



Title	Assessment of ultraviolet toxicity depending on DNA-damage using cyclobutane pyrimidine dimer formation in ultraviolet irradiated cells
Author(s)	Masuma, Runa
Citation	北海道大学. 博士(環境科学) 甲第11338号
Issue Date	2014-03-25
DOI	10.14943/doctoral.k11338
Doc URL	<a href="http://hdl.handle.net/2115/55365">http://hdl.handle.net/2115/55365</a>
Type	theses (doctoral)
File Information	Runa_Masuma.pdf



[Instructions for use](#)

**Assessment of Ultraviolet Toxicity depending  
on DNA-damage using Cyclobutane Pyrimidine  
Dimer Formation in Ultraviolet Irradiated Cells**

**Runa Masuma**

**Course in Environmental Adaptation Science  
Division of Environmental Science Development  
Graduate School of Environmental Science  
Hokkaido University**

# Contents

	Page No.
Contents	i
List of figures	v
Summary	viii
<b>Chapter I General Introduction</b>	<b>1</b>
1.1 Ultraviolet (UV) radiation	1
1.2 UV Toxicity	2
1.2.1 Direct DNA Damage	4
1.2.2 Indirect DNA Damage	5
1.2.3 UV radiation mediated apoptosis	6
1.3 Measurement of UV Toxicity	7
1.4 Objectives	9
Reference	11
<b>Chapter II Confirmation of analysis of UV toxicity</b>	<b>15</b>
2.1 PC12 cell	15
2.2 Analysis of UV toxicity	16
2.2.1 Cell viability	18
2.2.2 ELISA for quantification of CPD formation	19
Reference	21
<b>Chapter III Effects of UV wavelength on cell damages caused by UV irradiation in PC12 cells</b>	<b>24</b>
3.1 Introduction	24
3.2 Materials and methods	27

3.2.1 Materials	27
3.2.2 Cell culture	27
3.2.3 UV-irradiation	27
3.2.4 Cell viability	29
3.2.5 Genomic DNA isolation from PC12 cells	30
3.2.6 ELISA for quantification of CPD formation	30
3.2.7 Statistical analysis	31
3.3 Results	32
3.3.1 Cell viability evaluation	32
3.3.2 Determination of LD50 of the 250-, 270-, 290-, and 310-nm radiations	33
3.3.3 Quantification of CPD formation by ELISA	34
3.3.4 Time course for LD50 of 250-, 270-, 290-, and 310-nm irradiation: cell viability and CPD formation	36
3.4 Discussion	37
Reference	42
<b>Chapter IV Effect of <i>Tinospora cordifolia</i> on the reduction of ultraviolet radiation-induced cytotoxicity and DNA damage in PC12 Cells</b>	48
4.1 Introduction	48
4.2 Materials and Methods	51
4.2.1 Materials	51
4.2.2 Collection and preparation of <i>T. cordifolia</i> methanolic extract	52

4.2.3 Cell culture	52
4.2.4 Cell viability	53
4.2.5 Genomic DNA isolation from PC12 cells	53
4.2.6 Electrophoresis of the genomic DNA isolated from PC12 cells	54
4.2.7 Quantification of DNA fragmentation in the PC12 cells by using the TUNEL method	54
4.2.8 UV irradiation	55
4.2.9 Quantification of the formation of CPD by using ELISA	56
4.2.10 Statistical analysis	57
4.3 Results	57
4.3.1 Cell viability count	57
4.3.2 Agarose gel electrophoresis for the detection of DNA fragmentation	58
4.3.3 Quantitative determination of DNA fragmentation by the TUNEL method	59
4.3.4 Viability of UV-irradiated cells	60
4.3.5 Quantification of CPD formation by ELISA	61
4.4 Discussion	63
Reference	66
<b>Chapter V Discussion about cell sensitivity and mechanism of cell toxicity of UV radiation</b>	72
5.1 Introduction	72

5.2 Materials and Methods	74
5.2.1 Materials	74
5.2.2 Cell culture	75
5.2.3 UV-irradiation	76
5.2.4 Cell viability	77
5.2.5 Genomic DNA isolation and quantification of CPD formation by ELISA	78
5.2.6 Statistical analysis	79
5.3 Results	79
5.3.1 Determination of cell viability	79
5.3.2 Quantification of CPD formation	82
5.4 Discussion	86
Reference	87
<b>Chapter VI General Discussion and Conclusion</b>	91
<b>Acknowledgements</b>	94

## List of Figures

Figure no.	Figure Title	Page No.
Fig. 1.1	Structures of cyclobutane pyrimidine dimer (CPD) and Pyrimidine –pyrimidone (6-4) photopruducts	5
Fig. 2.1	Schematic diagram of the research design of chapter III	17
Fig. 2.2	Schematic diagram for the research design of chapter IV	18
Fig. 2.3	Proposed schematic diagram for the research design of chapter V	21
Fig. 3.1	Viability of PC12 cells exposed to 4 different wavelengths of UV radiations at different doses (2, 5, 10, 20, 100, and 200 mJ/cm <sup>2</sup> ) 24 h after irradiation	33
Fig. 3.2	LD <sub>50</sub> (mJ/cm <sup>2</sup> ) of 250, 270, 290, and 310 nm UV irradiated PC12 cells	34
Fig. 3.3 A, B	CPD formation in PC12 cells (A) 24 h cultured time after 250, 270, 290, and 310 nm UV radiations at doses of 2–200 mJ/cm <sup>2</sup> (B) Comparison of CPD formation at 100 mJ/cm <sup>2</sup> dose with 4 different wavelengths 24 h after irradiation	35
Fig. 3.4	Viability of PC12 cells at their LD <sub>50</sub> doses of different cultured time after UV irradiation with 4 different wavelengths	37
Fig. 3.5	Comparison of CPD formation in PC12 cells irradiated with 4 different wavelengths of UV radiation applied at their respective LD <sub>50</sub>	38

<b>Figure no.</b>	<b>Figure Title</b>	<b>Page No.</b>
Fig. 3.6	CPDs (%) remaining in PC12 cells irradiated with 4 different wavelengths of UV radiations at their respective LD <sub>50</sub> and cultured for different periods	40
Fig. 4.1 A, B	Viability of PC12 cells treated for 48 h with 0–100 µg/ml <i>T. cordifolia</i> methanol extract (TCME) cultured in (A) serum-rich (B) serum-deprived medium.	58
Fig. 4.2 A, B	Agarose gel electrophoresis of DNA extracted from PC12 cells treated with 0–100 µg/ml TCME and cultured for 2 days in (A) serum-rich (B) serum-deprived medium.	59
Fig. 4.3	Fragmented DNA from PC12 cells after treatment with 0–100 µg/ml TCME measured by TUNEL assay, incubated for 48 h in a serum-rich medium containing 5 µg/ml 6-OHDA	60
Fig. 4.4	Effect of TCME (10 ng, 100 ng, and 1 µg/ml) on viability of PC12 cells after UV irradiation with 250, 270, and 290 nm wavelengths, corresponding to doses of 120, 150, and 300 mJ/cm <sup>2</sup>	61
Fig. 4.5 A, B, C,	Effect of TCME on the formation of CPDs 24 h after UV irradiation at (A) 250 nm, (B) 270 nm, and (C) 290 nm corresponding to doses of 120, 150, and 300 mJ/cm <sup>2</sup>	62



<b>Figure no.</b>	<b>Figure Title</b>	<b>Page No.</b>
Fig. 5.1 A, B	Comparison between the cell viability of UV irradiated PC12 and CHO cells at <b>A)</b> 250 nm <b>B)</b> 290 nm wavelengths at 2-200 mJ/cm <sup>2</sup> exposure doses	80
Fig. 5.2 A, B	Comparison between the cell viability of UV irradiated PC12 and NHEK cells at <b>A)</b> 250 nm <b>B)</b> 270 nm wavelengths at 2, 5, 10, 20 and 100 mJ/cm <sup>2</sup> exposure doses	81
Fig. 5.3 A, B	Comparison between the cell viability of UV irradiated PC12 and HUVEC cells at <b>A)</b> 250 nm <b>B)</b> 270 nm wavelengths at 2, 5, 10, 20 and 100 mJ/cm <sup>2</sup> exposure doses	82
Fig. 5.4 A, B	Comparison between the CPDs formation of UV irradiated PC12 and CHO cells at <b>A)</b> 250 nm <b>B)</b> 290 nm wavelengths at 2, 5, 10, 20, 100 and 200 mJ/cm <sup>2</sup> exposure doses.	83
Fig. 5.5 A, B	Comparison between the CPDs formation of UV irradiated PC12 and NHEK cells at <b>A)</b> 250 nm <b>B)</b> 270 nm wavelengths at 2, 5, 10, 20 and 100 mJ/cm <sup>2</sup> exposure doses.	84
Fig. 5.6 A, B	Comparison between the CPDs formation of UV irradiated PC12 and HUVEC cells at <b>A)</b> 250 nm <b>B)</b> 270 nm wavelengths at 2, 5, 10, 20 and 100 mJ/cm <sup>2</sup> exposure doses	85

## Summary

Ultraviolet (UV) radiation is a markedly prominent environmental toxic agent mainly emits from sunlight. Depending on etiological effects, UV radiation can be subdivided into UVA (315-400 nm), UVB (280-315 nm) and UVC (200-280 nm). UV radiation leads numerous complications like sunburn, erythema, epidermis hyperplasia, photo-aging, immunosuppression and photodermatoses. Long term and continuous exposure to solar UV radiation causes gradual deterioration of cutaneous structure and function, which further leads to skin cancer. The harmful toxic effects mediated through UV radiation result from cumulative DNA damage. UV mediated DNA damage is mainly attributed directly by the formation of cyclobutane pyrimidine dimer (CPDs) between adjacent thymine (T) or cytosine (C) residues or to a lesser extent, formation of pyrimidine (6-4) pyrimidone photoproducts. Owing to its wavelengths, UVB and UVC are most proficient in induction of direct DNA damage through CPD formation. CPDs formation is responsible for 70-80% DNA damage and found to be crucial for induction of relevant toxic effects of UV radiation. In absence of efficient DNA repair, CPD formation in the genome may leads to deleterious mutations which further actuate cytotoxicity and mutagenesis. Indefinitely, measurement of CPD formation considered as most sensitive assay for the determination of UV induced DNA damage. To date, how different wavelengths of UV radiation affect the mechanism of toxicity caused by UV radiation has not been investigated. The whole study is carried out for three purposes (1) to elucidate the different UV wavelengths (250 nm, 270 nm, 290 nm and 310 nm) mediated cytotoxicity, DNA

damage and DNA repair on organisms by using PC12 cell system (2) to assess the protective potential of medicinal plant (*Tinospora cordifolia*) on UV-induced cytotoxicity and DNA damage in PC12 cell and (3) to find out the different cell sensitivity to UV radiation and molecular mechanism behind the UV sensitivity of CHO, NHEK and HUVEC cells compared to PC12 cell. To quantify cytotoxicity and DNA damage via CPDs formation, trypan blue exclusion assay and Enzyme linked immunosorbent assay (ELISA) were applied.

In first study, we evaluated cell viability for two purposes; one is to determine the cell killing ability of 4-different UV wavelengths at different exposure doses and another is to evaluate the lethal doses ( $LD_{50}$ ) of each tested UV wavelengths. On the other hand, quantification of CPD formation is implied to measure the DNA damage as well as DNA repair ability of 4 types of wavelengths of UV radiation. Cell survival rate was markedly decreased 24 h 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 ( $LD_{50}$ ) of 120 mJ/cm<sup>2</sup>. The  $LD_{50}$  gradually increased with increase in wavelength. Among the 4 wavelengths tested, the highest  $LD_{50}$  (6000 mJ/cm<sup>2</sup>) 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  $LD_{50}$  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.

