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Effects of UV wavelength on cell damages caused by UV irradiation in PC12 cells

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Abstract

Ultraviolet (UV) radiations present in sunlight are a major etiologic factor for many skin diseases and induce DNA damage through formation of cyclobutane pyrimidine dimer (CPD). This study was conducted to determine the toxicological effects of different wavelengths (250, 270, 290, and 310 nm) and doses of UV radiation on cell viability, DNA structure, and DNA damage repair mechanisms in a PC12 cell system. For this, we evaluated cell viability and CPD formation. Cell survival rate was markedly decreased 24 hours after UV irradiation in a dose-dependent manner at all wavelengths (except at 310 nm). Cell viability increased with increasing wavelength in the following order: 250 < 270 < 290 < 310 nm. UV radiation at 250 nm showed the highest cell killing ability, with a median lethal dose (LD50) of 120 mJ/cm². The LD50 gradually increased with increase in wavelength. Among the 4 wavelengths tested, the highest LD50 (6,000 mJ/cm²) was obtained for 310 nm. CPD formation decreased substantially with increasing wavelength. Among the 4 wavelengths, the proportion of CPD formation was highest at 250 nm and lowest at 310 nm. On the basis of LD50 values for each wavelength, PC12 cells irradiated with UV radiation of 290 nm showed maximum DNA repair ability, whereas those irradiated with the 310-nm radiation did not show any repair ability. Toxicity of UV radiation varied with wavelengths and exposure doses.

Keywords: Ultraviolet wavelengths; PC12 cell; cell viability; cyclobutane dimer formation; DNA damage; DNA repair

1. Introduction

Ultraviolet (UV) radiation induces basal and squamous cell carcinoma and cutaneous malignant melanoma in humans [1-3] and is therefore a major environmental concern. UV radiations exert their toxic effects by causing DNA damage, which depends mainly on the wavelength. The etiologically relevant UV wavelengths, which cause photoaging and photocarcinogenesis are UVA (315–400 nm), UVB (280–315 nm), and UVC (200–280 nm). The most energetic part of natural solar UV radiation is UVB [4], which is primarily responsible for induction of DNA damage [5]. DNA, which maximally absorbs radiation at wavelengths of 245–290 nm, is one of the main cellular targets of UV radiations [6,7]. Owing to its wavelength, which is within the absorption peak of DNA [8], UVC is the most proficient in induction of DNA damage. Although solar UVC radiation cannot reach the Earth's surface, these harmful radiations are generated from artificial light sources such as arc welding lights, bactericidal lamps, and photocuring devices [9]. In contrast to UVB and UVC radiation, the mutagenic and carcinogenic effects of UVA result from generation of reaction-oxygen species (ROS) through the process of photosensitization [10,11]. Researchers have mainly focused on UVA and UVB radiation, and UVC remains poorly studied. Bio-analysis of toxicity of different wavelengths is expected to clarify cancer mechanisms, providing information that will be helpful in the fields of photobiology, dermatology, ophthalmology, and cosmetology.

DNA damage in cells exposed to UV radiation plays significant roles in cell-cycle arrest, activation of DNA repair, cell death, mutation and neoplastic transformation, and

immunosuppression. These cellular processes are related to photoaging and photocarcinogenesis [12,13]. UV-mediated DNA damage is mainly attributed to the formation of cyclobutane pyrimidine dimers (CPDs) between adjacent thymine (T) or cytosine (C) residues, or to a lesser extent, formation of pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) via direct absorption of UV photons by the DNA bases [14,15]. Accumulation of these lesions can lead to mutations. Approximately 70–80% of UV-induced DNA damage is caused by production of CPDs, the levels of which vary at different wavelengths [16]. Biologically, compared to UVA, UVB and UVC are more potent inducers of photocarcinogenesis, which leads to generation of an aggrandized amount of CPDs, because nucleobases only weakly absorb (104-fold) radiation above 320 nm [8, 17-19]. CPD production levels at different wavelengths should be investigated to clarify the relationships between cell viability, production of CPDs, and nuclear excision repair.

