



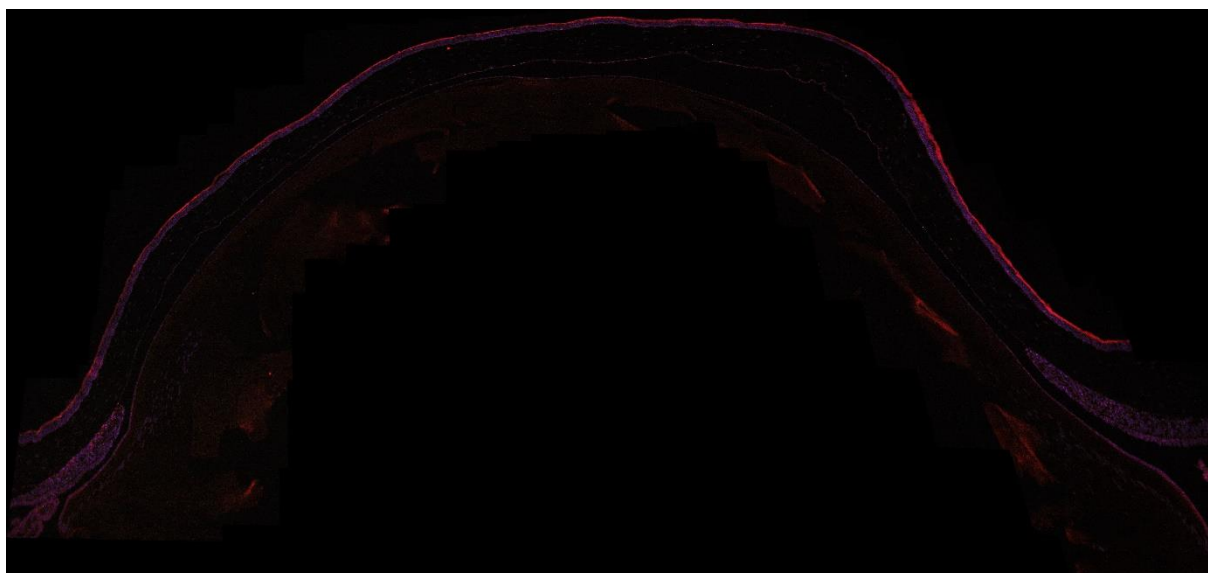
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SKRIFTLIG RAPPORT
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Time evolution of Cytochrome C release in the lens epithelial cells after in vivo exposure to UVR-300 nm

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Gråstarr

Gråstarr, grumlighet i ögats lins, är den främsta orsaken av blindhet i världen. Det finns ingen läkemedel för att bekämpa sjukdomen. Dagens behandling är kirurgi som inte är tillgängligt överallt i världen.

Exponering av solens UV-ljus är den största riskfaktorn för uppkomsten av sjukdomen. Studier har visat att denna exponering leder till programmerad celldöd i linsens celler som i sin tur leder till utveckling av gråstarr hos människor och djur. Igångsättning av den programmerade celldöden är beroende av ett signaleringssystem som börjar vid frisättning av ett protein som heter Cytokrom C. Genom att studera frisättningen av detta protein till följd av exponering till UV-ljus kan man bättre förstå sjukdomsmekanismen och möjligen utveckla läkemedelsbehandling.

För att undersöka frisättningen av Cytokrom C, användes 12 råttor. Råttornas vänstra ögon utsattes för UV-ljus av dosen 1 kJ/m^2 i 15 min medans de högra ögon bevarades utan exponering som kontroll. För att efterforska tidsberoendefrisättning av proteinet, delades djuren i 4 olika tidsgrupper och avlivades i olika tider av 1, 8, 16 och 24 timmar efter exponering. Frisättningen kontrollerades av markörer som binder till proteinet och släpper ut färg vilket går att se under mikroskop samt fotografera. Efter mikroskopering och fotografering gjordes, användes en färdig mjukvara för att granska färgskärpan på varje bild och skapa en siffra. Siffrorna jämfördes mellan olika bilder för att bedöma skillnaden mellan de utsatta och icke- utsatta ögon samt de 4 tidgrupperna mot varandra.

