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Single high-dose peroral caffeine intake inhibits ultraviolet radiation-induced apoptosis in human lens epithelial cells *in vitro*

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ABSTRACT.

Purpose: **The aim of the present study was to determine whether caffeine concentrations in human lens epithelial cells (LECs) achieved from acute peroral caffeine intake inhibit ultraviolet radiation-induced apoptosis *in vitro*.**

Methods: **Patients were planned for cataract surgery of both eyes with a caffeine abstinence of 2 weeks in total, starting 1 week before surgery of the first eye. The second eye was scheduled 1 week after the first eye. At the day of the second eye surgery, patients were given coffee containing 180 mg caffeine shortly before surgery. Lens capsules including LEC, harvested after capsulorhexis, were transferred to a cell culture dish and immediately exposed to close to threshold ultraviolet radiation (UVR). At 24 hr after UVR exposure, apoptotic LECs were analysed by TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining.**

Results: **TUNEL-positive cells were detected in UVR-exposed lens capsules both after caffeine intake and in controls. The mean difference in TUNEL-positive cells between caffeine intake and contralateral controls (no caffeine) resulted in a 95% CI 15.3 ± 10.4% (degrees of freedom: 16).**

Conclusion: **Peroral caffeine consumption significantly decreased UVR-induced apoptosis in LEC supporting epidemiological findings that caffeine delays the onset of cataract.**

Key words: caffeine – cataract – lens epithelial cells – ultraviolet radiation

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Introduction

Cataract is still the leading cause of blindness worldwide (Flaxman et al. 2017), and there is no approved drug that prevents cataractogenesis. However, epidemiological studies suggest that caffeine consumption retards cataract development. Varma (2016) reported that the incidence of age-related cataract is inversely associated with the amount of caffeine consumption, that is groups with higher caffeine intake have a lower cataract incidence. Rautiainen et al. (2014) observed that the risk of age-related cataract in middle-aged and elderly women was lower in a higher dietary total antioxidant capacity, including caffeine.

Caffeine and its ability to reduce oxidative damage in the lens have been described in previous experimental studies. Caffeine's general antioxidative ability was found to be similar to glutathione (the major antioxidant in the lens) (Giblin 2000) and significantly higher than ascorbic acid (Devasagayam et al. 1996). This finding was confirmed in experimentally UVR-induced cataract by comparing the protection factor (PF) of different candidate molecules. The PF is equivalent to the PF of sunscreens, that is the ratio between the threshold dose of toxic agent *in vivo* and the threshold dose without the toxic agent *in vivo* (Kronschläger et al. 2012). Caffeine achieved the highest protection against cataract formation for administered substances (PF: 1.24) (Kronschläger et al. 2013a) in comparison with vitamin E (PF: 1.14) (Söderberg et al. 2012) and vitamin C (PF: 1.0) (Mody et al. 2008) matching almost the PF of the glutaredoxin gene (PF: 1.3) (Kronschläger et al. 2012).

Ultraviolet radiation (UVR) is a major source of oxidative stress in the lens and the main risk factor contributing to age-related cataractogenesis (Delcourt et al. 2000). According to the World Health Organization (WHO), 20% of cataracts in the world are caused by overexposure to UVR (WHO 2019). Ultraviolet radiation (UVR) generates reactive oxygen species (ROS) damaging the lens via protein cross-linking, membrane damage and dysregulation of lens enzyme activity (Breadsell, Wegener & Breipohl 1994). At close to threshold doses, UVR induces apoptosis in the lens epithelial cells (LECs) (Michael et al. 1998), which are essential for keeping the lens transparent (Kronschläger et al. 2013a, 2013b). Lost LECs are

replaced by epithelial cell movement from the equator towards the lens sutures; however, LEC damage exceeding the individual repair capacity may lead to cataract (Meyer et al. 2014), eventually by the influx of calcium in the underlying fibre cells (Hightower 1995). Consequently, the absence/presence of ROS scavengers significantly modulates cataract formation.