Generally, cells have the ability to preserve genomic integrity. In mammalian cells, nucleotide excision repair (NER) is the most important mechanism for repairing UV-generated CPDs and (6-4) photoproducts and is used to remove bulky DNA damage from the genome [20-22]. However, if DNA damage is too severe and cannot be repaired, the apoptotic pathway is triggered to prevent propagation of the damage [23,24]. UV-induced apoptosis depends upon the mitochondrial pathway, where p53 plays a leading role by either up-regulating apoptosis-promoting genes such as Bax and Fas/Apo-1 or down-regulating expression of BCL-2-suppressing genes [25-28].

To date, how different wavelengths of UV radiation affect the mechanism of toxicity caused by UV radiation has not been investigated. To assess the toxicological effects of the different UV wavelengths on organisms, we compared the DNA damage and CPD formation caused by UV radiations of wavelength 250 nm, 270 nm, 290 nm, and 310 nm, administered at different doses, in a PC12 cell system. To confirm whether each wavelength influenced UV cytotoxicity and UV-induced DNA damage, we performed the trypan blue exclusion test and enzyme-linked immunosorbent assay (ELISA) on the PC12 cells.

2. Materials and Method

2.1. Materials

PC12 cells, a rat pheochromocytoma cell line, were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's Modified Eagle Medium (DMEM), streptavidin-conjugated peroxidase, ribonuclease A (RNase), ethidium bromide, and o-phenylenediamine dihydrochloride (OPD) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Rockville, MD, USA). Monoclonal antibody against CPDs was purchased from Cosmo-Bio Co., Ltd. (Japan). Biotinylated goat anti-mouse immunoglobulin was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). DNA markers were obtained from Promega (Madison, WI, USA), and proteinase K was obtained from Roche Diagnostics (Mannheim, Germany). Trypan blue stain solution (0.5%) was purchased from Nacalai Tesque (Kyoto, Japan). All the other

chemicals were of analytical reagent grade.

2.2 . Cell Culture

PC12 cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator with 5% CO₂ at 37°C. The cells were pre-incubated overnight in 35-mm tissue culture dishes, after which the medium was replaced with DMEM containing 10% FBS.

2.3. UV-irradiation

PC12 culture medium was changed by adding 2 mL phosphate-buffered saline, after washing twice with 2 mL of the same solution, because medium itself absorbed UV irradiation. Following addition of fresh medium, a xenon-lamp light source (MAX-301, Asahi Spectra, Tokyo, Japan), equipped with bandpass filters that isolate specific wavelength regions, was used as the UV source. Cells in the dishes were exposed to a narrow band with a width of approximately 10 nm at each different wavelength (250, 270, 290, and 310 nm) of UV radiation.

Before exposure to each wavelength, irradiance at the position of the target cells was measured using a radiometer (IL 1400 A, International Light Technologies, Peabody, USA) connected to a silicon-photodiode detector (SEL033, International Light Technologies), and exposure duration was determined by measuring the irradiate dose. Irradiation doses were then established using the following formula: irradiation dose (mJ/cm²) = exposure time (sec) × irradiance (mW/cm²).

To determine the dose–effect relationship, 6 doses (2, 5, 10, 20, 100, and 200 mJ/cm²) of each of the tested wavelengths were applied to the PC12 cells, and then the cell viability test and ELISA were performed on the irradiated cells. Cells that were not UV irradiated and were maintained at room temperature during irradiation were used as control cells. After UV irradiation, cells were cultured in medium containing serum for 24 h.

For determination of relative toxicity of the 4 different wavelengths, we quantified cell viability and the CPDs formed following UV irradiation with the 4 wavelengths, each applied at the same dose (100 mJ/cm²) and culturing for different periods—10 min, 30 min, 1 h, 2 h, 4 h, and 24 h—in serum-containing medium.