Resultatet visade ingen skillnad i färgskärpan mellan de utsatta och de icke-utsatta ögonen. Dessutom fanns inte heller någon skillnad i de 4 olika tidsgrupperna. Det talar för att dosen 1 kJ/m^2 i 15 min orsaker inte frisättning av Cytokrom C. Detta innebär att dosen och exponeringstiden är inte tillräcklig att ge upphov till den programmerade celldöden och gråstarr.

Abstract

The aim of this study was to investigate the time evolution of Cytochrome C (Cyt C) release in rat lens epithelial cells after in vivo exposure to 1 kJ/m² in 15 minutes of ultraviolet radiation type B (UVR-B). Twelve six-week-old female albino Sprague-Dawley rats were unilaterally exposed to 1 kJ/m² UVR-B at 300 nm. At a latency of 1, 8, 16 and 24 hours after exposure, both exposed and contralateral non-exposed eyes were enucleated and processed for immunohistochemistry. Three mid-sagittal sections from each lens were stained with antibodies. The stained lens epithelial cells were masked for estimation of mean pixel intensity. The results show no significant difference of Cyt C release among the post-exposure time groups (test statistic = 0.65, $f_{3,8} = 5.42$). There was no significant difference between exposed and contralateral non-exposed eyes (test statistic = 2.78, $f_{1,8} = 7.57$). No significant difference was indicated between exposed and contralateral non-exposed eyes at different postexposure time groups (test statistic = 0.23, $f_{3,8} = 5.42$). UVR-B of 1 kJ/m² at 300 nm in 15 minutes does not induce cytochrome C release from mitochondria into cytosol in the lens epithelial cells within 24 hours after exposure.

Key words: Cytochrome C - Apoptosis - Triton X-100 - UVR-B – Cataract – Lens

Introduction

Epidemiological (McCarty & Taylor, 2002) and experimental (Merriam et al. , 2000; Söderberg, 1988; Wang et al. , 2010) studies demonstrate that exposure to ultraviolet radiation (UVR) lead to cataract development. According to the World Health Organization, cataract is defined as clouding of the lens which blocks light transformation to the retina. Cataract is the major cause of blindness in the world (Resnikoff et al. , 2004). The current cure is surgical extraction of cataract and intraocular lens implantation. Over the next decades, demand for cataract surgery is expected to increase rapidly due to increased life expectancy. It has been estimated that the need for cataract surgery could be reduced by 50% if cataract onset is delayed by 10 years (Galichanin et al., 2012). To prevent, delay incidence of cataract and find new pharmaceutical treatments, it is essential to understand the mechanisms of cataract development in the lens.

The ocular lens is located behind the cornea and iris in the eyeball. The structures in the lens consist of the lens capsule, the lens epithelium, and the lens fibers. The capsule surrounds the lens providing a basement membrane anteriorly to the lens epithelium and lens fibers on the posterior surface (Danysh & Duncan, 2009). Laterally, the lens is suspended by ligaments known as zonular fibers. These fibers are produced by the ciliary body and are attached to the equatorial rim of the lens. For near and far vision of observed objects at various distance, the lens transmits and focuses the light onto the retina. During accommodation, the lens changes its shape through contraction and relaxation of ciliary muscles that modify the tension of the zonular fibers (Hejtmancik & Shiels, 2015). The anterior surface of the lens is covered by the lens epithelial cells. Cells in the central region (80% of the surface) have extremely low mitotic activity. Proliferation and division of the cells occur in the germinative zone surrounding the central region. The divided cells migrate to the equatorial region where they differentiate into fiber cells (Bhat, 2001; Lovicu & Robinson, 2004). The transparency of the lens is maintained by the particular arrangement of the lens cells and lack of light-scattering organelles (Bhat, 2001). Interruption in this homeostasis induced by UVR-B leads to pathological light scattering and cataractogenesis (Ayala et al., 2007; Kronschläger et al., 2013; Michael et al., 1998; Söderberg, 1988).