The safety of *Tinospora cordifolia* and its potential to protect against ultraviolet radiation-induced cytotoxicity and DNA damage in PC12 cells were investigated in second study. To evaluate the safety of *T. cordifolia*, cell viability, agarose gel electrophoresis, and TUNEL assay were carried out using PC12 cells treated with 0 to 100 µg/ml of methanol extract of *T. cordifolia*. To confirm the protective role against UV-induced damage, PC12 cells alone or in presence of 10 ng, 100 ng, or 1 µg/ml of *T. cordifolia* extract were exposed to 250, 270, and 290 nm of UV radiation, which corresponded to doses of 120, 150 and 300 mJ/cm<sup>2</sup>, respectively. *T. cordifolia* extracts significantly increased cell viability at 1 ng, 10 ng, and 1 µg/ml concentrations in serum-deprived medium compared to control. *T. cordifolia* extracts did not induce apoptosis, and they inhibited apoptosis induced by serum deprivation and 6-hydroxy dopamine. Treatment with *T. cordifolia* extracts significantly increased the cell survival rate irradiated at 290 nm. In addition, *T. cordifolia* extracts significantly reduced cyclobutane pyrimidine dimer formation induced by UV irradiation at all wavelengths. In conclusion, *T. cordifolia* is not toxic and safe for cells. Our findings can support its application as phototherapy in the medical sector.

In third study, the 4 types of cells (PC12, CHO, NHEK and HUVEC) were used to assess the cell sensitivity to UV radiation. The cells were exposed to 250, 270, and 290 nm of UV radiation at 2-200 mJ/cm<sup>2</sup> exposure doses. Results of cell viability

and CPDs formation were compared with PC12 cells results. The cell survival rate of CHO, NHEK and HUVEC were significantly lowered at each tested wavelengths compared to PC12 cell. CPD formation results also revealed the similar trends where NHEK showed the highest production of CPD within PC12, CHO, NHEK and HUVEC cells. On the contrary, CPD formation was higher or similar in case of CHO and similar in HUVEC compared to PC12 cell. The cell sensitivity sequences according to cell viability and CPD formation were  $NHEK \gg HUVEC \geq CHO \geq PC12$ . Different types of cell have distinctive cell sensitivity to UV radiation. Determination of prime causes of this discrepancy is great concern. To resolve this problem, molecular mechanism of UV radiated cells should be elucidated.

This study has three implications

(1) Researchers have mainly focused on UVA and UVB radiation, and UVC remains poorly studied. Bio-analysis of toxicity of different wavelengths is implicated to clarify cancer mechanisms and provide information that will be helpful in the fields of photobiology, dermatology, ophthalmology, and cosmetology.

(2) Identification and development of safe, non-toxic and effective radioprotective compounds are of enormous importance in mitigating the toxic effects of UV radiation. *Tinospora cordifolia* has aptitude to alleviate the DNA damage that is mediated through toxic, lowered wavelengths of UV radiation and open a possibility of using *Tinospora cordifolia* as photo-protective agent in medical sectors.

(3) Identification of exact reasons of different cell sensitivity will provide new era in photobiology field and further helps in the research of oncology, biology and medical field.

## **Chapter I**

### **General Introduction**

#### **1.1 Ultraviolet (UV) radiation**

UV radiation is the part of electromagnetic spectrum which is an invisible form of radiation. Sun light, the major source of UV radiation, is composed of continuous spectrum of electromagnetic radiation divided into three main regions of wavelengths: ultraviolet (200-400 nm), visible (400-700 nm) and infrared (700-5000 nm) (Matsumura and Ananthaswamy, 2004). UV radiation has shorter wavelength and higher energy than that of visible light. UV can be subdivided into three categories depending on their etiological and biological effects: UVA (315-400 nm), UVB (280-315 nm) and UVC (200-280 nm). Of these, UVC is of the highest energy and would be most damaging to biological tissues (Ridley et al., 2009). However, no solar UV radiation of wavelengths below 290 nm reaches the earth's surface because of the complete absorption through molecular oxygen and stratospheric ozone high up in the atmosphere. UVA constitutes the major remaining fraction of residual UVB (5-10%), which is not absorbed by passage through the stratospheric ozone layer (Besaratina et al., 2005; Ridley et al., 2009). Biologically, UVB is a few orders of magnitude more potent than UVA for inducing DNA damage and photocarcinogenesis (de Gruijl et al., 1993). Though UVC completely absorbed through ozone layer, artificial light such as welding, photo-curing, bactericidal and germicidal lamps, is the major detrimental sources

of UVC radiation. There is also growing concern about ongoing destruction of the ozone layer, which may eventually result in additional exposure of the UVC radiation on the earth surface (Lubin and Jensen, 1995)

## **1.2 UV Toxicity**

UV radiation is a markedly prominent environmental toxic agent. Skin and eyes are the only two organs of mammals that directly exposed to sunlight. Sunlight which is the main source of exposure to UV radiation causes erythema, photo-aging, sunburn, cataracts, ocular melanoma, immunosuppression and photo-dermatoses. Continuous exposure to UV radiation leads to numerous complication that are correlated with various pathological consequence of skin damage (Pallela et al., 2010). UV irradiation induces photo-damage of the skin by altering the composition of dermal extracellular matrix, resulting in wrinkles, laxity, coarseness, mottled pigmentation and histological changes include epidermal thickness and connective tissue alteration with photo-aging in dermal fibroblast (Kondo, 2000; Rittie and Fisher, 2002). The effects of UV radiation on the skin can be acute or chronic. Acute effects are related to sunburn, suntan, erythema, epidermal hyperplasia and inflammation whereas chronic effects associated with skin photo-aging, immunosuppression and carcinogenesis (Beissert and Granstein, 1996)

Long term and continuous exposure to solar UV radiation cause gradual deterioration of cutaneous structure and function. This apparently occurs as a



result of cumulative DNA damage resulting from acute DNA injury and effect of chronic inflammation, which further leads to the skin cancers. Three common types of skin cancer like basal cell, squamous cell carcinomas (collectively called nonmelanocytic skin cancer) and cutaneous malignant melanoma are produced by direct exposure to UV radiation (Armstrong and Krickler, 2001).

The broad solar UV radiation that passed through the earth was large deleterious to highly evolved organic molecules such as proteins and DNA. Though UV radiation does not break up molecules; however, it is very potent in starting up photo-chemical reactions through exciting the valence electrons. Conjugated bond in organic molecules absorb shorter wavelength of UV radiation (around 200 nm) but the absorption shift to longer wavelengths up to and over 300 nm in linear molecules or in ring structures (Feynman and Leighton, 1967). Proteins which contain tryptophan or tyrosine can absorb UV radiation and start up photo-chemical reactions and disturb signaling pathways. The DNA bases, all contain ring structures with an abundance of conjugated bonds, make DNA more obtrusive absorber of UV radiation in cells. Genes in cells are easily damaged upon UV irradiation, and mutations may subsequently occur through the signal transduction pathway in the cell. This genetic defect will be passed along to daughter cells, and corresponding defect in signal transduction will propagate. Thus the human skin exposed to sunlight will be under continuous threat of accumulating oncogenic damage and skin cancer. UV radiation induces a much wider range of DNA damage like protein-DNA crosslinks, oxidative base damage and single strand break, and contributes to UV carcinogenesis (de Gruijl et al.,

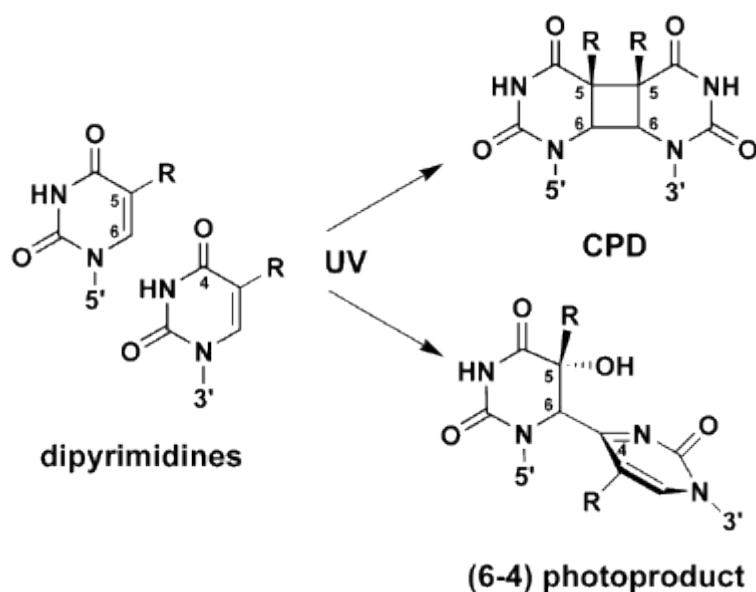
2001).

### **1.2.1 Direct DNA Damage**

UV radiation from 245 to 290 nm is absorbed maximally by DNA and proteins. Most energetic parts of natural solar UV radiation are the UVB region (Tornaletti and Pfeifer, 1996). Such a range of radiation is known to induce direct DNA damage. DNA damage can lead to oncogenic, and plays an important role in induction of human cancers.

#### **Mechanism behind UV radiation induced direct DNA damage**

UVB and UVC have the ability to produce promutagenic DNA lesions by forming dimeric photoproducts at bipyrimidine sites, i.e. cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (Douki et al., 1999; Cadet et al., 1992). CPDs are formed between C-4 and C-5 carbon atoms of any two adjacent pyrimidine, and the double bond becomes saturated to produce a 4-membered ring, where as (6-4) photoproducts formed between 5-prime 6 position and the 3-prime 4 position within TC and CC residues (Fig. 1.1). CPDs are produced three times than that of (6-4) photoproducts (Kanjilal and Ananthaswamy, 1996; Tornaletti and Pfeifer, 1996). Both lesions are known as 'hot spots' of UV-induced mutation and are potentially mutagenic. CPDs are supposed as major contributor for mutation to mammals compared to (6-4) photoproducts.



**Fig. 1.1** Structures of cyclobutane pyrimidine dimer (CPD) and Pyrimidine–pyrimidone (6-4) photoproducts.

### 1.2.2 Indirect DNA Damage

UVA radiation consists of longer wavelength and capable of penetrating into the deeper layers of the skin. Generally the poor absorption of UVA by DNA supports the notion that UVA indirectly triggers mutagenesis due to photosensitization reaction. UVA radiation, to which we are exposed, is thought to be weakly carcinogenic than either UVB and UVC, and to cause aging and wrinkling of the skin (Matsumura and Ananthaswamy, 2004). Exposure to UVA induces indirect DNA damage to basal layers of the epidermis by the formation of free radicals that exacerbate existing damage. Free radicals are highly unstable and reactive molecules, if left unchecked, they can cause oxidative injury by initiating chain reactions which further disrupt membranes, denature proteins, fragment DNA

and finally cause cell death, ageing and cancer (Halliwell and Gutteridge, 1997; Ames et al., 1995).

### **Mechanism behind UV radiation induced indirect DNA damage**

Presumably, UVA sensitizes intracellular chromophores, thereby generating reactive oxygen species (ROS), which in turns causes promutagenic DNA lesions. Such DNA lesions appear to converge on commonly inducing 8-oxo-7,8-dihydro-2' deoxyguanosine (8-oxo-dG), which is anticipated to cause signature mutations i.e. G and T transversions in fibroblast cells (Shibutani et al., 1991). This ROS-mediated DNA damage is more substantial with longer wavelength UVA than with UVB and UVC (Kielbassa et al., 1997).

### **1.2.3 UV radiation mediated apoptosis**

Apoptosis or programmed cell death is a distinct mode of cell destruction and represents a major regulatory mechanism in eliminating abundant and unwanted cells during embryonic development, growth, differentiation and normal cell turnover (Kerr et al., 1972).

Human cells have the ability to repair UV-induced DNA damage. If the DNA damage is too severe, DNA cannot be repaired. Apoptotic pathways are activated to eliminate the damaged cells. *p53* which acts as a transactivator of transcription, plays an important role in apoptotic pathways. *p53* protein induces apoptosis by

up-regulating the expression of apoptosis-promoting genes or by down-regulating apoptosis-suppressing genes such as Bcl-2. Wild type *p53* also activate *Fas* gene to its promoter sites (Mullauer et al., 2001). CPDs formation or ROS induced by oxidative stress as a result of long time UV exposure ultimately lead to apoptotic or necrotic cell death (Fiers et al., 1999). In sunburn, epidermal keratinocyte death occurs by apoptosis due to excessive exposure to solar UV radiation (Kligman, 1986).

.