The median lethal dose (LD50)—the dose required for killing 50% of the test cells—for 250, 270, 290, and 310 nm radiations was estimated. Next, the DNA-repair (recovery rate) ability of the cells was evaluated on the basis of cell viability and CPD formation. For this, PC12 cells were irradiated with the 4 wavelengths at their respective LD50 and cultured for 10 min, 30 min, 1 h, 2 h, 4 h, and 24 h after each irradiance. The recovery rate was then calculated by comparing the amount of CPDs remaining at the end of each culture time to that observed at the initial time (10 min). Recovery rate was calculated by using the following equation

$$\frac{(\text{Amount of CPDs at 10 min after UV irradiation} - \text{CPDs at final cultured time after UV irradiation})}{\text{Amount of CPDs at 10 min after the UV irradiation}} \times 100$$

Amount of CPDs at 10 min after the UV irradiation

2.4. Cell Viability

The trypan blue exclusion assay was used to evaluate cell viability. After UV irradiation, the cells were incubated in DMEM containing serum for different durations from 10 min up to 24 h. After incubation, the cells were stained with 0.25% trypan blue solution in phosphate-buffered saline. Total cells and trypan blue-stained cells were counted using a hemocytometer (TC10™ automated cell counter, Bio-Rad). Cell viability was expressed as a percentage of live cells against total cell number in each experiment. Each experiment was repeated 3–6 times. Cell viability was calculated using the following formula;

$$\text{Live cells (\%)} = 100 \times \text{Viable cells} / \text{Total cells}$$

2.5. Genomic DNA isolation from PC12 cells

Following UV irradiation of PC12 cells, genomic DNA was isolated using the procedure described by Yamanoshita et al. [29] with some modifications. Cells were washed with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and then harvested using a scraper. The harvested cells were centrifuged at 1,500 rpm for 5 min, and the supernatants were removed. Subsequently, cells were incubated with 10 mM Tris-HCl buffer (5 mM Tris), 0.1 M EDTA, 0.5% sodium dodecyl sulfate, and 20 µg/mL of pancreatic RNase for 15 min at 37°C. Proteinase K (100 µg/mL) was added to the suspension, and the enzyme reaction was carried out for 3 h at 50°C. The obtained DNA was resuspended in 89 mM Tris-HCl buffer (pH 8.1) containing 1 mM EDTA (1× TBE).

2.6. ELISA for quantification of CPD formation

To the extracted DNA samples, 1× TBE was added to adjust the DNA concentration to 1 µg/µL. Next, DNA was denatured by heating on a hot plate at 100°C for 10 min and chilling immediately on ice for 15 min. After denaturation, 46 µL/well of 1× TBE and 4 µL of denatured DNA were added to each well in a titer plate (2 wells for each sample), and the DNA solution was dried completely overnight at 37°C.

CPDs were quantified via ELISA, which was used for detecting the direct binding of monoclonal antibodies to antigens. After overnight incubation, a titer plate was washed 3 times with 40 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. To block unbound areas in each well of the titer plate, we added 100 µL of 2% blocking reagent in the same buffer to the wells, and the solution was incubated for 30 min at 37°C. First, antibodies against CPDs (diluted to 1:1,000 in the same buffer) were immobilized by incubation for 1 h at 37°C. Next, the plate was washed 3 times with the same buffer. The second antibody, a biotinylated anti-mouse immunoglobulin (diluted to 1:500 in the same buffer) was added, and the plate was incubated for 1 h at 37°C. Then, the plate was washed twice with the same buffer. After addition of streptavidin-conjugated peroxidase (diluted to 1:400 in the same buffer), the plate was incubated for 30 min at 37°C. After washing the well 5 times with the same buffer, 0.1% OPD in 50 mM phosphate-citrate buffer (pH 5.0) containing 0.03% sodium perborate was added into the wells. To stop the enzyme reaction, 6N HCl was added 5–10 min later. The absorbance at 495 nm was

measured using a Microplate Reader (model 450; Bio-Rad).

2.7. Statistical analysis

Each value is expressed as the mean $\pm$ SEM. Statistical analysis was performed using the unpaired *t*-test, followed by the *F*-test.