In the initiation of cataract disease in humans and animals, apoptosis plays a crucial role (Galichanin et al., 2012). Experimental studies show that UVR-B induces apoptosis in the lens epithelial cells (Galichanin et al. , 2010; W. C. Li et al. , 1995; Michael et al. , 1998). There are two well understood apoptotic pathways in mammalian cells, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Elmore, 2007). During the early stages of the mitochondrial pathway, Cyt C is released from the intermembrane space of mitochondria to the cytosol and interacts there with its adaptor molecule, Apaf-1, resulting in activation of pro-caspase-9 (P. Li et al. , 1997). Active caspase-9 cleaves and activates pro-caspase-3 and -7. These effector caspases are responsible for transition of the death signal that leads to morphological alterations presented in apoptosis (Robertson et al. , 2000). The morphological alterations are characterized by the condensation of both nuclear chromatin and cytoplasm. The process is followed by nucleus fragmentation and formation of apoptotic bodies containing nuclear material. Apoptotic bodies are eliminated by macrophages or adjacent cells through the process of phagocytosis (Elmore, 2007).

Release of Cyt C from the mitochondria is a key step in the apoptotic process. Between the inner and outer membrane of the mitochondria, Cyt C functions as an electron transfer mediator between complexes III and IV of the respiratory chain (Santucci et al. , 2019). Release of Cyt C to the cytosol can be Ca^{2+} -dependent or Ca^{2+} -independent. In the first case, The mitochondrial Ca^{2+} excess induces increased inner membrane permeability leading to matrix swelling, rupture of outer mitochondrial membrane and release of Cyt C. Ca^{2+} -independent release of Cyt C occurs by a two step-process including lipid peroxidation of its membrane-anchoring lipid, cardiolipin, and pro-apoptotic protein BAX promoted outer membrane permeabilization (Ott et al. , 2002).

Cyt c is detectable by immunofluorescence. In the methodology of immunohistochemistry, a commonly used membrane permeabilization agent is Triton X-100. It can at some concentrations lyse mitochondrial outer membranes (Gurtubay et al. , 1980), making Cyt C accessible to detection antibodies. In this study, a qualitative determination of different Triton concentrations allowed selection of a reliable Triton concentration.

Methods and materials

Animals

The experimental animals were six-week-old albino Sprague-Dawley (SD) female rats. All were kept and treated according to the ARVO (The association for research in vision and ophthalmology) statement for the use of Animals in Ophthalmic and Visual Research. Ethical approval was obtained from the Uppsala Ethics Committee on Animal Experiments, protocol number C 29/10.

Experimental design

Totally 12 rats were used in this study. The animals were divided into four post-exposure time groups and were sacrificed at 1, 8, 16 and 24 hours after exposure. Altogether, 24 rat lenses, were processed for immunohistochemistry. For each lens, three slides were stained to detect and compare release of Cytochrome C. For each slide, three different nuclei labelling were recorded.

Ultraviolet radiation source

UVR source

A high-pressure mercury lamp (model 6828; oriel, Stratford, CT, USA) generated UVR-B in the 300 nm wavelength region. The radiation was collimated and sequentially passed through a water filter, an interference filter ($\lambda_{\max} = 300$ nm; half bandwidth, 10 nm) and finally projected on the cornea of the left eye (Michael et al. , 2000). A custom-made photodiode was calibrated to a thermopile (model 818P-001-12; Newport, USA) and used to measure the irradiance of UVR in the corneal plane.

UVR exposure

The animals were anesthetized 15 minutes before the exposure by intraperitoneal injection, a mixture of 90 mg/kg ketamine and 10 mg/kg xylazine (Zhang et al. , 2007). The mydriaticum tropicamide 5 mg/ml was topically applied five minutes after the injection. The rats were exposed unilaterally to 1 kJ/m² UVR-B (UVR-300 nm) for 15 minutes while the contralateral eyes were protected during exposure and used as control (Dong et al., 2005).

Immunolocalization of Cytochrome C

Tissue fixation and dehydration

After UVR-B exposure, the animals were sacrificed with carbon dioxide (CO₂) followed by cervical dislocation. Both eyes were enucleated and placed in phosphate buffered saline (PBS, Medico, Sweden). The eyes were then fixed in 4% freshly prepared Paraformaldehyde (Paraformaldehyde, Sigma-Aldrich Co, St. Louis, MO, USA) for 20 minutes at room temperature followed by 30 minutes washing with PBS. The eyes were dehydrated in 30% sucrose overnight at 4°C and then positioned in a cup, embedded in Tissue-Tek® OCT™ Compound (Sakura Finetek, the Netherlands) and frozen at -70°C.