It was well established in experimental studies that caffeine, a potent ROS scavenger, protects the LEC from ROS, for example generated by UVR, and consequently from apoptosis (Devasagayam et al. 1996; Varma et al. 2008; Varma et al. 2010a; Kronschläger et al. 2013a). Moreover, caffeine experimentally inhibited selenite-induced cataract (Varma, Hegde & Kovtun 2010b) and kynurenine-induced cataract (Varma and Hedge, 2010), protected against high-sugar-induced transcription of microRNAs (Varma & Kovtun, 2013) in the lens and prevented galactose cataract (Varma et al. 2010c) supporting the protective potential of caffeine intake. Ultraviolet radiation (UVR) exposure consumes GSH content (Wang et al. 2010) and ascorbic acid content (Mody et al. 2008) in the lens. Caffeine administration was found to minimize the loss of GSH *in vivo* (Varma et al. 2010a) and *in vitro* (Varma et al. 2008). Further, coffee consumption also prevented the high-fat diet-induced decrease in the concentration of GSH and ascorbic acid (Nakazawa et al. 2018).

We demonstrated previously that caffeine eye drops administered to UVR-exposed rats protected against cataract development (Kronschläger et al. 2014). Moreover, we reported that caffeine from caffeine eye drops not only quickly accumulated in the rat lens but also showed a slow washout (Kronschläger et al. 2014). Varma showed that 5mM of caffeine in a culture dish inhibits UVR-induced apoptosis in LEC (Varma et al. 2008). We recently found that peroral caffeine consumption increased caffeine levels in lens capsule/epithelial cells. Drinking of 180 mg of caffeine resulted in 3.68 ± 1.86 caffeine ng/lens capsule/epithelium (Kronschläger et al. 2018). However, it remains unclear whether the caffeine dose perorally absorbed is sufficient in preventing UVR-induced LEC apoptosis *in vitro*. The aim of the present study was to investigate the effect of acute perorally accumulated caffeine on threshold UVR-induced LEC apoptosis *in vitro* and further to elucidate the role of perorally consumed caffeine on cataract prevention.

Methods

Ethics statement

The clinical prospective randomized controlled pilot study was approved by the local ethics committee of the city Vienna (protocol number: EK 18-041-0318). All the research complied with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to enrolment at the Hanusch Hospital.

Subjects

Study participants were recruited at the outpatient clinic of the Hanusch Hospital 1 week before cataract surgery of the first eye. Inclusion criteria comprised patients with bilateral cataract, who were older than 21 years and chose sedoanalgesia technique for surgery. Patients were excluded from the study if they suffered from pseudoexfoliation syndrome of the lens, had systolic hypertension of more than 160 mmHg at the day of surgery or were pregnant. After checking the inclusion and exclusion criteria, patients were included in the study and asked about their last caffeine intake, their caffeine habits and their weight. The study participants were then asked to avoid intake of caffeine-containing beverages (e.g. coffee, Coca-Cola, energy drinks, black or green tea, and dark chocolate) until cataract surgery of the second eye (2 weeks after the baseline visit). At this baseline visit, a standard ophthalmological examination including slit lamp biomicroscopy with LOCS II (Chylack et al. 1989) grading and retinal examination, visual acuity testing, biometry (IOL Master 700, Carl Zeiss Meditec AG, Jena, Germany) of both eyes and blood pressure measurement was carried out.

Study design

This was a prospective randomized controlled observer-blinded pilot study. Both eyes of each patient were included in the study; however, the first eye to be operated on was chosen by randomization. The time interval between surgery of the first and the second eye was 1 week. Shortly before surgery of the second eye, patients were asked to drink a cup of coffee containing 180 mg caffeine, which equals three cups of espresso. The laboratory personnel were masked concerning the patient's coffee consumption.

Study procedure

Patients were asked on both operation days if they had consumed any caffeine-containing beverages and, if so, they were excluded from the study. During surgery, heart activity, blood pressure and oxygen levels in the blood were monitored for each patient and stand-by anaesthesia was provided at all times. Standardized main incision and paracentesis were performed to harvest the anterior lens capsule and adhering LEC. After performing capsulorhexis (aimed for 5.5 mm), the lens capsule with the adhering LEC was removed with a forceps, transferred into a cell culture dish (STARLAB, Hamburg, Germany) and kept in sterile balanced salt solution (BSS). The lens capsules were then immediately exposed to 10 kJ/m^2 of UVR using the Bio-Link 312 UV irradiation system (Vilber Lourmat, Eberhardzell, Germany). All capsules were kept in one drop of BSS (about 50 μl BSS in 35×10 mm dishes) during UVR exposure just to cover the surface and keep the capsule wet. Hence, all of the capsules were kept in nearly the same amount of BSS during UVR exposure. During UVR exposure, capsules were floating in the dish. We found that the floating rhexis capsule with its typical incisions was almost as flat as mounted capsules. Further, we wanted to keep trauma to a minimum. Therefore, we decided not to mount the capsules on the bottom of the dish. We observed that threshold dose UVR exposure induced the same amount of apoptosis in cells up or cells down in the floating capsule. Moreover, we wanted to keep trauma to a minimum. Thus, UVR-exposed lens capsules were not checked whether cells were up or down. After UVR exposure, BSS was removed under a laminar-flow hood and replaced by culture medium (RPMI-1640) containing 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml

penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific Inc., Waltham, MA, USA). Lens capsules with adhering LECs were then cultured at 37°C with 5% CO_2 for 24 hr in a humified CO_2 incubator. On the next day, specimens were fixed in 4% formaldehyde solution (Carl Roth, Karlsruhe, Germany) for 30 min at 10°C and permeabilized in PBS containing 0.2% Triton X-100. Afterwards, lens capsules were stained using a commercial TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay kit (CF 594; TUNEL Assay Apoptosis Detection Kit; Biotium, Fremont, CA, USA). Specimens were incubated in TUNEL reaction buffer for 2 hr at 37°C (5% CO_2), counterstained with Hoechst (Hoechst 33258; Thermo Fisher Scientific Inc.) and photographed using a fluorescence microscope (Eclipse 80i; Nikon, Tokyo, Japan). Pictures of three sections of each lens capsule were taken to count the number of TUNEL- and Hoechst-positive cells in a 400 × 400 pixel area of each section [containing 244 ± 31 (SD) cells], and percentage of apoptotic cells was calculated. TUNEL-positive cells at the edge of a specimen were not included in the analysis, as death of these cells may have been triggered by mechanical damage during capsulorhexis.

UVR source and threshold dose

Ultraviolet radiation (UVR) was generated in the Bio-Link BLX system by 5 × 8 W illumination Vilber Lourmat© (Collégien, France) tubes. The spectral output (Fig. 1) includes mainly UVR-B (280–315 nm) with a defined wavelength peak at 312 nm. Additionally, the tubes emit a small fraction of UVR-A in a wavelength region from 315 to 370 nm. A programmable microprocessor constantly monitors the UV light emission. The UV light sensor is positioned above a well of light, in the top of the irradiation chamber. The irradiation stops automatically when the energy received matches the programmed energy. According to the manufacturer (Vilber Lourmat©), the device (Bio-Link 312 UV irradiation system) is calibrated at the time of production with a sensor that is calibrated and monitored by LNE (Laboratoire National de Métrologie et d'Essais, Paris, France). The sensor is returned to LNE every 24 months for checking. No further calibration is needed. Threshold dose was estimated by exposing LEC to 0, 5, 10, 15 and 20 kJ/m^2 UVR.