### **1.3 Measurement of UV Toxicity**

The biological effects of UV exposure of human skin are assorted. The isolation and identification of the CPD are considered as a breakthrough in DNA photochemistry. In induction of different toxic effects on human being through UV radiation, CPD formation plays an important role. Approximately 70-80% of UV-induced DNA damage depends on CPDs and the remaining was on 6-4PPs and its Dewar isomer.

UVB radiation, thought to be sufficient to induce erythema through the formation of about  $10^5$  CPDs per epidermal cell (Bykov et al., 1998). Different wavelengths of UV radiation absorbed by DNA and CPD have also shown to impair specific and non-specific immune responses in the skin. Cutaneous immune cells (antigen presenting cells, dendritic cells) are one of the cellular targets of DNA damage. Dendritic cells which produced CPDs after UV radiation, involved in the formation of T-suppressor cells, and deficient the antigen presenting activity (Vink et al.,

1997). There has an evidence that melanogenesis was also initiated by the formation of CPDs (Agar and Young, 2005).

Both photoproducts are accountable for mutagenesis and cancer, but CPD is believed to be the principal mutagenic lesion in mammalian cells because (6-4) Pyrimidone photoproducts are repaired more efficiently than CPDs (Brash, 1988; Yagi et al., 1995). Thymine-cytosine and cytosine-cytosine dimers are shown to be the most mutagenic among CPDs. UV induced cancer cells which mainly mediated through p53 gene, and mutations of thymine-cytosine/thymidine dimer and cytosine-cytosine/thymidine dimer are frequently found in this gene. In the absence of efficient DNA repair, CPD formation in the genome may lead to the introduction of deleterious mutations after DNA replication. DNA dimerization of pyrimidines induced by UV leads to the distortion of DNA structure, and further causes cytotoxicity, mutagenicity and the induction of cell signaling pathways. DNA replication, cell division and DNA transcription needed for the synthesis of mRNA, which is a crucial step for protein production, and cell survival can be blocked by pyrimidine dimers (Marrot and Meunier, 2008). Cell killing effects of UV light is associated with increased numbers of CPD formation (Mitchell, 1988).

Most of the UV related toxicity depends on the formation of CPDs. For this, measurement of UV induced CPDs in cellular DNA is a most acceptable and sensitive assay for the determination of DNA damage induced by UV radiation.

## 1.4 Objectives

Only a few studies have been performed on different wavelengths based on UV induced DNA damage in pheochromocytoma (PC12) cells. In this study, first, to elucidate UV-induced cytotoxicity and DNA damage depending on different wavelengths in PC12 cell, the cell viabilities and amounts of CPD formation after UV irradiation at 250, 270, 290 and 310 nm wavelengths at different exposure doses (2, 5, 10, 20, 100 and 200 mJ/cm<sup>2</sup>) were measured. The specific questions are as follows;

1. Depending on cell killing ability, are there any differences within the LD<sub>50</sub> of different wavelengths of UV radiation?
2. Which wavelengths have highest cytotoxicity and DNA damage capacity in PC12 cell system?
3. Does the rate of DNA-repair ability (determined by recovery rate of CPD formation) of different wavelengths of UV radiation at their LD<sub>50</sub> doses differ from each other and varied at different time interval?
4. What are the influence of UV exposure time on cell toxicity and DNA damage?

Second study based on the use of medicinal plant, which plays numerous roles in alleviation of different kinds of diseases in human being. *Tinospora cordifolia*, one of the most popular medicinal plant used in Ayurvedic medicine sector for its antipyretic, anti-inflammatory, anti-allergic, immunosuppressant, antioxidant and anti-carcinogenic activity. Still, study has not been conducted on *Tinospora cordifolia* against DNA damage induced by UV radiation. To evaluate the potency

of *Tinospora cordifolia* against DNA damage and cytotoxicity induced by UV radiation, two questions has raised and that are shown as follows;

- 1.** Is *Tinospora cordifolia* safe for cell?
- 2.** Does *Tinospora cordifolia* have any protective potentials against DNA damage and cytotoxicity induced by UV radiation?

For the determination of safety potential of *Tinospora cordifolia*, trypan blue exclusion assay, agarose gel electrophoresis and TdT-mediated-d-UTP biotin nick end labeling (TUNEL) methods were conducted. On the other hand, to quantify the protective potentials, cell viability through trypan blue exclusion assay and enzyme linked immune sorbent assay (ELISA) for CPD formation were performed in 250, 270 and 290 nm wavelengths UV irradiated PC12 cell.

Thirdly, to evaluate the cell sensitivity to UV radiation, 4 different cells [(PC12 cell, Chinese Hamster Ovary (CHO), Normal Human Epidermal Keratinocytes (NHEK) and Human Umbilical Vein Endothelial Cell (HUVEC)]. Evaluation of UV cytotoxicity and DNA damage were examined. For this third experiment, the specific questions are shown as follows;

- 1.** Does PC12 cell show strong UV-resistant capacity compared to other cells?
- 2.** Why different types of cells exhibit distinct sensitivity to UV radiation mediated toxicity?

Finally to find out the reason, the proposed research design is based on the elucidation of mechanism of cell toxicity by detection of m-TOR pathways



abnormalities through western blot analysis.

## **References**

- N. Agar, A.R. Young, Melanogenesis: a photoprotective response to DNA damage? *Mutat. Res.* 571 (2005) 121-132.
- B.N. Ames, L.S. Gold, W.C. Willet, The causes and prevention of cancer, *Proc. Natl. Acad. Sci. USA* 92 (1995) 5258-5265.
- B.K. Armstrong, A. Krickler, The epidemiology of UV induced skin cancer, *J. Photochem. Photobiol. B: Biol.* 63 (2001) 8-18.
- S. Beissert, R.D. Granstein, UV induced cutaneous photobiology, *Crit. Rev. Biochem. Mol. Biol.* 31 (1996) 381-404.
- A. Besaratinia, T.W. Synold, H.H. Chen, C. Chang, B. Xi, A.D. Riggs, G.P. Pfeifer, DNA lesions induced by UV A1 and B radiation in human cells: comparative analysis in the overall genome and in the p53 tumor suppressor gene, *Proc. Natl. Acad. Sci. USA* 102 (2005) 10058-10063.
- D.E. Brash, UV mutagenic photoproducts in *Escherichia coli* and human cells, A molecular genetics perspective on human skin cancer, *Photochem. Photobiol.* 48 (1988) 49-66.
- V.J. Bykov, C.T. Jansen, K. Hemminki, High levels of dipyrimidine dimers are induced in human skin by solar-stimulating UV radiation, *Cancer Epidemiol. Biomarkers Prevention* 7(1998) 199-202.
- J. Cadet, C. Anselmino, T. Douki, L. Voituriez, Photochemistry of nucleic acids in cells, *J. Photochem. Photobiol. B: Biol.* 15 (1992) 277-298.

- T. Douki, D. Perdiz, P. Grof, Z. Kuluncsics, E. Moustacchi, J. Cadet, E. Sage, Oxidation of guanine in cellular DNA by solar UV radiation: biological role, Photochem. Photobiol. 70 (1999) 184-190.
- R.P. Feynman, S.M. Leighton, in: The Feynman lectures on Physics, Addison-Wesley, Reading MA, 3 (1967) 15 (7- 12).
- W. Fiers, R. Bevaert, W. Declercq, P. Vandenabeele, More than one way to die: Apoptosis and necrosis and reactive oxygen damage, Oncogene 18 (1999) 7719-7730.
- F.R. de Gruijl, H.J. van Kranen, L.H.F. Mullenders, UV induced DNA damage, repair, mutations and oncogenic pathways in skin cancer, J. Photochem. Photobiol. B. Biol. 63 (2001) 19-27.
- F.R. de Gruijl, H.J.C.M. Sterenborg, P.D. Forbes, R.E. Davies, C. Cole, G. Keltken, H. van Weelden, H. Slaper, J.C. van der Leun, Wavelength dependence of skin cancer induction by ultra- violet irradiation of albino hairless mice, Cancer Res. 53 (1993) 53-60.
- B. Halliwell, J.M.C. Gutteridge (Eds.) (1997), FreeRadicals in Biology and Medicine, Oxford: Oxford University press.
- S. Kanjilal, H.N. Ananthaswamy, Molecular biology of skin carcinomas. In R. Weber, M. Miller, H. Goepfert (Eds.) (1996), Basal and squamous cell skin cancers of the head and neck, Williams and Wilkins. Baltimore, pp. 25-26
- J. F. Kerr, A.H. Wyllie, A.R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer. 26 (1972) 239-257.
- C. Kielbassa, L. Roza, B. Epe, Wavelength dependence of oxidative DNA damage induced by UV and Visible Light, Carcinogenesis 18 (1997) 811-816.

- A.M. Kligman, Photoaging in: *Dermatology in General Medicine: textbook and atlas* 3rd ed, T. B. Fitzpatrick, A.Z. Eisen, K. Wolff, I. M. Freedberg, K. F. Austen (Eds), McGraw Hill, New York (1986), pp. 1470-1475.
- S. Kondo, The roles of cytokines in photoaging, *J. Dermatol. Sci.* 23 (2000) 30-36.
- D. Lubin, E.H. Jensen, Effects of clouds and stratospheric ozone depletion on ultraviolet radiation trends, *Nature* 377 (1995) 710-713.
- L. Marrot, J.R. Meunier, Skin DNA photodamage and its biological consequences, *J. Ame. Aca. Dermatol.* 58 (2008) S139-S148.
- Y. Matsumura, H.N. Ananthaswamy, Toxic effects of ultraviolet radiation on the skin, *Toxicol. Appl. Pharmacol.* 195 (2004) 298-308.
- D.L. Mitchell, The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells, *Photochem. Photobiol.* 48 (1988) 51-57.
- L. Mullauer, P. Gruber, D. Seibinger, J. Buch, S. Wohlfart, A. Chott, Mutations in apoptosis genes: a pathogenetic factor for human disease, *Mutat. Res.* 488 (2001) 211-231.
- R. Pallela, Y. Na-Young, S.K. Kim, Anti-photoaging and photoprotective compounds derived from marine organisms, *Mar. Drugs* 8 (2010) 1189-1202.
- A.J. Ridley, J.R. Whiteside, T.J. McMillan, S.L. Allinson, Cellular and sub-cellular responses to UVA in relation to carcinogenesis, *Int. J. Radiant. Biol.* 85 (2009) 177-195.
- L. Rittie, G.J. Fisher, UV-light –induced signal cascades and skin aging, *Aging Res. Rev.* 1 (2002) 705-720.
- S. Shibutani, M Takeshita, A.P. Grollman, Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxo-G, *Nature* 349 (1991)

431-434.

- S. Tornaletti, G.P. Pfeifer, UV damage and repair mechanisms in mammalian cells, *BioEssays* 18 (1996) 221-228.
- A. A. Vink, A.M. Moodycliffe, V. Shreedhar, S.E. Ullrich, L. Roza, D.B. Yarosh, M.L. Kripke, the inhibition of antigen-presenting activity of dendritic cells resulting from UV-irradiation of murine skin is restored by *in vitro* photorepair of cyclobutyl pyrimidine dimers, *Proc. Natl. Acad. Sci. USA* 94 (1997) 5244-5260.
- T. Yagi, T. Morimoto, H. Takebe, Correlation of (6-4) photoproduct formation with transforming mutations in UV-irradiated *Ha-ras*, *Carcinogenesis* 16 (1995) 689-695.

