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Ultraviolet Action Spectrum for Cell Killing of Primary Porcine Lens Epithelial Cells

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Abstract: Ultraviolet Action Spectrum for Cell Killing of Primary Porcine Lens Epithelial Cells: Tsutomu Okuno, *et al*. Human Engineering and Risk Management Research Group, National Institute of Occupational Safety and Health, Japan—Objectives: The aim of this study was to determine ultraviolet (UV) action spectra for cell killing of primary porcine lens epithelial cells (LECs) that can be used to establish guidelines for evaluation of the hazard of cataract due to UV exposure in the workplace. **Methods:** Primary porcine LECs were exposed to different doses (radiant exposure) of UV at 17 different wavelengths from 235 nm to 311 nm. At 2 days after exposure, cell viability was assessed by measuring crystal violet staining of the cells and lactate dehydrogenase release into the culture medium. The exposure dose required to kill 50% of cells (LD_{50}) was determined from the dose-effect relationship obtained at each wavelength and was used to construct action spectra. **Results:** The action spectra had a broad minimum in the approximate range of 250–280 nm, indicating that UV is most hazardous to porcine LECs within this wavelength range. The spectra rose steeply at both longer and shorter wavelengths. These action spectra are consistent with the *in vivo* action spectra for opacities in the rabbit lens and for light scattering in the rat lens, taking the transmittance of the ocular media into account. **Conclusions:** These results will help to determine a UV hazard function for cataract formation, which can be used to draft guidelines for evaluation of the hazard of cataract due to UV exposure in the workplace.
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Key words: Action spectrum, Cataract, Cell killing, Hazard evaluation, Lens epithelial cell, Ultraviolet radiation

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Many workers are exposed to ultraviolet (UV) radiation from the sun, welding arcs, germicidal lamps and other sources. UV radiation can cause a variety of health problems, including photokeratitis, photoconjunctivitis, cataract, pterygium, erythema, skin aging and skin cancer[1, 2]. Indeed, photokeratitis[3, 4] and erythema[3] often occur in workplaces where arc welding is performed, and cataract[5, 6] and skin cancer[7–10] are prevalent among outdoor workers who are likely to be exposed to a large amount of solar UV radiation. As a first step toward protecting workers from these health problems, UV hazards should be evaluated in workplaces.

Because UV radiation of different wavelengths has different effects in regard to health problems, the wavelength dependence of effectivity is crucial for the evaluation of UV hazards. This wavelength dependence is represented by action spectra or hazard functions.

An action spectrum is a plot of the exposure dose of monochromatic optical radiation such as UV radiation that induces a specific photochemical or photobiological effect as a function of wavelength. Action spectra for adverse health effects in humans that are used for hazard evaluation are specifically called hazard functions. To evaluate the hazard of a specific health effect, the hazard function for that particular health effect is required because hazard functions generally differ for different health effects.

A hazard function has been developed from action spectra for photokeratitis in animals and UV-induced erythema in humans[11], and based on this hazard function, several guidelines have been established for evaluation of the combined hazards of photokeratitis and erythema[12–14]. These guidelines have enabled

evaluation of the UV hazard of these acute injuries in the workplace. For other UV-induced health effects, however, hazard functions have not been established due to a lack of action spectrum data, and therefore, the UV hazards cannot be evaluated.

Action spectra for effects on animals, cultured cells and biological molecules are important for the development of hazard functions because human action spectra are often difficult or impossible to measure directly. Nonhuman action spectra are also useful in that they can be accurately determined in carefully designed and controlled experiments. However, there are still insufficient data on nonhuman action spectra from which to derive reliable hazard functions. More studies are needed on action spectra related to UV hazards.

As part of a project to study action spectra that can be used for UV hazard evaluation, we previously determined the action spectrum for cell killing of a human lens epithelial cell line within the wavelength range of 260–300 nm by measuring the viability of cells after exposure to narrowband UV radiation at different wavelengths in carefully designed and controlled experiments[15]. In the present study, we similarly determined the action spectra for killing primary porcine lens epithelial cells (LECs) within a wider wavelength range of 235–311 nm. These results, combined with the previous results, will contribute to the development of hazard functions, particularly that for cataract formation, because it has been suggested that UV-induced damage to the lens epithelium in humans leads to cataract formation[16].