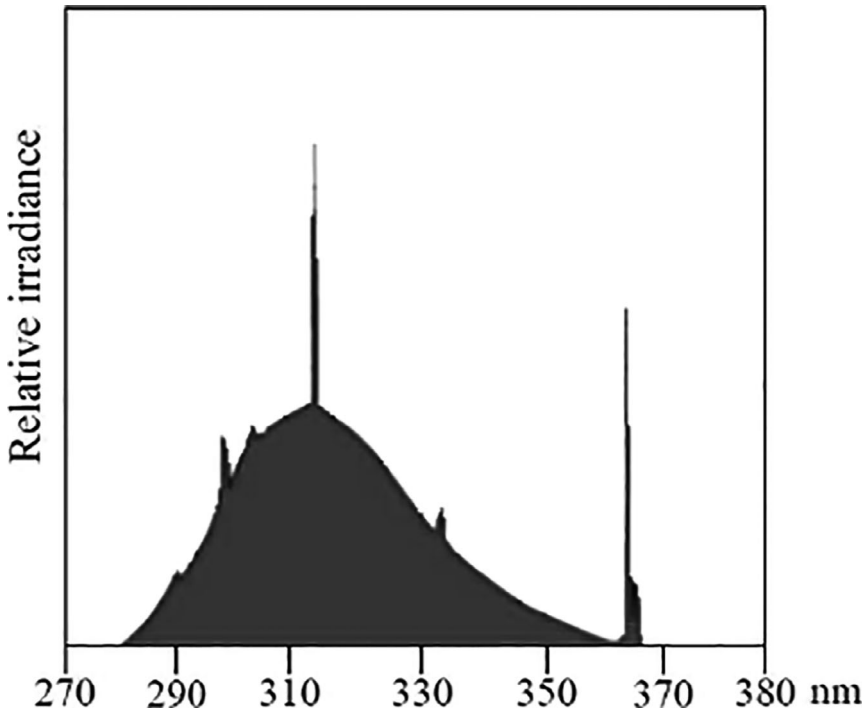

Fig. 1. Spectral output of the Bio-Link BLX system.

Caffeine

For making espresso, we used Nespresso© (Lausanne, Switzerland) standard capsules. Each standard capsule delivers 60 mg of caffeine (range 55–65 mg) per pour. We used three standard capsules to achieve 180 mg of caffeine.

Statistics

Missing data were excluded from the analysis. Cell counting was done using ImageJ 1.52a (National Institutes of Health, Bethesda, MD, USA). The significance level and confidence coefficients were set at 0.05 and 0.95, respectively, considering sample size.

Results

A total of 40 eyes of 20 patients were included in this study. Three patients had to be excluded because they did not comply with the protocol and drank coffee during the course of the study. Hence, 34 lens capsules with adhering LECs from 17 patients were used for analysis. Demographic data of the patients and the time between caffeine intake and surgery of the second eye are summarized in Table 1.

The included patients presented mostly bilateral mixed cataract (13 patients) or bilateral nuclear cataract (four patients) with no patient displaying only cortical or posterior subcapsular cataract (PSC; Fig. 2).

LOCS II grading of all patients analysed is shown in Table 2.

There was no significant difference in LOCS II grading between right and left eyes (paired samples Wilcoxon's test: $p > 0.05$).

Before analysing the protective effect of peroral caffeine intake on lens capsules, we estimated the lowest amount of UVR exposure necessary to induce apoptosis in LEC (non-caffeine group). Therefore, we exposed specimens to UVR of 0, 5, 10, 15 and 20 kJ/m^2, followed by staining with Hoechst and

Table 1. Demographic data of study participants and time between caffeine intake and surgery.

n	17
Age ± SD (years)	73 ± 6.1
Weight ± SD (kg)	81.8 ± 15.2
Female:male (%)	59:41
Average cup of coffee/ day ± SD	1.6 ± 0.7
Time between caffeine intake and surgery ± SD (min)	165 ± 89.7