3. Results

3.1. Cell viability evaluation

To examine whether UV irradiation caused cell toxicity, we evaluated cell viability by staining irradiated PC12 cells with trypan blue 24 h after exposure to 4 different wavelengths of UV radiations at irradiation doses of 2–200 mJ/cm² (Fig. 1). Compared to the cell viability of the control, viability of irradiated cells was significantly lower at all irradiation doses (except 310 nm irradiation). The viability of PC12 cells was significantly reduced following exposure to high irradiation doses (10, 100, and 200 mJ/cm²) of the 290-nm radiation; however, cell viability was not significantly affected following exposure to the 310-nm radiation, irrespective of the dosage (Fig. 1).

In addition, to determine the relative toxicity of each wavelength tested, we quantified the viabilities of cells irradiated with the 4 different wavelengths at 100 mJ/cm² and incubated for 10 min up to 24 h. Interestingly, irrespective of the wavelength used, the viabilities did not change significantly among the cells cultured for 10 min up to 4 h (data not shown). For cells cultured for

24 h, the viability of cells irradiated with 250-nm radiation was lower than that of cells exposed to 270-, 290-, and 310-nm radiations. Out of the cells irradiated with the 4 tested wavelengths, cells exposed to 310-nm radiation showed the highest survival rate.

3.2. Determination of LD50 of the 250-, 270-, 290-, and 310-nm radiations

For determining the LD50 of the 4 tested wavelengths, we exposed PC12 cells to 25–800 mJ/cm² of 250-, 270-, and 290-nm radiations and to 125–8000 mJ/cm² of the 310-nm radiation. After irradiation, the cell viabilities were quantified. The LD50 values were calculated from the results of the cell viability assay and were 120, 150, 300, and 6000 mJ/cm² for 250, 270, 290, and 310 nm, respectively (Fig. 2).

3.3. Quantification of CPD formation by ELISA

CPD formation in irradiated PC12 cells was analyzed using ELISA. As shown in Fig. 3A, CPD numbers were higher in the irradiated cells than in the control cell at 24 h cultured time after UV irradiation at 2–200 mJ/cm² irradiation doses, irrespective of the wavelength used. Among the 4 wavelengths tested, incidence of CPD formation was highest for the 250-nm radiation, and this increase in the amounts of CPDs formed was found to be statistically significant. Similar to the results for the 270- and 290-nm radiations, the 250-nm radiation induced high incidence of CPD formation, whereas the 310-nm radiation did not induce significant CPD formation at doses up to 200 mJ/cm². These results indicate that CPD formation increased in an irradiance-dependent

manner. For determining relative CPD-inducing ability of the different wavelengths of UV radiation at different cultured time, we exposed PC12 cells to the 4 tested wavelengths at 100 mJ/cm² and incubated these irradiated cells for 10 min to 24 h. By comparing the amount of CPDs formed in cells irradiated with the different wavelength, the magnitude of CPD formation was found to be the highest at 250 nm and the lowest at 310 nm at 24 h after UV irradiation, and CPD incidence at each tested wavelength showed the following order: 250 nm > 290 nm > 270 nm > 310 nm (Fig. 3B).

3.4. Time course for LD50 of 250-, 270-, 290-, and 310-nm irradiation: cell viability and CPD formation

Cell viability gradually decreased for each wavelength with increasing culture time after UV irradiation at LD50 (Fig. 4). Among the 4 wavelengths, 310 nm at its LD50 significantly reduced the number of live cells at each culture time. The decrease in cell viability caused by the different wavelengths at their corresponding LD50 showed the following order: 310 nm > 290 nm > 250 nm > 270 nm.

As shown in Fig. 5, CPD formation was significantly less with 270 nm and 290 nm over a 24-h culture time compared with that observed at the initial time (10 min after the UV irradiation). The recovery rate was approximately one-fifth-fold for 250-nm, two-third-fold for 270-nm, and half-fold for 290-nm radiations (Fig. 5). Cells irradiated with 310-nm UV radiation at its LD50 did not show any DNA repair ability.