Immunohistochemistry

The frozen eyes were cryosectioned in 10- μ m-thick mid-sagittal slices and mounted on slides. Three slides were discarded between sequential slides to avoid staining the same nuclei in different slides. Slides were rehydrated in PBS for 15 minutes and then, were blocked and permeabilized in blocking buffer (1XPBS/5% normal serum/ 0.09% Triton X-100). Primary rabbit polyclonal-cleaved cytochrome C antibody (Asp175 9661; Cell Signaling Technology, Inc., Danvers, MA, USA) was diluted in antibody dilution buffer (1XPBS/1% BSA). After applying the primary antibody, the slides were incubated in a humidifying chamber at +4°C overnight. The slides were rinsed three times in 1xPBS for 5 minutes each and incubated in secondary antibody (Anti-Rabbit IgG Alexa flour 488 Conjugate) for 2 hours at room temperature in the dark. After rinsing three times in 1xPBS for 5 minutes each, the slides were finally mounted with mounting medium containing DAPI (4', 6-diamidino-2-phenylindole, Vectashield; Vector Laboratories, Burlingame, CA, USA) and allowed to cure overnight at room temperature. The slides were then photographed using a fluorescence microscope (Universal Microscope Axioplan 2 Imaging; Carl Zeiss, Thornwood, NY, USA).

Applying a standard green pass filter matched to fluorescein (FITC), Cyt C labelled lens epithelial cells were observable in red and DAPI stained cell nuclei were visible in blue. Images of each slide were captured with the standard green pass filter during a 0.3 second exposure time. The images were stitched using Image Composite Editor (ICE) and CorelDraw to obtain an image of the whole lens epithelial cell line. The region of interest, the epithelial cell line, was then manually masked in CorelDraw software, using freehand tool under RGB model of R 255 G 0 B 0. Both original and masked image were saved as jpg files in one folder. The files were finally loaded on MATLAB to read the differences in the mean light

intensity of each image. Each image represented a light intensity value that was used for statistical analysis.

Determination of Triton X-100 Concentration

The contralateral non-exposed eyes were used to detect a reliable concentration of Triton X-100. Each slide was treated with different Triton X-100 concentrations of respectively 0.00%, 0.05%, 0.09%, 0.16% and 0.3%. All slides were incubated for 30 minutes. After applying the Cyt C primary and secondary antibodies and given incubation time, the slides were photographed in a fluorescence microscope.

Statistical Analysis

The mean pixel intensity value and CI for each group was calculated. Analysis of variance (ANOVA) was used to assess the difference between the time groups and the difference against the contralateral non-exposed eyes. The significance level and the confidence coefficient were respectively set to 0.05 and 0.95.

Results

UVR-B induced Cytochrome C Release

Figure 1 shows the mean pixel intensity of the exposed vs non-exposed eyes (baseline) in different time groups with its respective CI. The CI of each time groups crosses zero which indicates that there is no difference between the exposed and non-exposed eyes in different times groups.

The outcome of ANOVA indicated no significant difference between the post-exposure time groups (test statistic = 0.65, $f_{3,8} = 5.42$). There was no significant difference between exposed and contralateral non-exposed eyes (test statistic = 2.78, $f_{1,8} = 7.57$). Finally, there was no significant difference was between exposed and contralateral non-exposed eyes at different post-exposure time groups (test statistic = 0.23, $f_{3,8} = 5.42$).

There was no difference observed in the pixel intensity indicating Cyt C release within 24 hours after in vivo exposure to UVR-B (UVR-300 nm) (Figure 2).