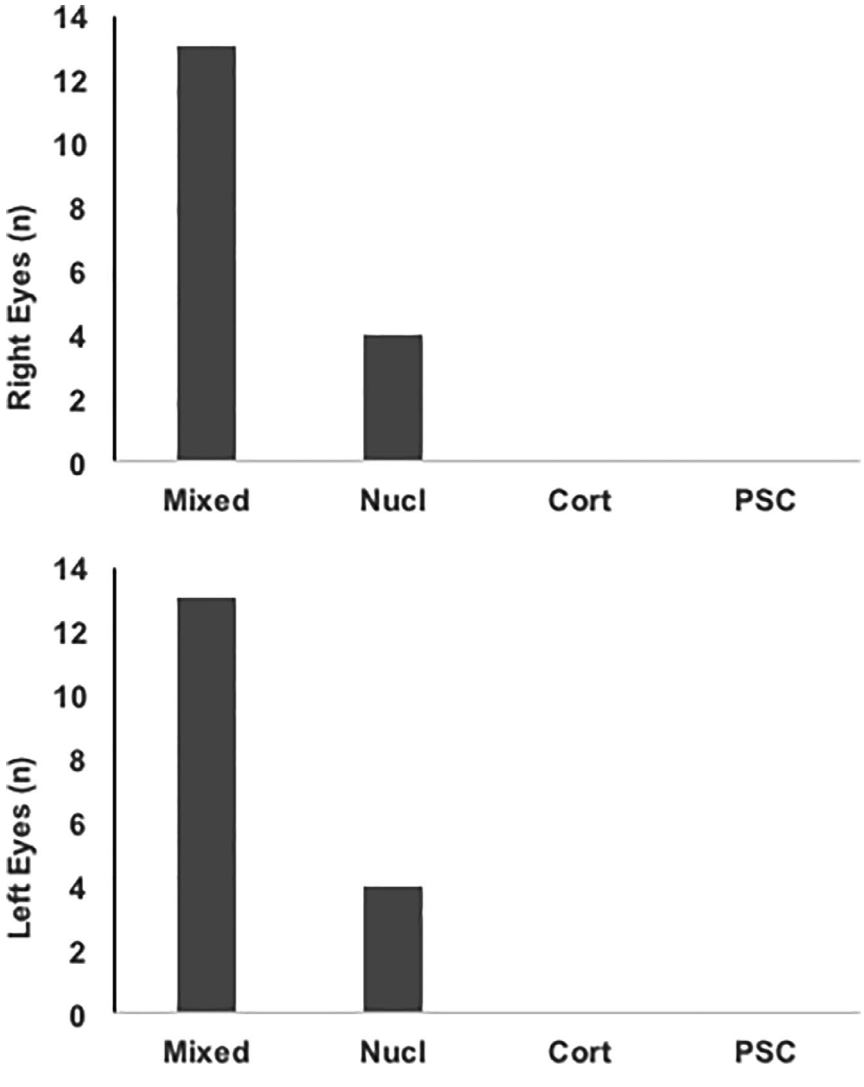

Fig. 2. Cataract types of participants, right and left eyes. Cort = cortical cataract, Mixed = mixed cataract, Nucl = nuclear cataract, PSC = posterior subcapsular cataract.

Table 2. LOCS II grading of study participants.

Participant	Right eye	Left eye
1	nucl II, cort III	nucl II, cort III
2	nucl III	nucl III
3	nucl II	nucl II
4	nucl II, psc I	nucl II, psc III
5	nucl II, cort I, psc II	nucl II, cort I, psc II
6	nucl II, cort II	nucl II, cort II
7	nucl II, cort II	nucl II, cort II
8	nucl III, psc I	nucl III, psc I
9	nucl II	nucl II
10	nucl III, cort II	nucl II, cort II
11	nucl II, cort III, psc III	nucl II, cort III, psc III
12	nucl II, cort II, psc I	nucl II, cort I
13	nucl III, cort III, psc II	nucl III, cort III, psc III
14	nucl II, cort III	nucl II, cort III
15	nucl I	nucl I
16	nucl II, cort II	nucl II, cort II
17	nucl II, cort II, psc III	nucl II, cort II, psc II

TUNEL assay. To confirm the findings of the first experiment, we repeated the experiment with a second set of lens capsules. We found that 10 kJ/m^2 was the lowest amount of UVR necessary to induce apoptosis in LEC (Fig. 3).

This was independent of cells up or down during UVR exposure. Hence, in all of the following experiments 10 kJ/m^2 was employed as threshold dose to induce apoptosis. The exposure time was 2 min 35 seconds.

No difference in cell sizes could be detected in each of the sections used for analysis.

Threshold UVR exposure induced TUNEL staining of LEC in both the treatment and control groups (Fig. 4).

However, LEC after caffeine intake showed significantly less TUNEL staining than LEC without caffeine intake (Fig. 4) as indicated by a 95% CI for the mean of the difference between the caffeine intake eye and the contralateral control eye [CI (0.95) = 15.3 ± 10.4%, degrees of freedom: 16; Fig. 5].

Additionally, a paired *t*-test of TUNEL-positive cells (%) in LEC between caffeine intake eye and contralateral control eye resulted in a p-value of 0.011.

Discussion

This study demonstrates for the first time the protective effect of acute perorally consumed caffeine on UVR-induced apoptosis in LEC.

Caffeine concentrations in lens capsule/epithelium were not analysed as we already studied the pharmacokinetics of acute peroral caffeine intake. An acute peroral intake of 180 mg caffeine resulted in 3.68 ± 1.86 caffeine ng/lens capsule/epithelium (Kronschläger et al. 2018). The caffeine abstinence interval of 1 week was chosen based on data of our previous study that after 1 week of caffeine abstinence no caffeine was detectable in the lens (Kronschläger et al. 2018).