4. Discussion

The PC12 cell line is derived from rat pheochromocytoma tumor cells and is used to investigate cell toxicity of trace amounts of chemical substances. Many studies support this [30-32]. The PC12 cell line system is widely used in research because of its ability for rapid division, differentiation ability, and the presence of Na, K, and Ca channels and other membrane receptors [33,34]. PC12 cells have stronger UV resistance compared to other cell lines. Our results showed that PC12 cells could tolerate high LD50 (ranging from 120 to 6000 mJ/cm²) of 250–310 nm wavelengths of UV radiations (Fig. 2). In contrast, for normal human epidermal keratinocytes (NHEKs) and porcine lens epithelial cells (LECs), the minimum LD50 values for 269-nm and 261.7-nm radiations were 1.64 and 1.77 mJ/cm², respectively, and for human LECs the minimum LD50 for the 267.5-nm radiation was 8.86 mJ/cm² [35-37]. The LD50 of radiation for the PC12 cells was 50 times that for NHEKs and LECs.

The present study evaluated cell survival rate by using the PC12 cell system to clarify the relationship between cell viability and different wavelengths of UV radiation with respect to the irradiation dose and culture time after UV irradiation. Cell viability was significantly decreased at 250-, 270- and 290-nm radiations (Fig. 1). A previous report proposed that shorter wavelengths induce DNA damage directly through formation of CPDs or 6-4 photoproducts and that the cell killing effect of UV light is associated with the amount of pyrimidine dimers formed [11,38]. As previously reported, the cell killing action spectrum is close to the DNA absorption spectrum [39],

and we hypothesized that DNA more prominently absorbed shorter wavelengths that directly induced DNA damage, which was responsible for the lethal effect of UVC irradiation on cells. We found that cell death depended on incubation time after UV irradiation, and cell death after irradiation was not significantly reduced until 24 h at all wavelengths. Cell death occurs from irreparable DNA damage that remains 24–48 h after UV exposure [19,40].

In this study, we confirmed that UV radiations induced DNA damage via CPD formation, an effect that was wavelength dependent. No wavelength-based chronological sequence was found as shown in Figs. 3A and B. The highest amount of CPDs was generated following exposure to the 250-nm radiation. CPD formation was more with the 290-nm than with the 270-nm radiation. DNA and proteins absorb is maximally at 260 nm and 280 nm, respectively [8,41]. UV light is absorbed by tryptophan and tyrosine in proteins at 310 nm and 290 nm, respectively [42,43]. The most effective wavelength, within the UVB range, for inducing DNA photoproducts in the basal layer of the epidermis is 300 nm [41]. The relationship between CPD formation and the irradiation dose was consistent with our expectation: the magnitude of CPD formation increased in a dose-dependent manner. This finding was supported by the results of Kubota et al. [19].

To determine DNA-repair ability of the PC12 cells, the LD50 values of the 4 tested wavelengths were determined from the results of the cell viability assays (Fig. 2). In this study, the LD50 values for 310 nm were 50, 40, and 20 times the LD50 values for 250, 270, and 290 nm, respectively. It was previously reported that compared to UVC, UVA absorption by DNA is 105-fold times weak [44], and UVA is therefore proportionately far less effective in reducing cell

viability. Our findings regarding the DNA-repair ability demonstrated that compared to the CPDs detected at 10 min after UV irradiation, the CPD numbers dramatically decreased during other culture times for all wavelengths (except 310 nm) administered at their respective LD50 (Fig. 5). NER is the most important mechanism for elimination of structurally unrelated DNA lesions such as CPD from the genome within 24–48 h of UV irradiation [22,19]. In this study, cells irradiated with 290- and 270-nm radiations at their corresponding LD50 showed superior repair ability, whereas no repair was observed in cells irradiated with 310-nm radiation (Fig. 6). CPD Repair rates depend on genes and vary along the same gene sequence between adjacent base positions [45,46]. The exact reason behind the different repair rates following exposure to different wavelengths is still unclear. DNA-repair ability, assessed via a cytotoxicity assay, could not be explained on the basis of the cell viability results (Fig. 4).