## **Chapter II**

### **Confirmation of analysis of UV toxicity**

#### **2.1 PC12 cell**

PC12 cells, a cell line of rat pheochromocytoma, are a tumor arising from chromaffin cells of the adrenal medulla. When the cells cultured under normal conditions, PC12 cells resemble adrenal chromaffin cells in morphology, physiology and biochemistry. Due to special characteristics, PC12 cell considered as a useful model for studying toxicity of environmental chemicals. PC12 cell line exhibited as a helpful model for studying the mechanism of induction and inhibition of apoptosis, even cultured in a serum-deprived medium (Sun et al., 2012). Serum-deprived PC12 cells manifest endonuclease activity that causes internucleosomal cleavage of cellular DNA (Batistatou and green, 1991). Caspase (caspase-3) like activity which is one of the reasons for cell death is induced in PC12 cell (Stefanis et al., 1996; Haviv et al., 1997). PC12 cells have proven to be a useful system for studying the effects of trace amounts of chemical substances like 2,4,5-trichlorophenoxyacetic, nonylphenol and copper on apoptosis (Yamanoshita et al., 2001; Aoki et al., 2004; Kawakami et al., 2008; Sun et al., 2012). It is a mature neuroendocrine cell model for studying diseases of the nervous and endocrine system (Chestnut et al., 2011). PC12 cells provide an excellent model for studying chemical disruption of processes related with neuronal differentiation, synthesis, storage and release of neurotransmitters,

function and regulation of ion channels and interactions of compounds with membrane bound receptors (Shafer and Atchison, 1991)

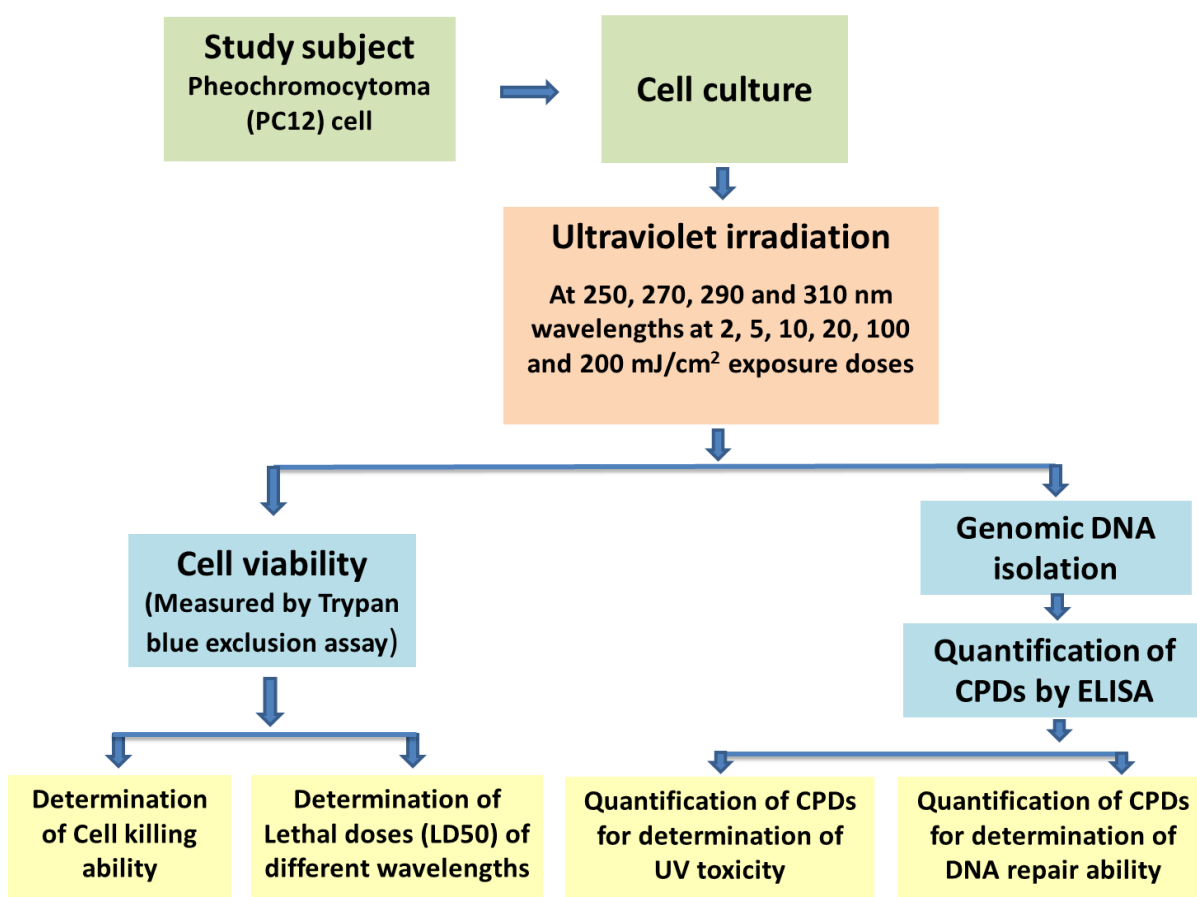
### **Important features of PC12 cells**

- Rapidly dividing cell and having dense-core secretory granules (Dumen et al., 2008)
- Distinct responses of differentiation, proliferation and survival can all be assessed independently
- There are few growth factors, hormones and neurotrophins to which it does not respond (Vaudry et al., 2002).
- Physiological and biochemical changes upon differentiation are used for experiments.
- Depending on conditions, upon stimulation PC12 cells release dopamine, norepinephrine and acetylcholine
- It contains Na, K and Ca channels and other membranes receptors like as G-protein coupled receptors.
- In response to toxic compound and environmental toxicant, it allows the expressions of certain channels and proteins (Shafer and Atchison, 1991).

### **2.2. Analysis of UV toxicity**

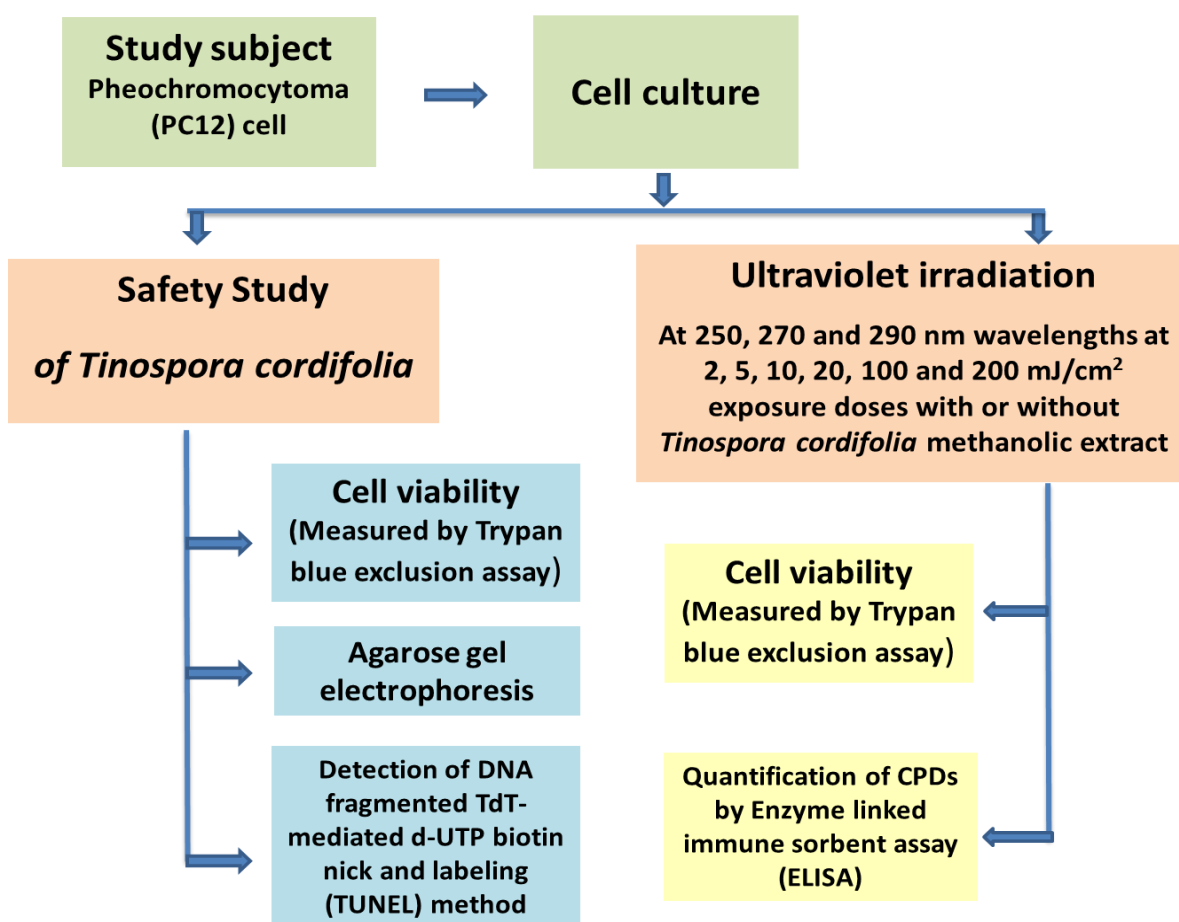
The biological toxic effects of UV radiation depend on the DNA damage which occur mainly the formation of CPDs at their dipyrimidine sides. This DNA damage

further plays significant roles in cell-cycle arrest, cell killing, mutation and apoptosis. So quantification of cell viability and CPD formation are the fundamental methods to determine the toxicity of UV radiation (Fig. 2.1). Besides this, different wavelength of UV radiation has distinct toxicity on cell. Toxicity also depends on exposure time. Based on wavelength (250 nm, 270 nm, 290 nm and 310 nm) and exposure degree (2, 5, 10, 20, 100 and 200 mJ/cm<sup>2</sup>) of UV radiation in a PC12 cell system, the toxicity was determined. To confirm whether each wavelength influenced UV cytotoxicity and UV-induced DNA damage, the trypan blue exclusion test and ELISA on the PC12 cells were performed.



**Fig. 2.1** Schematic diagram of the research design of chapter III

Despite, in this research, to know the protective role of *Tinospora cordifolia* on UV-induced cell toxicity and DNA damage, same investigations were done in the PC12 cells (Fig. 2.2). In that case, cells (containing different doses of *Tinospora cordifolia*) had been irradiated with 3 different UV wavelengths (250, 270 and 290 nm) corresponding to exposure doses of 120, 150 and 300 mJ/cm<sup>2</sup>.



**Fig. 2.2** Schematic diagram for the research design of chapter IV

### 2.2.1 Cell viability

Basically UV radiation kills cells because of the accumulation of DNA damage. *P53* which is responsible for slowing cell cycle and checking for damage, directs cells to



die in case of extreme damage occurred by UV exposure.

The trypan blue exclusion assay was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude the dye (trypan blue), whereas dead cells do not (Strober, 2001). After UV irradiation, the cells were incubated for different durations from 10 min up to 24 h, and then 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.

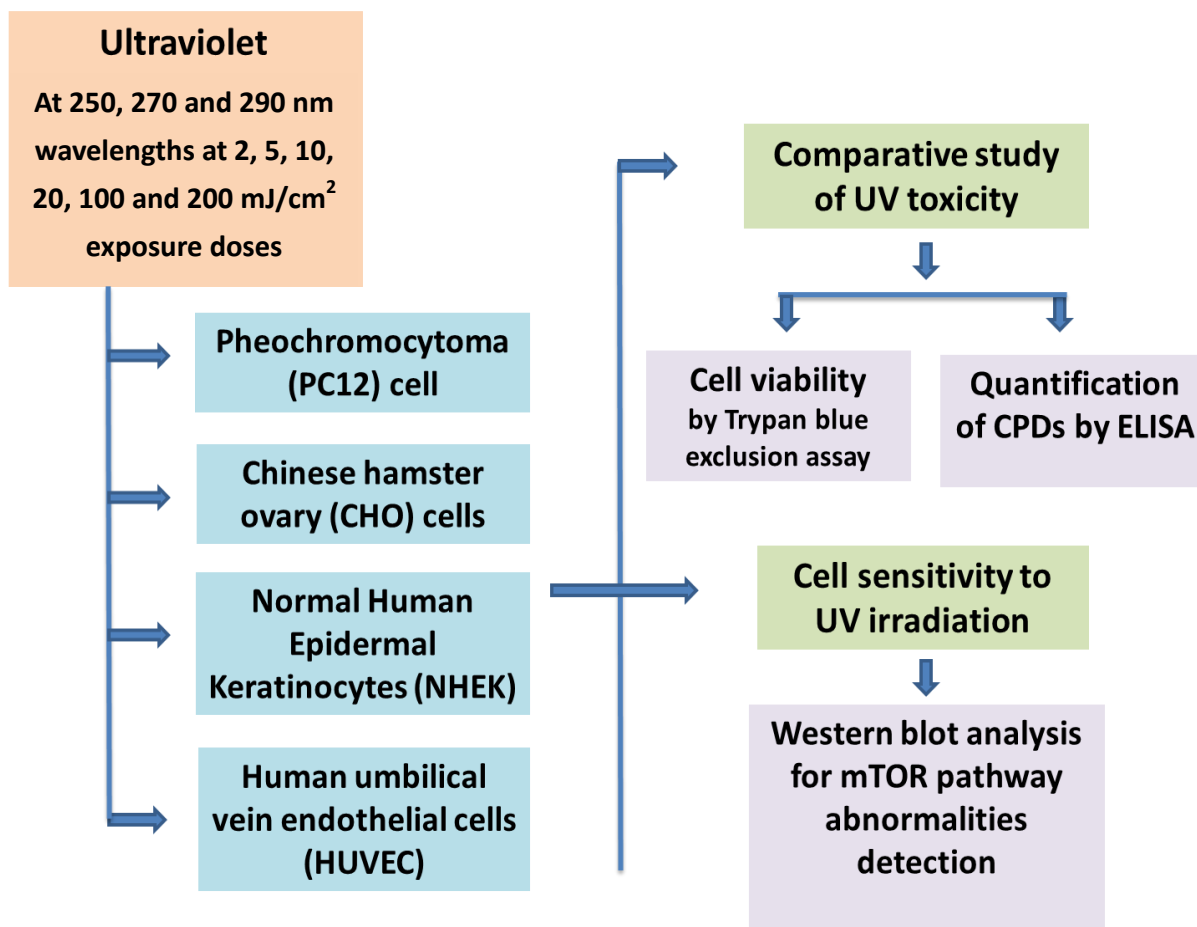
### **2.2.2 ELISA for quantification of CPD formation**