We chose to investigate apoptosis of LEC as apoptotic death of LEC precedes the appearance of cortical opacities (Li et al. 1995; Zhou et al. 2007). Moreover, LEC apoptosis has been convincingly linked to cortical cataract in animal and human studies (Hightower 1995; Li et al. 1995; Spector et al. 1995; Michael et al. 2000; Charakidas et al. 2005). We performed LOCS II grading because of the fact that apoptosis from cortical cataract could interfere with our study results, that is in patients with asymmetric cataract. However, patients included in our study did not show any significant difference in LOCS II grading between

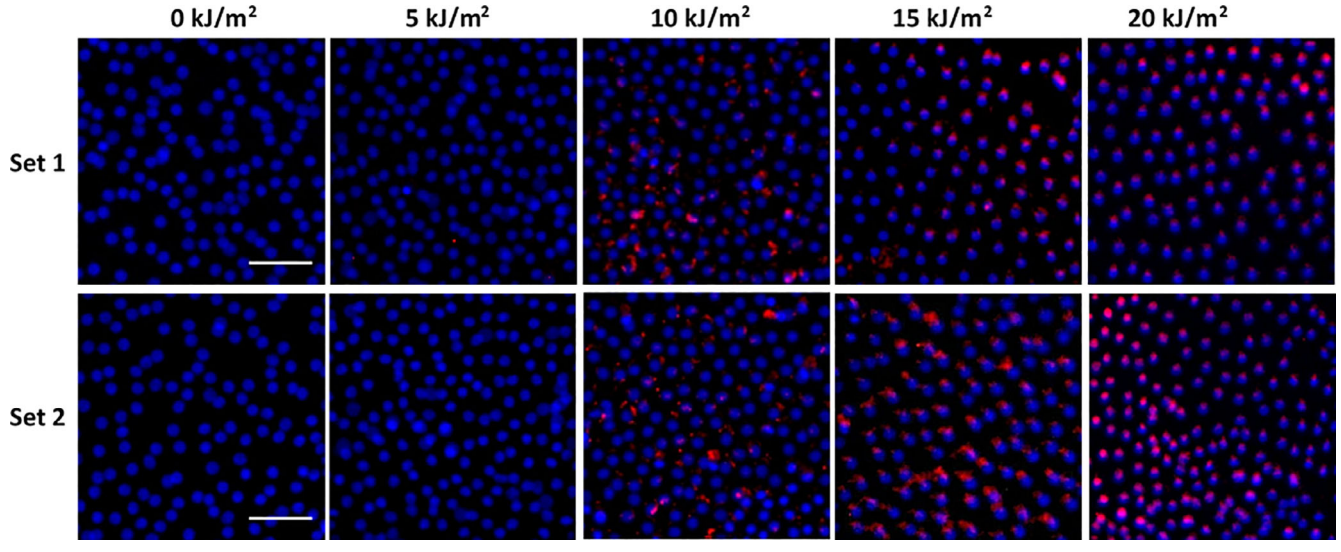

Fig. 3. Lens epithelial cells of two respective lens capsules (set 1, set 2) were exposed to 0, 5, 10, 15 and 20 kJ/m^2 of ultraviolet radiation to test for the minimal amount of energy necessary to induce apoptosis. Scale bar is 50 μm.

right and left lenses (Fig. 2, Table 2) and analysis included always both lenses of a patient, one lens acting as a control lens.

Apoptosis of LEC was experimentally induced by UVR in our study and analysed 24 hr after exposure. Interestingly, UVR exposure, which is typically associated with cortical cataract, is also linked to nuclear cataract (Neale et al. 2003) and posterior subcapsular cataract (Bochow et al. 1989). The time interval of 24 hr was selected because we have previously shown that LEC apoptosis peaks 24 hr after UVR exposure (Kronschläger et al. 2013b). The TUNEL method was chosen to detect apoptosis as it has been applied in several LEC studies (Michael et al. 1998; Kronschläger et al. 2013b). Additionally, Michael et al. reported that at threshold dose UVR exposure the occurrence of apoptotic LEC using TUNEL staining was verified by transmission electron microscopy (Michael et al. 1998). The composition of the culture medium in our study was identical to the culture medium of Sundelin et al (2014). The TUNEL assay kit worked well. LECs were in place adherent to the lens capsule showing clear staining (Figs 3 and 4). Cells face up or face down in the floating lens capsule during threshold UVR exposure induced the same amount of apoptosis. This might be due to the transmittance of the lens capsule of >90 % for the spectral output of the Bio-Link BLX system used in this study (Söderberg et al. 1996).