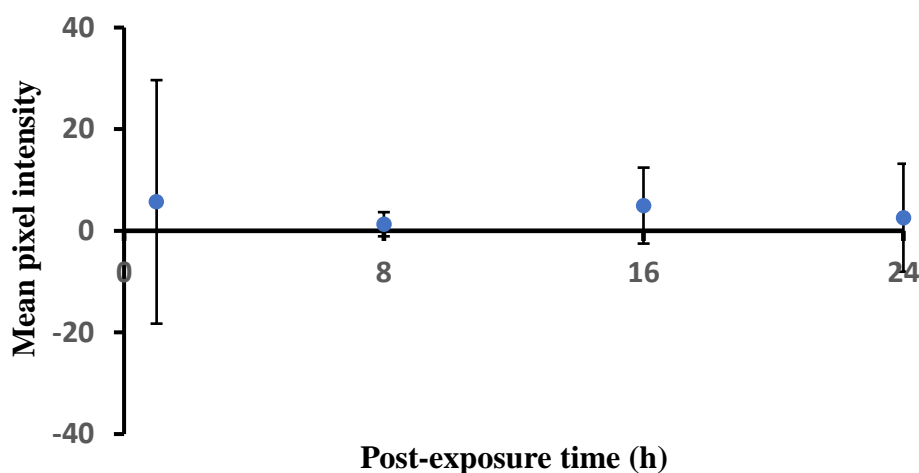


Figure 1 The mean difference of Cyt C release between the exposed and contralateral non-exposed lens (Baseline) after in vivo exposure to UVR-B (UVR-300 nm) as a function of post-exposure time. Bars are 95% CI for the mean difference.