Materials and Methods

Cell preparation

Approximately 15 porcine eyes (Tokyo Shibaura Zoki, Tokyo, Japan) were purchased and used for each cell preparation within several hours of slaughter. After removing the cornea from the eye, the lens was extracted. The posterior capsule of the lens was incised, and the lens cortex and nucleus were removed to obtain the lens epithelium. Two or three pieces of the lens epithelium were incubated in a dish to grow LECs. Isolated LECs were cultured for three passages before use.

Cells were seeded at a density of 40,000 cell/m*l* in 96-well plates in Dulbecco's Modified Eagle's Medium (DMEM/F-12, Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies), 100 U/m*l* penicillin (Gibco, Life Technologies) and 100 μg/m*l* streptomycin (Gibco, Life Technologies) and were grown to subconfluence at 37°C in a humidified atmosphere of 5% CO_2. Cells were then cultured in low-serum medium containing 0.2% FBS for 1 day to inhibit cell growth, which tends to be irregular. Inhibiting cell growth also serves to prevent UV-induced cell growth inhibition from affecting the results. Immediately after the medium was replaced with phosphate-buffered saline (PBS), which is transparent to UV in the wavelength region studied, cells were exposed to UV radiation. After exposure, cells were continuously cultured in low-serum medium for 2 days, during which UV-induced cell death occurred, and cell viability was assessed.

UV exposure

A xenon lamp light source (MAX-301, Asahi Spectra, Tokyo, Japan) was used for UV exposure. This apparatus is equipped with bandpass filters that isolate specific wavelength regions of UV radiation. The UV output was delivered to the cells with a uniform irradiance by a quartz light guide (Asahi Spectra) and a quartz collimating lens (Asahi Spectra).

Cells in 96-well plates were exposed to narrowband UV radiation with a bandwidth of approximately 10 nm at 17 different wavelengths from 235 nm to 311 nm (Table 1). The dose-effect relationship was determined by exposure of cells in two 96-well plates to 9 different doses of radiant exposure at each wavelength. Doses were applied at regular intervals ranging from 0 to approximately 2 times the dose expected to produce a surviving cell fraction of 50%. Four wells were used for each of the nonzero doses, and 8 wells were used for the zero doses used as controls. As positive controls, 8 wells were exposed to 30 mJ/cm^2 of 270-nm UV radiation, which killed all of the cells.

Before exposure at each wavelength, irradiance was measured at the position of the target cells with a radiometer (IL1400A, International Light Technologies, Peabody, MA, USA) connected to a silicon photodiode detector (SEL033, International Light Technologies), and exposure durations were determined by dividing the target doses by the measured irradiance. After exposure, irradiance was remeasured, averaged with the first measurement and used to calculate accurate exposure doses. The radiometer was calibrated prior to the experiments.

Cell viability assessment

Cell viability was assessed by measuring crystal violet (CV) staining of the cells and lactate dehydrogenase (LDH) release into the culture medium.

After removing the medium from the wells, cells in 96-well plates were washed with saline and lightly dried. Cells were then stained by incubation at room temperature for 15 min with 50 μ*l*/well of 0.1% crystal violet (Wako Pure Chemical Industries, Osaka, Japan) and 0.1% methanol in PBS, and the cells were

Table 1. UV exposure

Center wavelength (nm)	Bandwidth* (nm)	Irradiance (mW/cm^2)	Exposure duration (s)	Exposure dose (radiant exposure) (mJ/cm^2)
235.0	8.3	0.313–0.386	<50.3	9 doses in the range 0–16
237.8	9.4	0.480–0.598	<26.9	9 doses in the range 0–14
241.8	10.4	0.265–0.435	<33.2	9 doses in the range 0–8.8
247.0	11.1	0.252–0.345	<26.8	9 doses in the range 0–7.2
250.4	10.4	0.308–0.322	<20.3	9 doses in the range 0–6.4
256.6	9.2	0.404–0.491	<13.8	9 doses in the range 0–5.6
261.7	10.0	0.351–0.388	<16.0	9 doses in the range 0–5.7
266.6	8.5	0.390–0.517	<12.3	9 doses in the range 0–4.8
270.3	10.7	0.224–0.261	<21.4	9 doses in the range 0–4.8
277.5	9.4	0.461–0.467	<10.4	9 doses in the range 0–4.8
280.7	9.5	0.276–0.324	<20.2	9 doses in the range 0–5.6
285.9	8.2	0.909–0.919	<10.5	9 doses in the range 0–9.6
292.7	10.5	0.961–1.065	<20.7	9 doses in the range 0–20
296.7	8.8	1.22–1.59	<23.0	9 doses in the range 0–28
301.2	10.9	2.79–3.09	<28.7	9 doses in the range 0–80
306.4	11.3	2.88–4.48	<69.4	9 doses in the range 0–210
311.3	12.1	3.60–3.85	<249	9 doses in the range 0–960

*Full width at half maximum.

then washed with water and dried. After adding sodium dodecyl sulfate (Wako Pure Chemicals Industries) to the wells, their absorbance at 570 nm, which was expected to be proportional to the number of living cells, was measured using a microplate reader (Model 680 XR, Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was calculated from this absorbance by linear interpolation between the mean absorbances obtained for the negative and positive controls, which correspond to cell viabilities of 0 and 100%, respectively.

