the cornea. Further, the mydriatic tropicamide was topically applied 10 min before exposure to wash away any excess amount of methylcellulose before the exposure to UV-B. We were not able to see any spectral shift of absorbance of the caffeine dissolved in methylcellulose for topical administration. To make sure a potential effect of caffeine was not due to methylcellulose shielding, solvent only was used as a control and the PF is a comparison of the threshold for UV-B-induced cataract between solvent and caffeine, and solvent only.

Therefore, we believe that the protection of caffeine observed is caused by caffeine and due to reactive oxygen scavenging. Considering that caffeine is a powerful antioxidant (Shi et al., 1991), that is easily available and well tolerated (Ashton, 1987; Curatolo and Robertson, 1983) our finding implicates that caffeine may be a clinically useful anticataract agent.

On cell level we showed that caffeine protects from caspase-3 activation (Figure 7), thereby inhibiting an apoptotic pathway and confirming the findings of Varma (Varma SD, 2010b, 2011), and our own finding that apoptosis plays a major part in UV-B radiation cataractogenesis (Michael et al., 1998).

Orally supplied caffeine, even more than 3 coffee cup equivalents per day, seems to have no effect on age-related cataract (Klein et al., 2003). However, it is possible that topically administered, caffeine may reach a lenticular caffeine concentration sufficient for cataract protection without systemic effects.

In conclusion, topical caffeine protects against UV-B-induced lens opacification and is a promising candidate molecule for delay of onset of ultraviolet radiation cataract.

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Highlights

- Topically applied caffeine protects against UV-B-induced cataract in the rat.
- The protection factor (PF) for caffeine was estimated to 1.23, which is higher than what we previously found with the same technique for α -tocopherol, PF = 1.14, and for ascorbate, PF = 0.
- Caffeine eye drops prevent ultraviolet radiation induced cataract by inhibiting apoptosis of lens epithelial cells.

Figure 1.
Relative spectral UV-B irradiance from the monochromator-filtered Mercury high-pressure arc lamp output.

Figure 2.
Darkfield illumination photographs of 8 kJ/m² UV-B-exposed and control lenses from caffeine and placebo treated rats.

Figure 3.
Mean light scattering difference between caffeine and placebo at one week after exposure to 8 kJ/m² of UV-B radiation. Bars are 95% CI.

Figure 4. Mean difference of GSH/protein between exposed and contralateral unexposed lens, between topically administered, caffeine in vehicle and vehicle only, at one week after unilateral in vivo exposure to 8 kJ/m². Bars are 95% CI.

Figure 5.
Lens morphology after in vivo exposure to 0, 2.6, 3.7, 4.5 or 5.2 kJ/m² UV-B radiation in rats pre-treated with placebo or caffeine eye drops.

Figure 6.

Lens light scattering at one week after various UV-B radiant exposures. Grey colour corresponds to lenses from the caffeine group and black colour corresponds to lenses from the placebo group. The solid lines represent least square fit for each group. Vertical lines are the $MTD_{2.3:16}$ of the two eye drop groups.

Figure 7.
Immunostaining of activated caspase-3 in lens epithelial cells of lenses expose to 8 kJ/m² UV-B and control lenses from caffeine and vehicle and vehicle only treated rats.