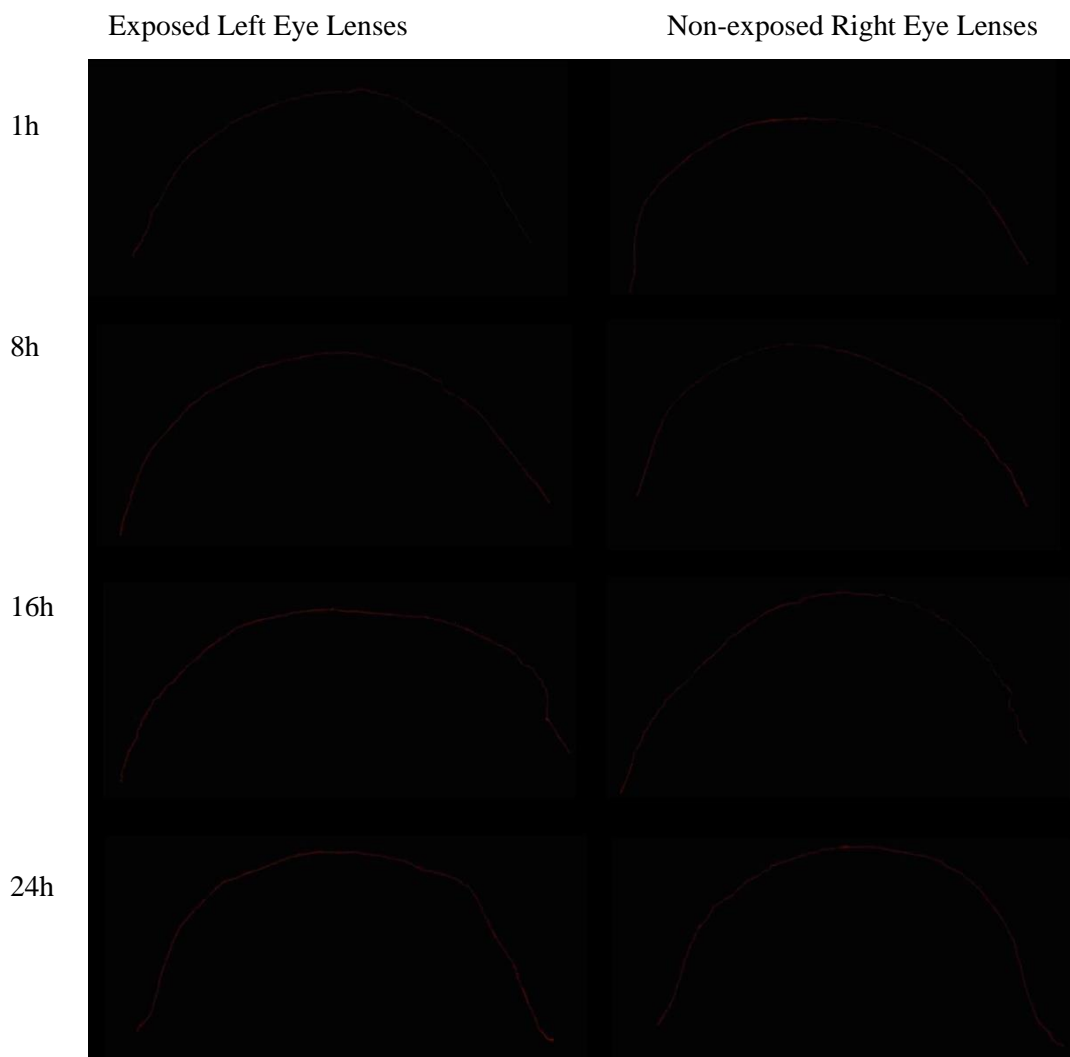


Figure 1 Cyt C staining for the whole epithelium cell line after 15 minutes in vivo exposure to 1 kJ/m² UVR-B in the exposed and contralateral non-exposed eyes

Qualitative determination of Triton X-100 concentration

Cytochrome C staining in the contralateral non-exposed lens epithelium cells at different concentrations of Triton X-100 was observed. At the concentrations of 0.00%, 0.05%, 0.09%, 0.16%, 0.3%, the pixel intensity increased as Triton X-100 concentration increased (Figure 3). A 0,09% concentration of Triton X-100 was selected and used during analysis.

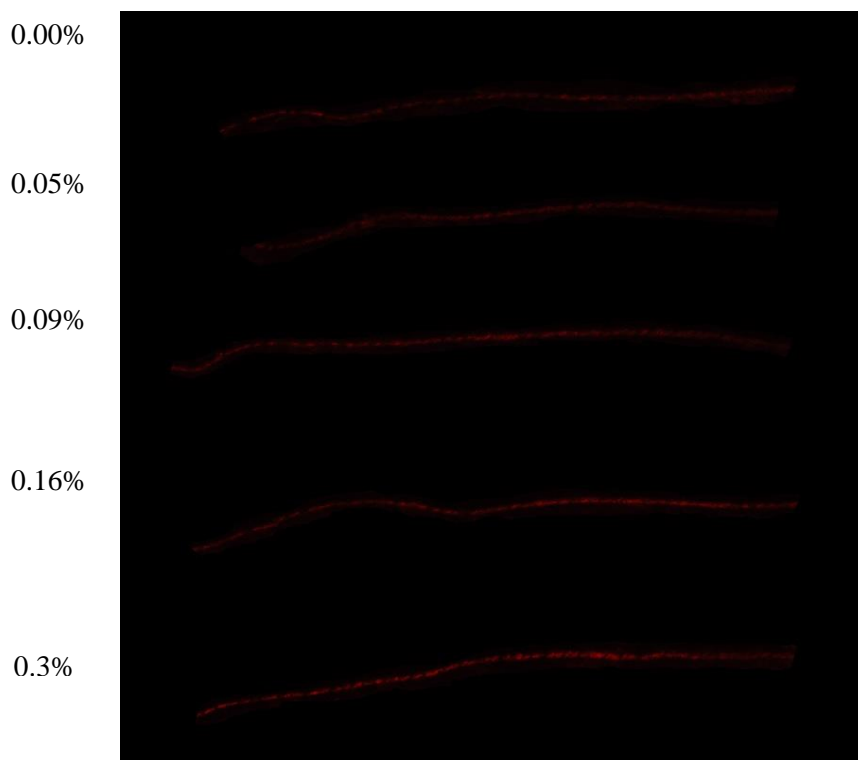


Figure 3 Cytochrome C staining in the non-exposed lens epithelium cells at different concentrations of Triton X-100.

Discussion

This study was designed to investigate the release and time evolution of Cyt C after 15 minutes of in vivo exposure to 1 kJ/m^2 UVR-B. The result of this study shows that exposure at this dose does not induce Cyt C release from mitochondria in the lens epithelial cells within 24 hours post-exposure.