LDH release into the culture medium was measured using a cytotoxicity kit (MTX-LDH, Kyokuto Pharmaceutical Industrial, Tokyo, Japan). The culture medium collected from wells (50 μl/well) was transferred to another 96-well plate and incubated at room temperature for 45 min with a detection reagent containing nitrotetrazolium blue. After adding the stop agent to the wells, their absorbance at 570 nm, which was expected to be proportional to the number of dead cells, was measured using the microplate reader. Cell viability was calculated from this absorbance by linear interpolation between the mean absorbances obtained for the negative and positive controls, which correspond to cell viabilities of 0% and 100%, respectively.

Derivation of LD_{50} and action spectra

The cell viability data, measured at each wavelength as a function of the dose of radiant exposure, were fitted with a cumulative lognormal distribution function using the least squares method. The exposure dose required to kill 50% of cells (LD_{50}) was calculated using the best-fit function.

The experiment was repeated more than three times at each wavelength, and the mean and standard deviation of LD_{50} were calculated. Action spectra were constructed from the mean LD_{50}.

Results

We measured cell viability as a function of UV dose at different wavelengths. At each exposure wavelength, cell viability, assessed by CV staining and LDH release, decreased with increasing radiant exposure in a manner described by a cumulative lognormal distribution function (Fig. 1). The LD_{50} was then calculated from this function (Table 2). The LD_{50} assessed using CV staining was lower than that assessed using LDH release by 18–58%. The LD_{50} varied greatly with wavelength, ranging from 1.77 to 319 mJ/cm^2 as assessed using CV staining and from 2.39 to 424 mJ/cm^2 as assessed using LDH release (Table 2).

Two action spectra were constructed by plotting the LD_{50} obtained using the CV staining or LDH release assay against wavelength (Fig. 2). These spectra have a broad minimum in the approximate range of 250–280 nm, indicating that UV is most hazardous to cells within this range. The spectra rise steeply at both longer and shorter wavelengths.

Discussion

In this study, we developed UV action spectra for cell killing of primary porcine LECs. It was shown that UV is most effective in killing LECs in the

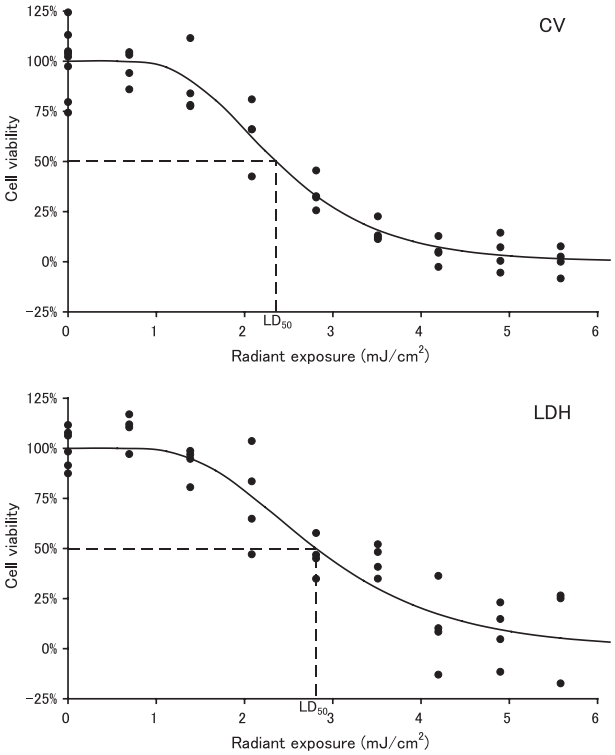

Fig. 1. Cell viability as a function of radiant exposure. Cell viability was assessed by crystal violet (CV) staining and lactate dehydrogenase (LDH) release after the indicated exposure to 256.6-nm UV. The solid curve represents the cumulative lognormal distribution function best fitted to the data, from which the LD_{50} is derived.

approximate range of 250–280 nm. This implies that UV absorption by both DNA and protein leads to cell death because DNA and protein have strong absorption around 260 nm and 280 nm, respectively[17].