In conclusion, the cytotoxicity of and DNA damage induction by 4 different wavelengths of UV radiation, administered at different irradiation doses, were found to be related. Among the 4 tested wavelengths, 250 nm and 310 nm showed the highest and lowest abilities for reduction of cell survival rate and induction of CPD formation, respectively. These results suggested that cells respond to different wavelengths of UV radiation via different mechanisms. Repair ability was higher at 290 nm and 270 nm than at 250 nm, each administered at their respective LD50. Although 310 nm was less lethal to cells and induced less DNA damage than the other tested wavelength, long-term exposure (at the LD50) might cause severe and irreparable damage. Further investigation is needed to clarify the precise mechanism of CPD formation by each

wavelength.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Caption of Figures

Fig. 1 Fig. 1 Viability of PC12 cells exposed to 4 different wavelengths of UV radiations at different doses (2, 5, 10, 20, 100, and 200 mJ/cm²) 24 h after irradiation. Error bars indicate SEM ($n = 3$). Asterisks denote values that are significantly different (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) from the control value.

Fig. 2 LD50 (mJ/cm²) of 250, 270, 290, and 310 nm UV radiations, which were determined from the number of viable PC12 cells present 24 h after UV irradiation with different doses. The LD50 values were 120, 150, 300, and 6000 mJ/cm² for 250, 270, 290, and 310 nm, respectively. Error bars indicate SEM ($n = 3-4$).

Fig. 3 CPD formation (A) 24 h after UV irradiation with 250, 270, 290, and 310 nm UV radiations at doses of 2–200 mJ/cm² doses ($n = 3$). Asterisks denote values significantly different (* $p < 0.05$, *** $p < 0.001$) from the control value. (B) Comparison of CPD formation in cells irradiated with 4 different wavelengths of UV radiation (100 mJ/cm²) 24 h after irradiation ($n = 4$). Asterisks denote significantly different values (* $p < 0.05$, *** $p < 0.001$). Error bars indicate SEM.

Fig. 4 Viability of PC12 cells after UV irradiation with 4 different wavelengths at their LD50 doses. Error bars indicate SEM ($n = 3$). PC12 cells were cultured for different durations after UV irradiation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate significant differences from the control value.

Fig. 5 Comparison of CPD formation in PC12 cells irradiated with 4 different wavelengths of UV radiation applied at their respective LD50 (120 mJ/cm² for 250 nm, 150 mJ/cm² for 270 nm, 300 mJ/cm² for 290 nm, and 6000 mJ/cm² for 310 nm). Error bars indicate SEM ($n = 3$). Asterisks denote values significantly different (* $p < 0.05$) from the initial time (10 min).

Fig. 6 CPDs (%) remaining in PC12 cells irradiated with 4 different wavelengths of UV radiations at their respective LD50 and cultured for different periods. Here, a 10-min culture period is considered the initial time (0% recovery), and recovery rate was evaluated by calculating the percentage of CPDs remaining at the end of each culture period, which was then compared to the CPD percentage observed at initial time.