Following UV irradiation of PC12 cells, genomic DNA was isolated using the procedure described by Yamanoshita et al. (2000) with some modifications. 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. Mori et al. (1991) have established monoclonal antibodies specific for CPDs. This antibody enable to quantified photoproduct in DNA purified from cultured cells or from the skin epidermis using ELISA.

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  $\mu$ L 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 1:1000 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 antimouse 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, 6 N HCl was added 5–10 min later. The absorbance at 495 nm was measured using a Microplate Reader (model 450; Bio-Rad). This technique contributes to understand the molecular mechanism of cellular responses to UV and DNA damage in many research including cancer research, photobiology, dermatology, immunology and cosmetology.



**Fig. 2.3** Proposed schematic diagram for the research design of chapter V

## References

- M. Aoki, M. Kurasaki, T. Saito, S. Seki, T. Hosokawa, Y. Tahakahashi, H. Fujita, T. Iwakuma, Nonylphenol enhances apoptosis induced by serum deprivation in PC12 cells, *Life Sci.* 74 (2004) 2301–2312.
- A. Batistatau, L. A. Greene, Aurintricarboxylic Acid Rescues PC12 cells and Sympathetic Neurons from Cell Death caused by nerve growth factor deprivation: correlation with suppression of endonuclease activity, *J. Cell Biol.* 115 (1991) 461–471.

- B.A. Chestnut, Q. Chang, A. Price, C. Lesuisse, M. Wong, L.J. Martin, Epigenetic regulation of motor neuron cell death through DNA methylation, *J. Neurosci.* (2011) 16619-16636.
- J. G. Duman, L. Chen, B. Hille, Calcium transport mechanisms of PC12 cells, *J. Gen. Physiol.* 131 (2008) 307-323.
- R. Haviv, L. Lindenboim, H. Li, J. Yuan, R. Stein, Need for caspases in apoptosis of trophic factor-derived PC12 cells, *J. Neurosci. Res.* 50 (1997) 69-80.
- M. Kawakami, R. Inagawa, T. Hosokawa, T. Saito, M. Kurasaki, Mechanism of apoptosis induced by copper in PC12 cells, *Food Chem. Toxicol.* 46 (2008) 2157-2164.
- T. Mori, M. Nakane, T. Hattori, T. Matsunaga, M. Ihara, O. Nikaido, Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6-4) photoproduct from the same mouse immunized with ultraviolet-irradiated DNA, *Photochem. Photobiol.* 54 (1991) 225-232.
- T. J. Shafer, W. D. Atchison, Transmitter, ion channels and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies, *Neurotoxicology* 12 (1991) 473-492.
- L. Stefanis, D.S. Park, C.Y. Yan, S. E. Farinelli, C. M. Troy, M. L. Shelanski, L. A. Greene, Induction of CPP32-like activity in PC12 cells by withdrawal of trophic support, dissociation from apoptosis, *J. Biol. Chem.* 271 (1996) 30663-30671.
- W. Strober, Trypan blue exclusion test of cell viability, *Curr. Protoc. Immunol.* 2001, Appendix 3: appendix 3B, doi: 10.1002/0471142735.ima03bs21.
- Y. Sun, K. Takahashi, T. Hosokawa, T. Saito, M. Kurasaki, Diethyl phthalate enhances apoptosis induced by serum deprivation in PC12 cells, *Basic Clin.*

- Pharmacol. Toxicol. 111 (2012) 113-119.
- Y. Sun, Z. Guo, S. Iku, T. Saito, M. Kurasaki, Diethyl phthalate enhances expression of SIRT1 and DNMT3a during apoptosis in PC12 cells, J. App. Toxicol. 33 (2012) 1484-1492.
- D. Vaudry, P. J. S. Stork, .P. Lazarovici, L.E. Eiden. Signaling pathways for PC12 cell differentiation: making the right connection, Science 296 (2002) 1648-1649.
- O. Yamanoshita, T. Saito, K. Takahashi, T. Hosokawa, M. Okabe, K. Ito, M. Kurasaki, 2,4,5-Trichlorophenoxyacetic acid inhibits apoptosis in PC12 cells, Life Sci. 69 (2001) 403-408.

## **Chapter III**

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

### **3.1 Introduction**

UV radiation induces basal and squamous cell carcinoma and cutaneous malignant melanoma in humans (Woodhead and Setlow, 1996; Jhappan et al., 2003; Armstrong and Krickler, 2001) 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 (Kvam and Tyrrell, 1997), which is primarily responsible for induction of DNA damage (Freeman et al., 1989). DNA, which maximally absorbs radiation at wavelengths of 245–290 nm, is one of the main cellular targets of UV radiations (Peak and Peak, 1989; Tornaletti and Pfeifer, 1996). Owing to its wavelength, which is within the absorption peak of DNA (Mallet and Rochette, 2011), 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 (Matsumura and Ananthaswamy, 2004). In

contrast to UVB and UVC radiation, the mutagenic and carcinogenic effects of UVA result from generation of ROS through the process of photosensitization (Pygmalion et al., 2010; de Gruijl, 2002). 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 (Pallela et al., 2010; Tanaka et al., 2005). UV-mediated DNA damage is mainly attributed to the formation of 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 (Douki et al., 1999; Rochette et al., 2003). 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 (Vink and Roza, 2001). 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 (Mallet and Rochette, 2011; Besaratinia et al., 2005; Cadet et al., 2005; Kubota et al., 2001). 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 (Mu et al., 1996; Li et al., 1997; Boer and Hoeijmakers, 2000). However, if DNA damage is too severe and cannot be repaired, the apoptotic pathway is triggered to prevent propagation of the damage (Gniadecki et al., 1997; Matsumura and Ananthaswamy, 2002). 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 (Mullauer et al., 2001; Tsujimoto and Shimizu, 2000; Adams and Cory, 2001; Zeigler et al., 1994).

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 ELISA on the PC12 cells.



## **3.2 Materials and methods**

### **3.2.1 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). Proteinase K was obtained from Roche Diagnostica (Mannheim, Germany). Trypan blue stain solution (0.5%) was purchased from Nacalai Tesque (Kyoto, Japan). All the other chemicals were of analytical reagent grade.

### **3.2.2 Cell culture**

PC12 cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator with 5% CO<sub>2</sub> 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.

### **3.2.3 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 ( $\text{mJ}/\text{cm}^2$ ) = exposure time (s)  $\times$  irradiance ( $\text{mW}/\text{cm}^2$ ).

To determine the dose-effect relationship, 6 doses (2, 5, 10, 20, 100 and 200  $\text{mJ}/\text{cm}^2$ ) 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  $\text{mJ}/\text{cm}^2$ ) 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 (LD<sub>50</sub>)—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 LD<sub>50</sub> 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:

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

#### **3.2.4 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:

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

### **3.2.5 Genomic DNA isolation from PC12 cells**

Following UV irradiation of PC12 cells, genomic DNA was isolated using the procedure described by Yamanoshita et al. (2000) 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 1500 rpm for 5 min, and the supernatants were removed. Subsequently, cells were incubated with 10 mM Tris-HCl buffer, 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).

### **3.2.6 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  $\mu$ L 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 1:1000 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 antimouse 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, 6 N HCl was added 5–10 min later. The absorbance at 495 nm was measured using a Microplate Reader (model 450; Bio-Rad).

### **3.2.7 Statistical analysis**

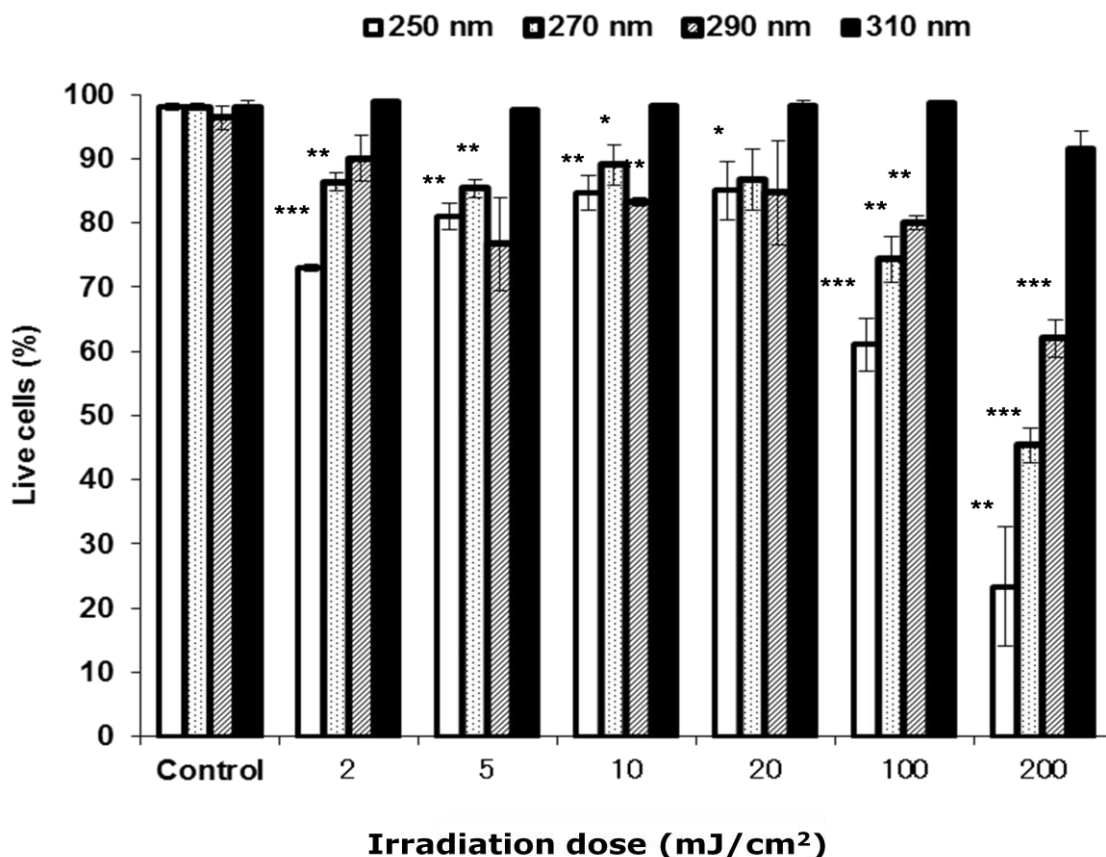
Each value is expressed as the mean $\pm$ SEM. Statistical analysis was performed using the unpaired t-test, followed by the F-test.