The dose 1 kJ/m^2 is a subthreshold dose and does not induce light scattering (Söderberg et al. , 2002). However, the expression of p53 and active caspase-3 after exposure to 1 kJ/m^2 was demonstrated by several authors (Galichanin, 2017; Galichanin et al. , 2014; Galichanin, Svedlund, et al. , 2012; Talebizadeh et al. , 2014). Active caspase-3 is downstream to Cyt C in the cascade of apoptotic proteins. Release of Cyt C is essential for the activation of caspase-3 (Santucci et al. , 2019). The maximum tolerable dose (MTD) of UVR-B at 300 nm in rat was estimated at 3.65 kJ/m^2 . Doses above this threshold cause lens opacification that leads to light scattering (Söderberg et al., 2002). Above MTD, apoptotic features and mediator proteins including p53 and active caspase-3 are demonstrated in studies done by several investigators

(Ayala et al., 2007; Galichanin et al., 2010; Galichanin, Svedlund, et al., 2012; Michael et al., 1998; Söderberg, 1988). Subthreshold doses have additive effects and can lead to doses above MTD, causing development of cataract (Galichanin, et al., 2014).

Both animal and epidemiological studies in countries with excessive light, show that exposure to UVR-B leads to production of reactive oxygen species (ROS) (Varma et al. , 2011). ROS is capable of releasing nearly 85% of Cyt C a cell (Ott et al. , 2002). In the current study, no release was revealed in comparison to the contralateral non-exposed lens (Fig. 2), (Fig. 3). A subthreshold dose of 1 kJ/m² is not enough for the release of Cyt C, although (Galichanin, 2017; Talebizadeh et al. , 2014) demonstrated expression of active caspase-3 at the same dose. However, a study by (Garcia-Faroldi et al., 2013) showed the presence of active caspase-3 in granule-like compartments of mast cells in mice. Garcia found caspase-3-like enzymatic activities in the cytosol after the permeabilization of the granule membrane. This indicates the possibility of Cyt C independent active caspase-3 existence in the cytosol of lens epithelial cells. The granule membrane can be permeabilized when cells are treated with Triton X-100. The extrinsic apoptotic pathway can also activate caspase 3. Nevertheless, it is unknown if exposure to UVR can lead to extrinsic pathway induced apoptosis. Further research is needed to elucidate this matter.

Determination of Triton X-100 concentration for the process of immunohistochemistry was essential. Triton at different concentrations can solubilize membranes and proteins. Cell membrane lysis is detectable already at 0.05% Triton. Solubilization and disintegration of mitochondrial membranes occur above 0.1% Triton. When Triton concentration exceeds 0.5%, the granular material of the inner membrane consists mostly of protein-rich aggregates. Above 0.5% Triton more than 30% of Cyt C is solubilized and solubilization increases as Triton concentration increase (Gurtubay et al. , 1980). The disintegration of the mitochondrial membrane facilitates accessibility of Cyt C to its primary and secondary antibodies leading to false results. The purpose of this study was to investigate Cyt C release due to UVR-B induced apoptosis, not Triton facilitated Cyt C-antibody interaction which would be the false results. The result of qualitative determination of Cyt C concentration proved that more antibodies access Cyt C as Triton induced membrane disintegration occur (Fig.1). However, above 0.5% Triton, protein-rich aggregates form and could trap antibodies leading to a false result. A reasonable concentration of Triton was therefore set to 0.09%. The signal at 0.00% Triton might be due to extramitochondrial localization of Cyt C (Soltys et al. , 2001).

Immunofluorescence supplied information about the release and location of Cyt C. Western blotting detection of Cyt C might be possible but tissue processing for western blotting could cause destruction of Cyt C. Immunohistochemistry, utilized in the current study is associated with problems of non-specific bindings that generate false-positive signals and background noise. This problem could have affected the Matlab program that read the mean pixel intensity of the samples.

Conclusions

Ultraviolet radiation at 1 kJ/m^2 does not induce Cyt C release from mitochondria in the lens epithelial cells within 24 hours after exposure.

Disclosures

The authors have no financial conflicts of interest.

Data availability

Data will be made available on request.

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