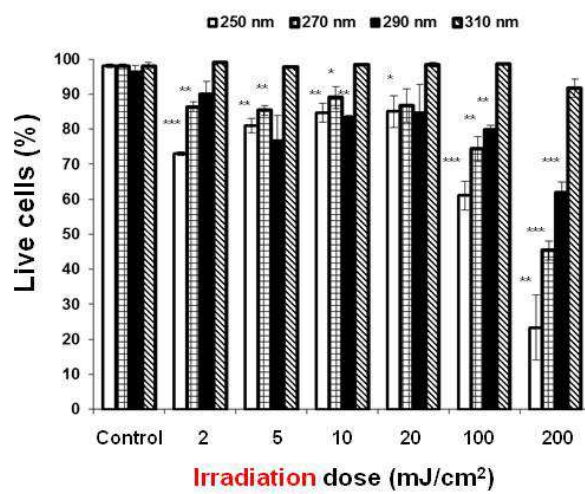


Fig.1

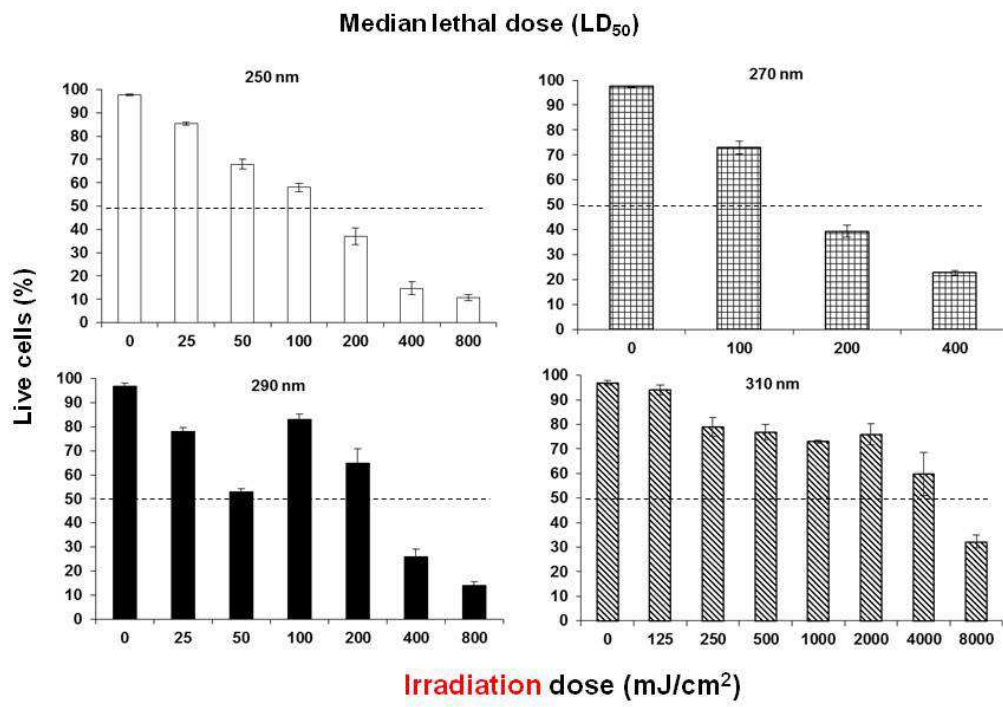


Fig. 2

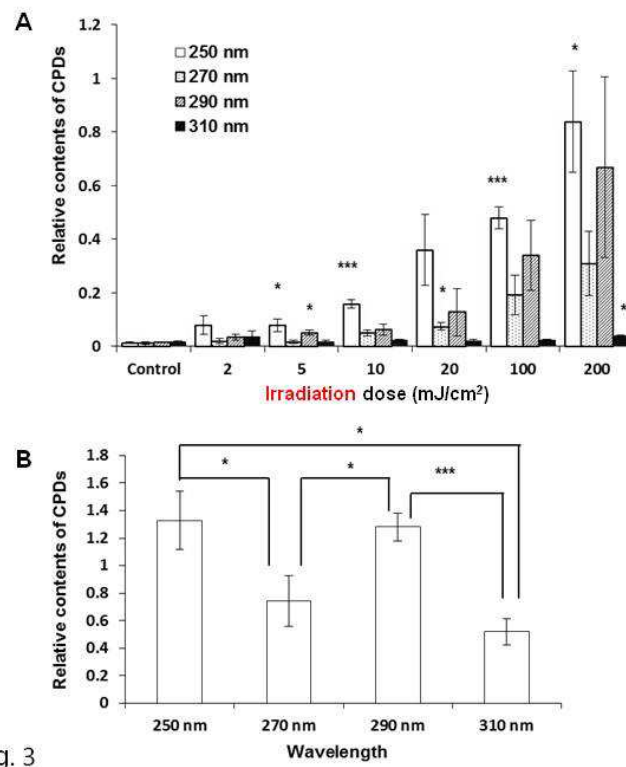


Fig. 3