Regarding UVR-exposed lenses, radiation exposure is expressed as cataract threshold dose equivalent (maximum tolerable dose – $MTD_{2.3:16}$). The concept of the $MTD_{2.3:16}$ was described in detail by Söderberg et al. (2002), for example the $MTD_{2.3:16}$ of the pigmented mouse was calculated to 2.9 kJ/m^2 for UVR in the 300 nm range (Meyer et al. 2007). Ultraviolet radiation (UVR) in the 300 nm range is most toxic to the lens (Merriam et al. 2000). The UVR tubes used in our study have a maximum peak of 312 nm and a broad spectrum output including also UVR-A. Therefore, the comparability to the Söderberg irradiation system is only given by adjusting the energy dose of the Bio-Link BLX system. Threshold UVR dose applied in this study was 10 kJ/m^2. This dose is similar to the estimated threshold dose used in Gross et al. (2018), implementing the same UVR tubes. Ultraviolet intensity and effects are determined by the nature of the ultraviolet emitting device. It is difficult to compare those doses to real sunlight exposure, as sunlight is composed of different spectra of light and is generally referred to erythemal UV dose (IARC 2011). The erythemal UV dose is the total amount of UV radiation absorbed by the human skin during the day, for example a completely cloud-free day in June 2002 in De Bilt, the Netherlands, resulted in a cumulative erythemal ultraviolet dose of 4 kJ/m^2 (UV radiation monitoring: UV index and UV dose n.d.). Low-dose UVR exposure, however, is essential for finding a protective effect. UVR generates ROS and high-dose UVR exposure might overwhelm the protection given by caffeine concentrations in the ng range (Kronschläger et al. 2018). Therefore, UVR exposure was set to threshold dose.

The protective effect of caffeine in our study might have been boosted by other antioxidant compounds of coffee. Coffee consists of many antioxidant compounds. The main antioxidant compounds are caffeine and chlorogenic acids (Komes & Bušić 2014). Chlorogenic acids though are destroyed during the roasting process. Pyrocatechol, which is generated during the roasting process, inhibits selenite-induced cataract (Ishimori et al. 2017). However, pyrocatechol was not tested in UVR-induced cataract and its pharmacokinetics and stability during UVR exposure remain unclear. Therefore, it is anticipated that caffeine is the most important antioxidant of coffee in preventing UVR-induced cataract.

The time interval between caffeine intake and surgery varied (Table 1). Previous data, however, suggest that even 12 min after peroral caffeine consumption significant caffeine levels have accumulated in the lens capsule/epithelial cells (Kronschläger et al. 2018) and in addition caffeine is only slowly washed out of the lens (Kronschläger et al. 2014). Considering pharmacokinetics and the protective effect, we would suggest that caffeine be consumed just before sun exposure similar to sunscreens.