### **3.3 Results**

#### **3.3.1 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/cm<sup>2</sup> (Fig. 3.1). 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/cm<sup>2</sup>) of the 290-nm radiation; however, cell viability was not significantly affected following exposure to the 310-nm radiation, irrespective of the dosage (Fig. 3.1).

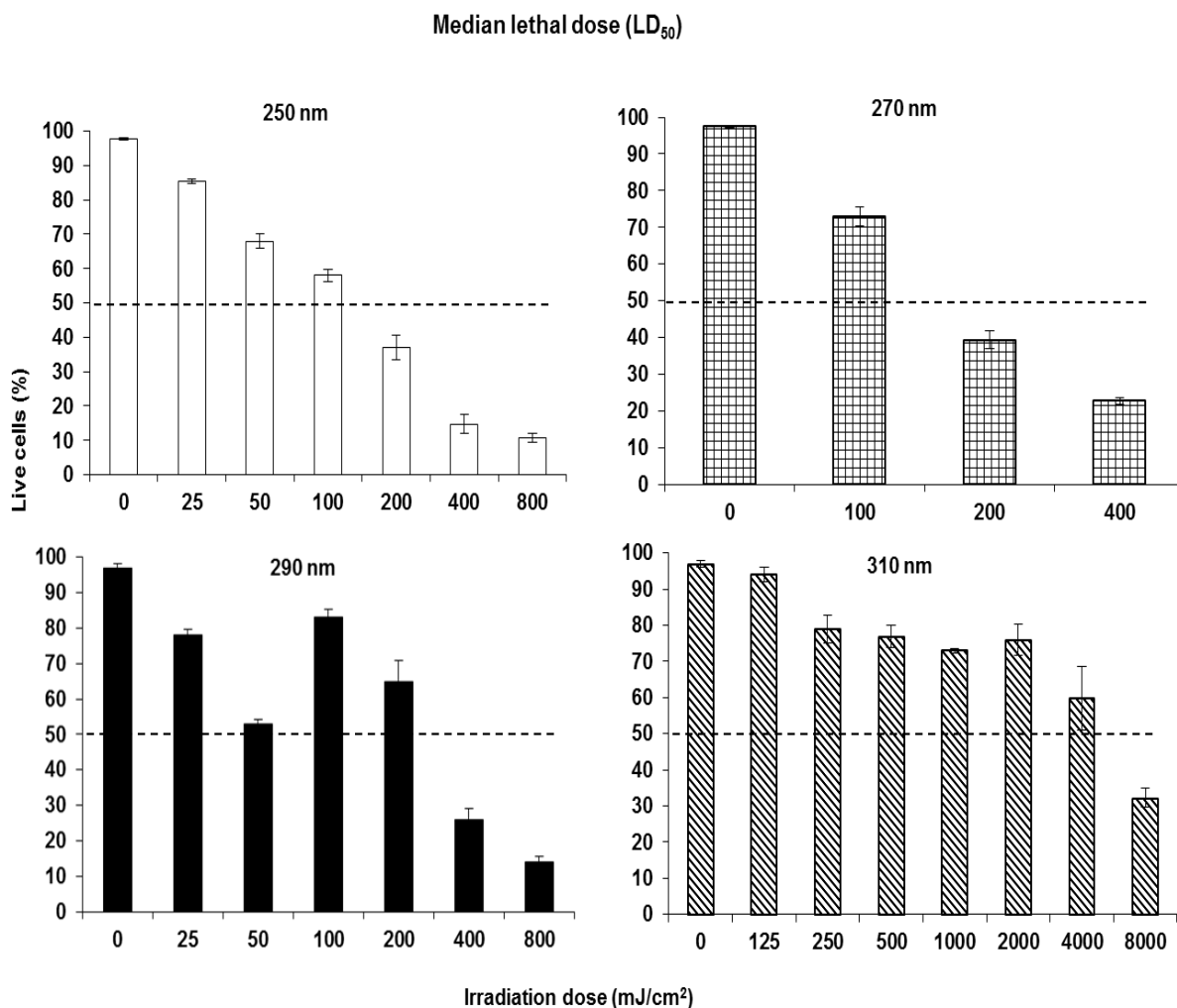
In addition, to determine the relative toxicity of each wavelength tested, we quantified the viabilities of cells irradiated with the 4 different wavelengths at 100 mJ/cm<sup>2</sup> and incubated for 10 min up to 24 h. Interestingly, irrespective of the wavelength used, the viabilities did not change 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.



**Fig. 3.1** Viability of PC12 cells exposed to 4 different wavelengths of UV radiations at different doses (2, 5, 10, 20, 100, and 200 mJ/cm<sup>2</sup>) 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.

### 3.3.2 Determination of LD<sub>50</sub> of the 250-, 270-, 290-, and 310-nm radiations

For determining the LD<sub>50</sub> of the 4 tested wavelengths, we exposed PC12 cells to 25-800 mJ/cm<sup>2</sup> of 250-, 270-, and 290-nm radiations and to 125-8000 mJ/cm<sup>2</sup> of the 310-nm radiation. After irradiation, the cell viabilities were quantified. The LD<sub>50</sub> values were calculated from the results of the cell viability assay and were 120, 150, 300, and 6000 mJ/cm<sup>2</sup> for 250, 270, 290, and 310 nm, respectively (Fig. 3. 2).

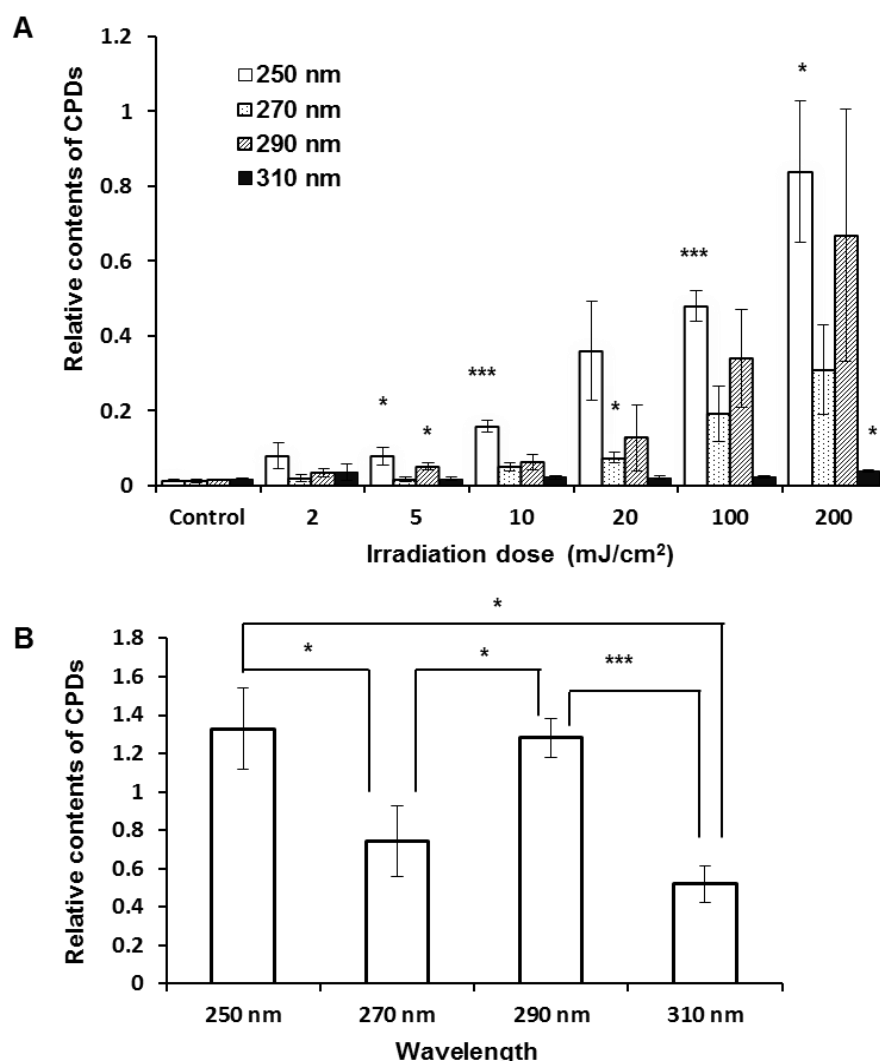


**Fig. 3.2** LD<sub>50</sub> (mJ/cm<sup>2</sup>) of 250, 270, 290, and 310 nm UV radiations, which were determined from the number of viable PC12 cells present 24 h after UV irradiation with different doses. The LD<sub>50</sub> values were 120, 150, 300, and 6000 mJ/cm<sup>2</sup> for 250, 270, 290, and 310 nm, respectively. Error bars indicate SEM (n = 3–4).

### 3.3.3 Quantification of CPD formation by ELISA

CPD formation in irradiated PC12 cells was analyzed using ELISA. As shown in Fig. 3.3A, 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/cm<sup>2</sup> 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





**Fig. 3.3** CPD formation (A) 24 h after UV irradiation with 250, 270, 290, and 310 nm UV radiations at doses of 2–200 mJ/cm<sup>2</sup> 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/cm<sup>2</sup>) 24 h after irradiation (n = 4). Asterisks denote significantly different values (\*p < 0.05, \*\*\*p < 0.001). Error bars indicate SEM.

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/cm<sup>2</sup>. These results indicate that

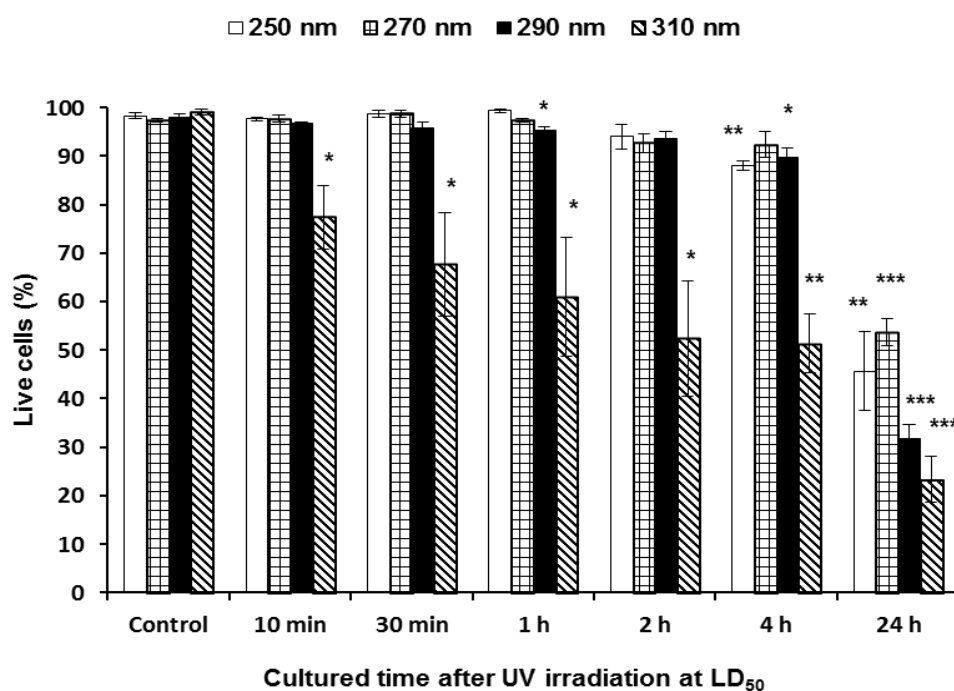
CPD formation increased in an irradiance-dependent manner. For determining relative CPD-inducing ability of the different wavelengths of UV radiation at different cultured time, we exposed PC12 cells to the 4 tested wavelengths at 100 mJ/cm<sup>2</sup> and incubated these irradiated cells for 10 min 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. 3.3B).

#### **3.3.4 Time course for LD<sub>50</sub> 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 LD<sub>50</sub> (Fig. 3.4). Among the 4 wavelengths, 310 nm at its LD<sub>50</sub> significantly reduced the number of live cells at each culture time. The decrease in cell viability caused by the different wavelengths at their corresponding LD<sub>50</sub> showed the following order: 310 nm > 290 nm > 250 nm > 270 nm.

As shown in Fig. 3.5, 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 LD<sub>50</sub> did not show any DNA repair ability.