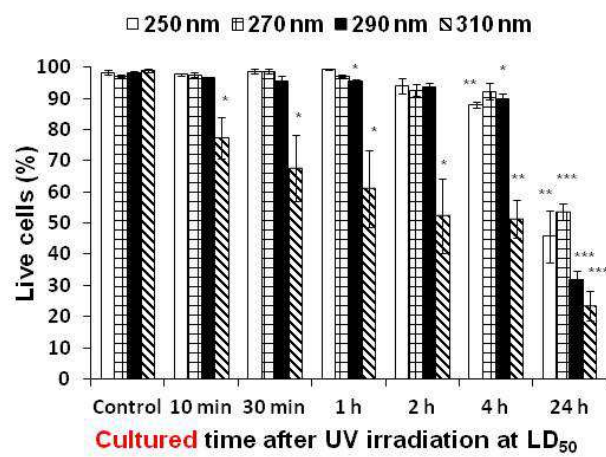


Fig. 4

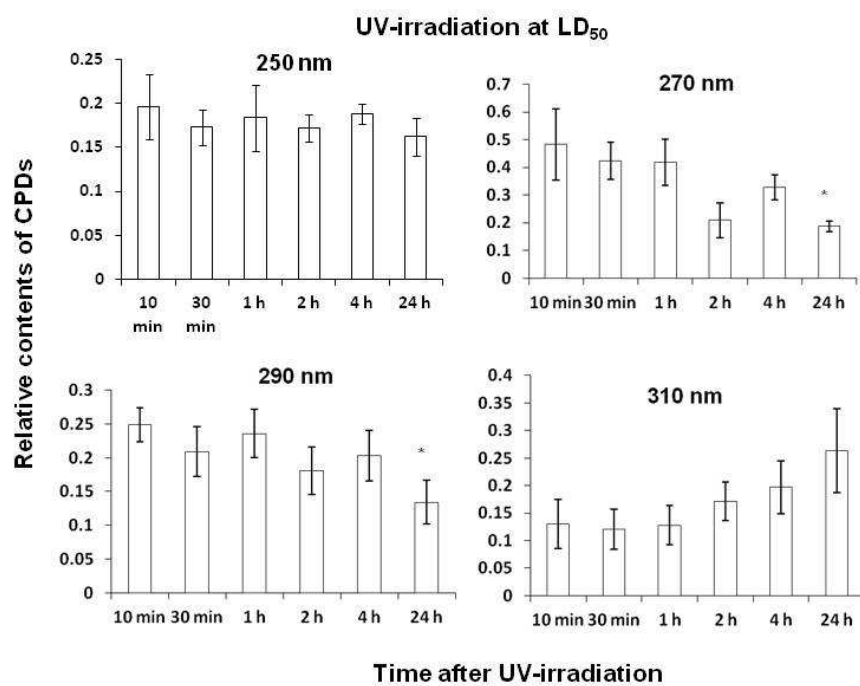


Fig. 5

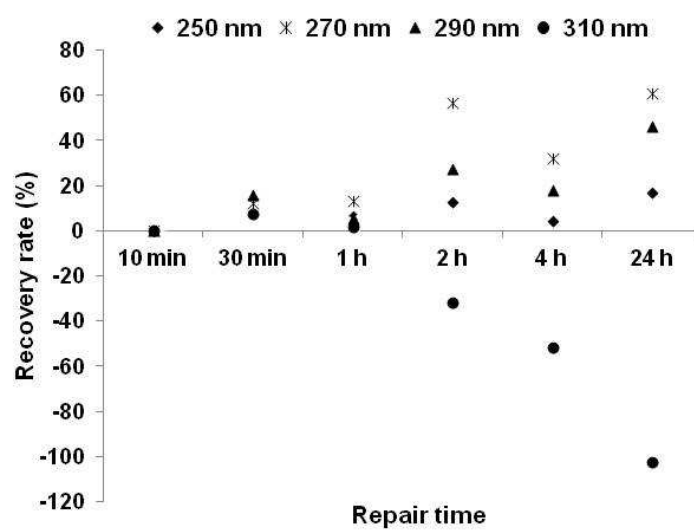


Fig. 6