The CV staining and LDH release assays used to determine cell viability after UV exposure showed some discrepancies in cell viability despite the use of both positive and negative controls. This means that the CV staining of the cells or LDH release into the culture medium or both are not linearly correlated with cell viability.

There have been a few reports of UV action spectra for killing LECs *in vitro*. We previously deter-

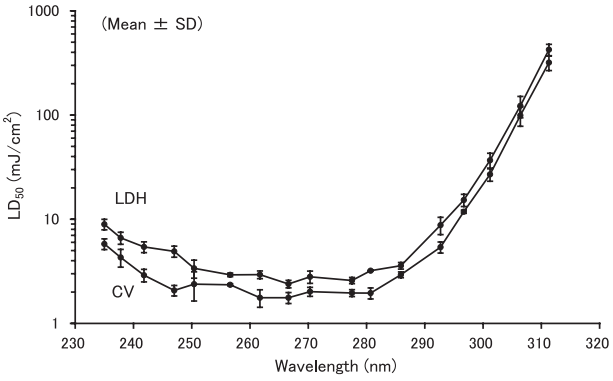

Fig. 2. Action spectra constructed from the LD_{50} assessed by crystal violet (CV) staining and lactate dehydrogenase (LDH) release. The obtained LD_{50} was plotted against the indicated UV wavelength.

Table 2. LD_{50}

	LD_{50} assessed by crystal violet staining			LD_{50} assessed by lactate dehydrogenase release		
Wavelength (nm)	Mean (mW/cm^2)	SD* (mW/cm^2)	N**	Mean (mW/cm^2)	SD* (mW/cm^2)	N**
235.0	5.80	0.68	4	8.95	1.0	4
237.8	4.30	0.83	5	6.62	0.88	3
241.8	2.90	0.40	3	5.42	0.65	3
247.0	2.07	0.24	4	4.92	0.60	3
250.4	2.38	0.74	4	3.38	0.67	4
256.6	2.35	0.05	3	2.93	0.11	3
261.7	1.77	0.34	3	2.94	0.25	3
266.6	1.77	0.21	4	2.39	0.20	3
270.3	2.02	0.20	3	2.80	0.38	3
277.5	1.96	0.15	4	2.58	0.18	3
280.7	1.96	0.24	5	3.21	0.02	3
285.9	2.93	0.19	3	3.58	0.26	3
292.7	5.39	0.66	3	8.77	1.7	3
296.7	11.8	0.42	4	15.3	2.0	3
301.2	27.0	3.8	4	36.8	6.3	3
306.4	99.2	21	4	123	28	4
311.3	319	52	3	424	53	3

*Standard deviation. **Number of experiments.

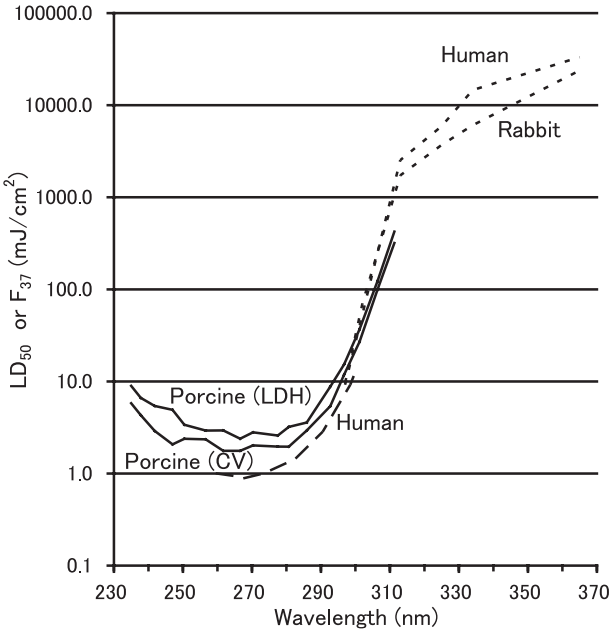

Fig. 3. Action spectra for cell killing of different lens epithelial cells. Solid lines: LD_{50} for primary porcine cells determined using crystal violet (CV) staining and lactate dehydrogenase (LDH) release assays in the present study. Dotted lines: F_{37} for the human cell line (N/N1003A) and the rabbit cell line (HLE B-3) determined using a clonogenic assay[18, 19]. Dashed line: LD_{50} for the human cell line (SRA 01/04) determined using a CV staining assay in a previous study[15].