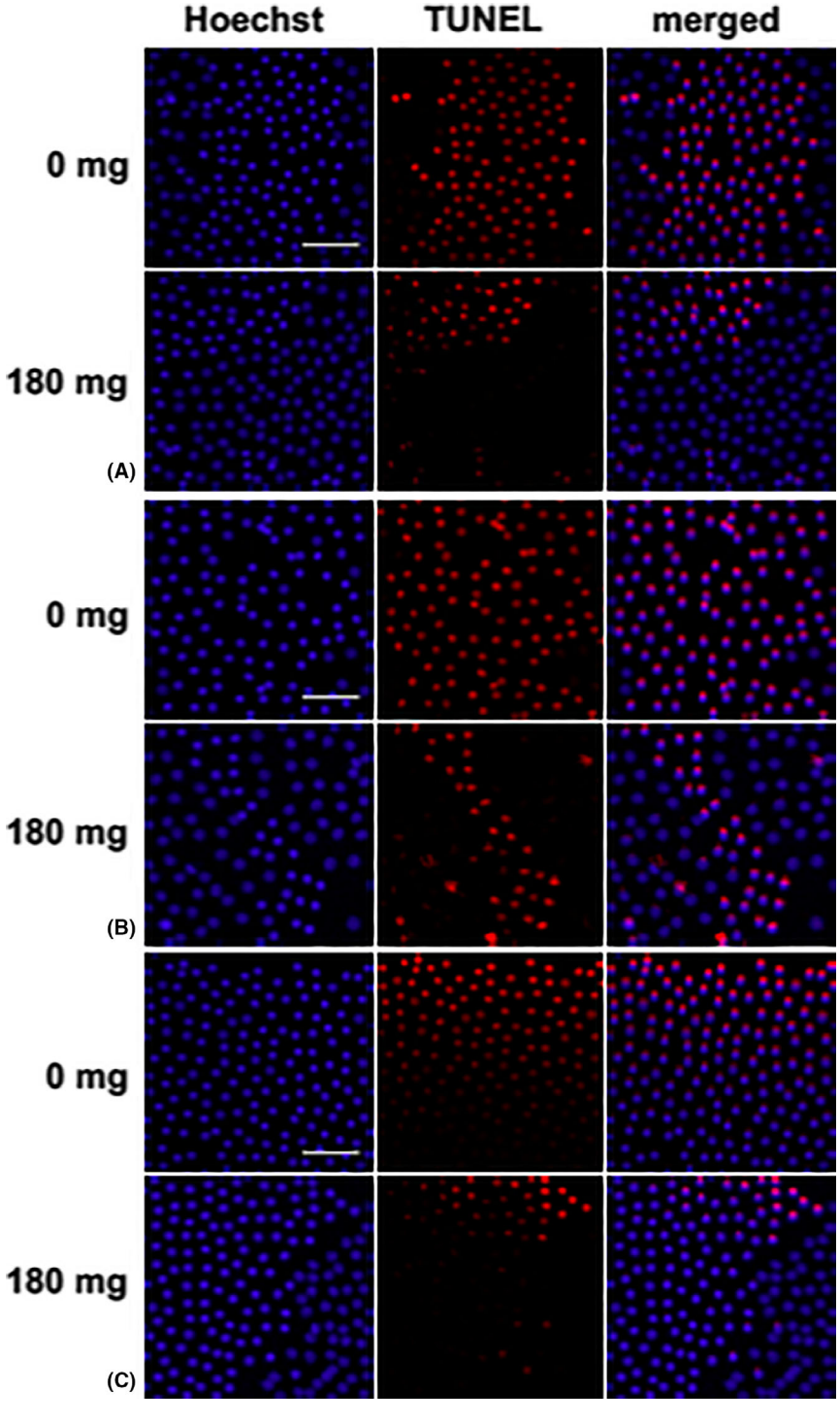

Fig. 4. Examples of Hoechst and TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining of lens epithelial cells in both eyes from three patients (A, B, C) after intake of 0 or 180 mg caffeine and threshold ultraviolet radiation exposure. Scale bar is 50 μm.

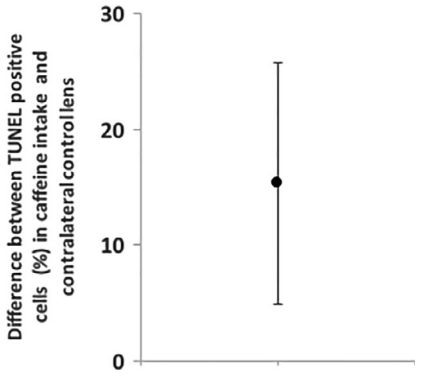

Fig. 5. Mean difference in TdT-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells (%) in lens epithelial cells between caffeine intake eye and contralateral control eye. Bars are 95% CI.

On the contrary, Klein et al. (2003) did not show the inverse relationship between caffeine consumption and onset of cataract. Klein et al. applied subjective scores for cataract grading. Objective quantitative data analysis using density of cataract measurements obtained by swept-source OCT for nuclear cataract and area of lens opacity measurements for cortical and posterior subcapsular cataract might be more precise in detecting significant lens opacity change.

In conclusion, we demonstrated that peroral caffeine intake inhibits UVR-induced apoptosis in LEC, backing results from epidemiological studies that reported a protective effect of caffeine consumption on age-related cataract. Caffeine is omnipresent in many beverages and chocolate-based food products and is therefore a major dietary antioxidant. Consequently, caffeine might have a significant impact on delaying the onset of age-related cataract. Further epidemiological studies are needed to confirm our findings.

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