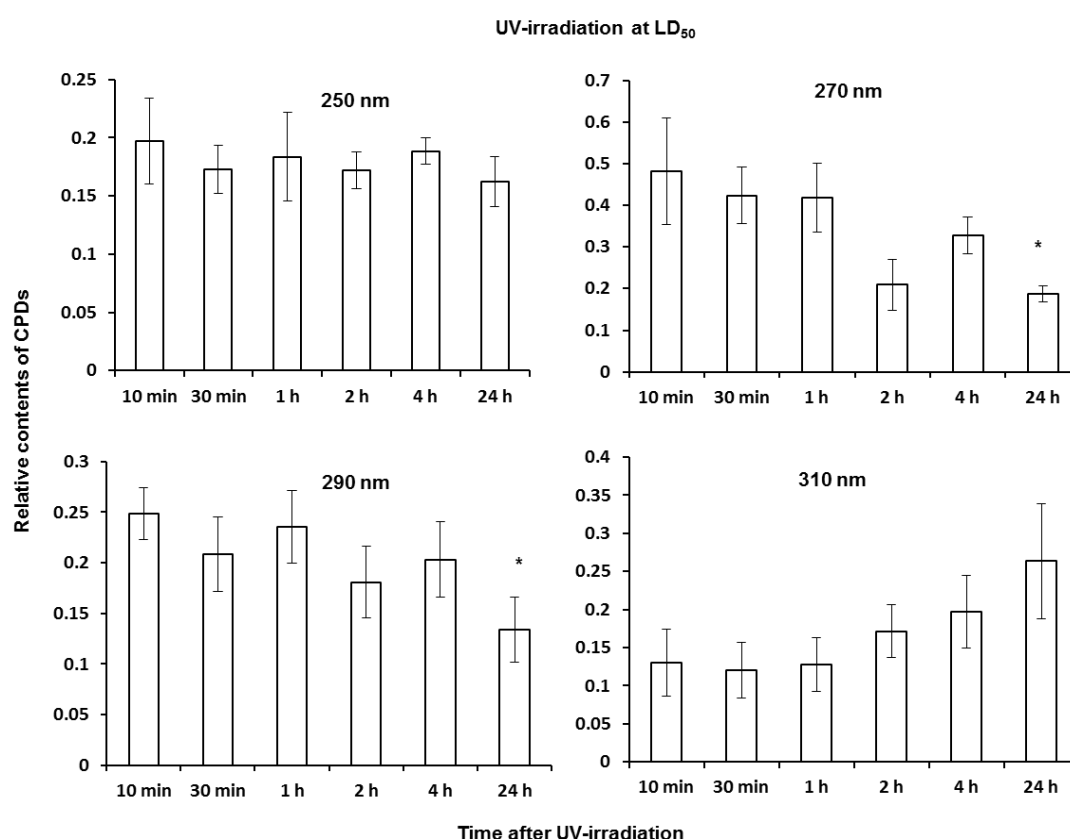


**Fig. 3.4** Viability of PC12 cells after UV irradiation with 4 different wavelengths at their LD<sub>50</sub> doses. Error bars indicate SEM (n = 3). PC12 cells were cultured for different durations after UV irradiation. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 indicate significant differences from the control value.

### 3.4 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 (Yamanoshita et al., 2001; Sun et al., 2012; Aoki et al., 2004). 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 (Duman et al., 2008; Shafer et al., 1991). PC12 cells

have stronger UV resistance compared to other cell lines. Our results showed that PC12 cells could tolerate high LD<sub>50</sub> (ranging from 120 to 6000 mJ/cm<sup>2</sup>) of 250–310 nm wavelengths of UV radiations (Fig. 3.2). In contrast, for normal human epidermal keratinocytes (NHEKs) and porcine lens epithelial cells (LECs), the minimum LD<sub>50</sub> values for 269-nm and 261.7-nm radiations were 1.64 and 1.77 mJ/cm<sup>2</sup>, respectively, and for human LECs the minimum LD<sub>50</sub> for the 267.5-nm radiation was 8.86 mJ/cm<sup>2</sup> (Aoki et al., 2011; Okuno et al., 2012; Okuno, 2007). The LD<sub>50</sub> of radiation for the PC12 cells was 50 times that for NHEKs and LECs.

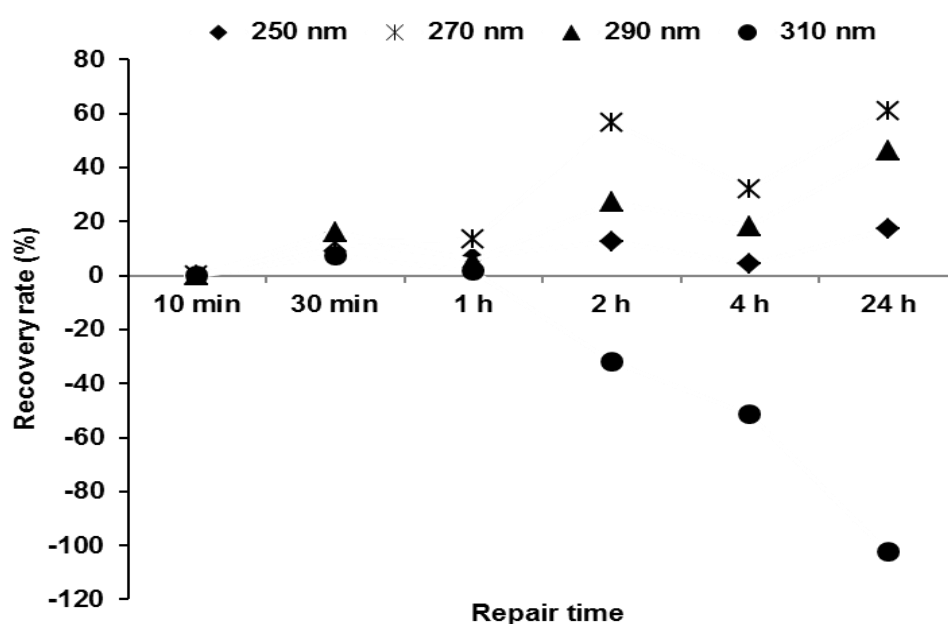


**Fig. 3.5** Comparison of CPD formation in PC12 cells irradiated with 4 different wavelengths of UV radiation applied at their respective LD<sub>50</sub> (120 mJ/cm<sup>2</sup> for 250 nm, 150 mJ/cm<sup>2</sup> for 270 nm, 300 mJ/cm<sup>2</sup> for 290 nm, and 6000 mJ/cm<sup>2</sup> for 310 nm). Error bars indicate SEM (n=3). Asterisks denote values significantly different (\*p < 0.05) from the initial time (10 min).

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. 3.1). 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 (de Gruijl, 2002; Mitchell, 1988). As previously reported, the cell killing action spectrum is close to the DNA absorption spectrum (Coohill et al., 1987), 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 (Kubota et al., 2001; Borges et al., 2008).

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 Fig. 3.3A 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 (Mallet and Rochette, 2011; Runger and Kappes, 2008). UV light is absorbed by tryptophan and tyrosine in proteins at 310 nm and 290 nm, respectively (Creed, 1984a;

Creed, 1984b). The most effective wavelength, within the UVB range, for inducing DNA photoproducts in the basal layer of the epidermis is 300 nm (Runger and Kappes, 2008). 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. (2001).



**Fig. 3.6** CPDs (%) remaining in PC12 cells irradiated with 4 different wavelengths of UV radiations at their respective LD<sub>50</sub> 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.

To determine DNA-repair ability of the PC12 cells, the LD<sub>50</sub> values of the 4 tested wavelengths were determined from the results of the cell viability assays (Fig. 3.2). In this study, the LD<sub>50</sub> values for 310 nm were 50, 40, and 20 times the LD<sub>50</sub>

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 (Sutherland and Griffin, 1980), 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 LD<sub>50</sub> (Fig. 3.5). 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 (Boer and Hoeijmakers, 2000; Kubota et al., 2001). In this study, cells irradiated with 290- and 270-nm radiations at their corresponding LD<sub>50</sub> showed superior repair ability, whereas no repair was observed in cells irradiated with 310-nm radiation (Fig. 3.6). CPD Repair rates depend on genes and vary along the same gene sequence between adjacent base positions (Gao et al., 1994; Tornaletti, 1994). 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. 3.4).

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 LD<sub>50</sub>. Although 310 nm was less lethal to cells and induced less DNA damage than the other tested wavelength, long-term exposure (at the LD<sub>50</sub>) might cause severe and irreparable damage. Further investigation is needed to clarify the precise mechanism of CPD formation by each wavelength.

## **References**

- J.M. Adams, S. Cory, Life-or-death decisions by the Bcl-2 protein family, *Trends Biochem. Sci.* 26 (2001) 61-66.
- K. Aoki, T. Nakanishi-Ueda, M. Tsuji, T. Okuno, Y. Saito, H. Yasuhara, Ultraviolet action spectrum and effect of EPC-K1 on ultraviolet radiation-induced injury in cultured normal human epidermal keratinocytes, *Showa Univ. J. Med. Sci.* 23 (2011) 1-10.
- M. Aoki, M. Kurasaki, T. Saito, S. Seki, T. Hosokawa, Y. Takahashi, H. Fujita, T. Iwakuma, Nonylphenol enhances apoptosis induced by serum deprivation in PC12 cells, *Life Sci.* 74 (2004) 2301-2312.
- B.K. Armstrong, A. Krickler, The epidemiology of UV induced skin cancer, *J. Photochem. Photobiol. B* 63 (2001) 8-18.
- A. Besaratinia, T.W. Synold, H.H. Chen, C. Chang, B. Xi, A.D. Riggs, G.P. Pfeifer, DNA lesions induced by UV A1 and B radiation in human cells: comparative analysis in the overall genome and in the p53 tumor suppressor gene, *Proc. Natl. Acad. Sci. USA* 102 (2005) 10058-10063.
- J. de Boer, J.H.J. Hoeijmakers, Nucleotide excision repair and human



- syndromes, *Carcinogenesis* 21 (2000) 453-460.
- H.L. Borges, R. Linden, J.Y.J. Wang, DNA damage-induced cell death: lessons from the central nervous system, *Cell Res.* 18 (2008) 17-26.
- J. Cadet, E. Sage, T. Douki, Ultraviolet radiation-mediated damage to cellular DNA, *Mutation Res.* 571 (2005) 3-17.
- T.P. Coohill, M.J. Peak, G.P. Peak, The effects of the ultraviolet wavelengths of radiation present in sunlight on human cells in vitro, *Photochem. Photobiol.* 46 (1987) 1043-1050.
- D. Creed, The photophysics and photochemistry of the near UV-absorbing amino acids. I. Tryptophane and its simple derivatives, *Photochem. Photobiol.* 39 (1984a) 537-562.
- D. Creed, The photophysics and photochemistry of the near UV-absorbing amino acids. II. Tyrosine and its simple derivatives, *Photochem. Photobiol.* 39 (1984b) 563-565.
- T. Douki, D. Perdiz, P. Grof, Z. Kuluncsics, E. Moustacchi, J. Cadet, E. Sage, Oxidation of guanine in cellular DNA by solar UV radiation: biological role, *Photochem. Photobiol.* 70 (1999) 184-190.
- J.G. Duman, L. Chen, B. Hille, Calcium transport mechanisms of PC12 cells, *J. Gen. Physiol.* 131 (2008) 307-323.
- S.E. Freeman, H. Hacham, R.W. Gange, D.J. Maytum, J.C. Sutherland, B.M. Sutherland, Wavelength dependence of pyrimidine dimer formation in DNA of human skin irradiated in situ with ultraviolet light, *Proc. Natl. Acad. Sci. USA* 86 (1989) 5605-5609.
- S. Gao, R. Drouin, G.P. Holmquist, DNA repair rates mapped along the human

- PGK-1 gene at nucleotide resolution, *Science* 263 (1994) 1438-1440.
- R. Gniadecki, M. Hansen, H.C. Wulf, Two pathways for induction of apoptosis by ultraviolet radiation in cultured human keratinocytes, *The Soc. Invest. Dermatol.* 109 (1997) 163-169.
- F.R. de Gruijl, Photocarcinogenesis: UVA vs. UVB radiation, *Skin. Pharmacol. Appl. Skin. Physiol.* 15 (2002) 316-320.
- C. Jhappan, F.P. Noonan, G. Merlino, Ultraviolet radiation and cutaneous malignant melanoma, *Oncogene* 22 (2003) 3099-3112.
- Y. Kubota, C. Niwa, T. Ohnuma, Y. Ohko, T. Tatsuma, T. Mori, A. Fujishima, Protective effect of TiO<sub>2</sub> particles on UV light induced pyrimidine dimer formation, *J. Photochem. Photobiol. A Chem.* 141 (2001) 225-230.
- E. Kvam, R.M. Tyrrell, Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation, *Carcinogenesis* 18 (1997) 2379-2384.
- G. Li, V.C. Ho, D.L. Mitchell, M.J. Trotter, V.A. Tron, Differentiation-dependent p53 regulation of nucleotide excision repair of keratinocytes, *Am. J. Pathol.* 150 (1997) 1457-1464.
- J.D. Mallet, P.J. Rochette, Ultraviolet light-induced cyclobutane pyrimidine dimers in rabbit eyes, *Photochem. Photobiol.* 87 (2011) 1363-1368.
- Y. Matsumura, H.N. Ananthaswamy, Molecular mechanisms of photocarcinogenesis, *Front. Biosci.* 7 (2002) D765-D783.
- Y. Matsumura, H.N. Ananthaswamy, Toxic effects of ultraviolet radiation on the skin, *Toxicol. Appl. Pharmacol.* 195 (2004) 298-308.
- D.L. Mitchell, The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells, *Photochem. Photobiol.* 48 (1988) 51-57.