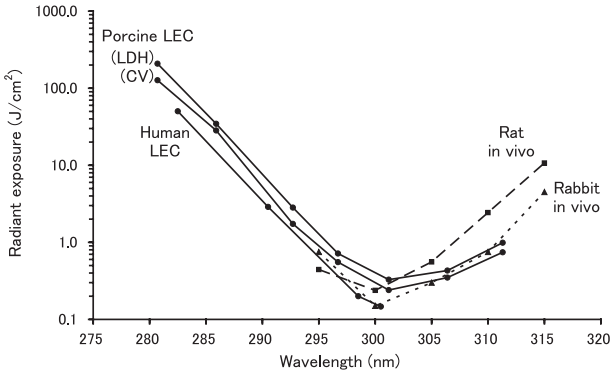

Fig. 4. *In vivo* action spectra. Solid lines: action spectra for the killing of lens epithelial cells (LECs) in humans estimated from the *in vitro* action spectra for primary porcine LECs determined using the crystal violet (CV) staining and lactate dehydrogenase (LDH) release assays in the present study and for the human LEC line (SRA 01/04) determined using the CV staining assay in a previous study[15]. Dotted line: *in vivo* action spectrum for opacities in the rabbit lens based on threshold values[21]. Dashed line: *in vivo* action spectrum for light scattering in the rat lens based on the "maximum acceptable dose"[22].

mined an action spectrum for cell killing of a human LEC line (SRA 01/04) within the wavelength range of 260–300 nm using a similar CV staining assay[15]. The obtained LD_{50} was somewhat lower than that in the present study (Fig. 3), presumably because cells were not cultured in low-serum medium to inhibit cell growth in the previous study. Thus, UV should have induced cell growth inhibition in addition to cell death, which decreased cell viability values and then led to the lower LD_{50}.

Andley *et al*. determined action spectra within the wavelength range of 297–365 nm for cell killing of a rabbit LEC line (N/N1003A)[18] and a human LEC line (HLE B-3)[19] using a clonogenic assay (Fig. 3). Their action spectra cannot be directly compared with ours because they were constructed using an exposure dose that results in a cell surviving fraction of 37% (F_{37}). Nevertheless, all of these action spectra have similar shapes, suggesting that all types of LECs *in vitro* may have the same UV action spectrum for cell killing.

The action spectra obtained in this study are important in terms of evaluation of the hazard of cataract due to UV exposure, since they will help to derive the action spectrum for LECs *in vivo*, which can then be used to develop a hazard function for cataract formation. LECs *in vivo* are exposed to attenuated ambient UV because ambient UV is partially absorbed as it travels through the cornea and anterior chamber before reaching the LECs. This means that the action spectrum for LECs *in vivo* is calculated by dividing the action spectrum for naked LECs by the combined spectral transmittance of the cornea and anterior chamber.

Thus, assuming that naked human LECs respond to UV in the same way as porcine LECs *in vitro*, we calculated the action spectra for killing human LECs *in vivo* by dividing the *in vitro* action spectra obtained in this study by the spectral transmittance of the human cornea and aqueous humor (Fig. 4). The spectral transmittance was estimated from the thicknesses of the human cornea and aqueous humor and the spectral absorption coefficients of the cornea and aqueous humor of the rhesus monkey, which were measured very carefully by Maher[20] and are considered a good baseline for the primate eye. If UV-induced death of LEC leads to cataract formation, the *in vivo* action spectra obtained in this study (Fig. 4) should represent the action spectrum for cataract formation.

The *in vivo* action spectra (Fig. 4), unlike the *in vitro* action spectra (Fig. 3), have a distinct minimum around 300 nm and rise steeply at both longer and shorter wavelengths. This shape suggests that UV at around 300 nm is particularly effective in causing cataract.

The *in vivo* action spectra derived from the *in vitro*

action spectra in both the present and previous studies[15] and the *in vivo* action spectra for opacities in the rabbit lens[21] and for light scattering in the rat lens[22] agree well with one another (Fig. 4), even though they were determined using different materials and/or methods. This result indicates the reliability of all of these action spectra. Thus, it may be possible to develop a hazard function for cataract formation using these action spectra and to establish guidelines for evaluation of the UV hazard of cataract in the workplace based on this hazard function.

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