- D. Mu, D.S. Hsu, A. Sancar, Reaction mechanism of human DNA repair excision nuclease, *J. Biol. Chem.* 271 (1996) 8285-8294.
- L. Mullauer, P. Gruber, D. Seibinger, J. Buch, S. Wohlfart, A. Chott, Mutations in apoptosis genes: a pathogenetic factor for human disease, *Mutat. Res.* 488 (2001) 211-231.
- T. Okuno, Ultraviolet action spectrum for cell killing in a human lens epithelial cell line, *Ind. Health* 45 (2007) 137-142.
- T. Okuno, T. Nakanishi-Ueda, T. Ueda, H. Yasuhara, R. Koide, Ultraviolet action spectrum for cell killing of primary porcine lens epithelial cells, *J. Occ. Health* 54 (2012) 181-186.
- R. Pallela, Y. Na-Young, S.K. Kim, Anti-photoaging and photoprotective compounds derived from marine organisms, *Mar. Drugs* 8 (2010) 1189-1202.
- M.J. Peak, J.G. Peak, Solar-ultraviolet-induced damage to DNA, *photodermatology* 6 (1989) 1-15.
- M.I. Pygmalion, L. Ruiz, E. Popovic, J. Gizard, P. Portes, X. Marat, K. Lucet-Levannier, B. Mullet, J.P. Galey, Skin cell protection against UVA by Sideroxyl, a new antioxidant complementary to sunscreens, *Free Rad. Biol. Med.* 49 (2010) 1629-1637.
- P.J. Rochette, J.P. Therrien, R. Drouin, D. Perdiz, N. Bastein, E.A. Drobetsky, E. Sage, UVA-induced cyclobutane pyrimidine dimers form predominantly at thymine-thymine dipyrimidines and correlate with the mutation spectrum in rodent cells, *Nucl. Acids Res.* 31 (2003) 2786-2794.
- T.M. Runger, U.P. Kappes, Mechanisms of mutation formation with long-wave ultraviolet light (UVA), *Photodermatol. Photoimmunol. Photomed.* 24 (2008)

2-10.

- T.J. Shafer, W.D. Atchison, Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies, *Neurotoxicology* 12 (1991) 473-492.
- Y. Sun, K. Takahashi, T. Hosokawa, T. Saito, M. Kurasaki, Diethyl phthalate enhances apoptosis induced by serum deprivation in PC12 cells, *Bas. Clin. Pharmacol. Toxicol.* 111 (2012) 113-119.
- J.C. Sutherland, K.P. Griffin, Absorption spectrum of DNA for wavelengths greater than 300 nm, *Radiat. Res.* 86 (1980) 399-409.
- K. Tanaka, J. Hasegawa, K. Asamitsu, T. Okamoto, Prevention of the ultraviolet B-mediated skin photoaging by a nuclear factor  $\kappa$ B inhibitor, parthenolide, *J. Pharmacol. Exp. Therap.* 315 (2005) 624-630.
- S. Tornaletti, G.P. Pfeifer, Slow repair of pyrimidine dimers at p53 mutations hotspots in skin cancer, *Science* 263 (1994) 1436-1438.
- S. Tornaletti, G.P. Pfeifer, UV damage and repair mechanisms in mammalian cells, *BioEssays* 18 (1996) 221-228.
- Y. Tsujimoto, S. Shimizu, Bcl-2 family: life-or-death switch, *Febs. Lett.* 466 (2000) 6-10.
- A.A. Vink, L. Roza, Biological consequences of cyclobutane pyrimidine dimers, *J. Photochem. Photobiol., B* 65 (2001) 101-104.
- A.D. Woodhead, R.B. Setlow, M. Tanaka, Environmental factors in nonmelanoma and melanoma skin cancer, *J. Epidemiol.* 9 (1996) S102-114.
- O. Yamanoshita, M. Kurasaki, T. Saito, K. Takahashi, H. Sasaki, T. Hosokawa, M. Okabe, J. Mochida, T. Iwakuma, Diverse effect of tributyltin on apoptosis in

PC12 cells, Biochem. Biophys. Res. Commun. 272 (2000) 557-562

O. Yamanoshita, T. Saito, K. Takahashi, T. Hosokawa, M. Okabe, K. Ito, M.

Kurasaki, 2,4,5-Trichlorophenoxyacetic acid inhibits apoptosis in PC12 cells, Life Sci. 69 (2001) 403-408.

A. Zeigler, A.S. Jonson, D.j. Leffell, J.A. Simon, H.W. Sharma, J. Kimmelman, L.

Remington, T. Jacks, D.E. Brash, Sunburn and p53 in the onset of skin cancer, Nature 372 (1994) 773-776.

## **Chapter IV**

# **Effect of *Tinospora cordifolia* on the reduction of ultraviolet radiation-induced cytotoxicity and DNA damage in PC12 Cells**

### **4.1 Introduction**

The study of human safety related to medicinal plant extracts has been gaining immense interest. Plants that have traditionally provided cures for various human ailments have been a major source of medicine. According to the WHO, up to 80% of people rely on medicinal plants for their ailments (Tripathi and Tripathi, 2003). *Tinospora cordifolia*, which belongs to the *Menispermaceae* family, is a large, glabrous, deciduous, succulent, climbing shrub, which is distributed throughout the tropical regions of Indian subcontinent and China. The *T. cordifolia* extract has been reported to be rich in several bioactive components such as alkaloids; diterpenoid lactones; glycosides; steroids; sesquiterpenoid, phenolic, and aliphatic compounds; and polysaccharides (Kapil and Sharma, 1997). Methanol extracts of *T. cordifolia* leaves are abundance of phenolic and flavonoid contents that exhibit anti-oxidant as well as superoxide radical scavenging activity (Premanath and Lakshmidhevi, 2010). Antioxidant saponarin, characterized as  $\alpha$ -glucosidase inhibitor has been isolated from *T. cordifolia* methanol extracts show anti-diabetic property (Sengupta et al., 2009). The therapeutic value of *T.*

*cordifolia* is varied. It has been used in several indigenous drug preparations and exhibits anti-allergic (Badar et al., 2005), anti-inflammatory (Wesley et al., 2008), and hepatoprotective activity (Karkal and Bairy, 2007). Furthermore, Alcoholic extracts of the plants has shown immunomodulatory, antitumor, and anti-neoplastic activity, and has been found to increase bone marrow precursor cells in tumor-bearing hosts (More and Pai, 2011; Singh et al., 2006). However, little information exists on the effects of *T. cordifolia* extract on apoptosis or DNA damage.

Because *T. cordifolia* contains different kinds of bioactive components, the evaluation of its protective effect against photo-induced DNA damage is of great importance. Exposure to UV radiation present in sunlight is a major risk factor for the development of skin diseases such as erythema, edema, sunburn, hyperpigmentation, melanoma, and non-melanoma forms of skin cancer (Aguilera et al., 2012; Choi et al., 2002). UV radiation-subdivided into UVA (315-400 nm), UVB (280-315 nm), and UVC (200-280 nm)-is directly absorbed by DNA, and damages it (Freeman et al., 1989). A significant amount of UVA and UVB radiation reaches the surface of the Earth, but UVC radiation is completely absorbed by the stratospheric ozone layer (Beissert and Granstein, 1995). Artificial light such as that from arc welding, photocuring, and bactericidal lamps, as well as the depletion of the ozone layer, are the major sources of UVC radiation (Matsumura and Ananthaswamy, 2004). UVB and UVC radiation damage DNA by forming CPDs between adjacent thymine (T) and cytosine (C) residues, and to a lesser extent, pyrimidine (6-4) pyrimidone photo products. On the other hand,

UVA radiation alters the antioxidant status and generates ROS by triggering ROS signaling pathways (Pygmalion et al., 2010). The UV-induced damages are associated with cell-cycle arrest, cell death, mutation, neoplastic transformation, immunosuppression, and finally, photo aging and photocarcinogenesis (Pallela et al., 2010). Most of the mutagenic and carcinogenic effects of UV radiation have long been attributed to the shorter wavelengths of UVB and UVC (Runger and Kappes, 2008). Many plant products rich in bioactive molecules capable of protecting DNA from radiation-induced damage have been investigated (Baliga et al., 2004). Natural medicines have gained popularity over synthetic drugs because of their safety and abundant therapeutic characteristics. Identification and development of safe, non-toxic, and effective radioprotective compounds are of enormous importance in mitigating the toxic effect of UV radiation-induced DNA damage.

Apoptosis is the programmed cell death that occurs as a normal developmental response to a definable physiological stimulus. The unneeded cells damaged by apoptosis are eliminated without any local inflammation due to leakage of their contents (Fulda and Debatin, 2004). Apoptosis, through cellular destruction, is carried out by intracellular proteases called caspases, which-upon activation of the intrinsic or extrinsic pathways-are regulated by expression of the BCL-2 superfamily (Aravind et al., 1999). Exposure to UV radiation triggers the apoptotic mitochondrial pathway if damage to the DNA is severe (Matsumura and Ananthaswamy, 2002). PC12 cell line has been shown to be a very useful model for studying the mechanism of induction and inhibition of apoptosis. Furthermore,



apoptosis with DNA fragmentation is induced in PC12 cells when they are cultured in a serum-free medium. The PC12 cell system makes it possible to investigate the effect of trace amounts of chemical substances on the apoptotic reaction. Many studies deal with this aspect of apoptosis (Gollapaudi and Oblinger, 1999; Sun et al., 2012). Few studies have used a PC12 cell system to investigate the protective effect of *T. cordifolia* against UV radiation-induced DNA damage.

The first aim of this study was to investigate the toxic effects of different concentrations of *T. cordifolia* on cell viability, apoptosis, and DNA fragmentation in PC12 cells. For this purpose, we used the trypan blue exclusion assay, agarose gel electrophoresis, and the TdT-mediated d-UTP-biotin nick end labeling (TUNEL) methods. The second aim was to elucidate the protective effects of *T. cordifolia* on UV-induced cell toxicity and DNA damage by using the cell viability and ELISA in PC12 cells that had been irradiated with 3 different UV wavelengths (250, 270 and 290 nm) corresponding to doses of 120, 150 and 300 mJ/cm<sup>2</sup>.

## **4.2 Materials and Methods**

### **4.2.1 Materials**

PC12 cells were purchased from the American Type Culture Collection (Manassas, VA). FBS was obtained from Hyclone (Rockville, MD, USA); DMEM, streptavidin-conjugated peroxidase, RNase, OPD and ethidium bromide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Biotin-16-2'-deoxyuridine 5'-triphosphate (biotinylated dUTP), proteinase K, and blocking reagent were

obtained from Roche Diagnostics (Mannheim, Germany). Terminal deoxynucleotidyl transferase (TdT) was obtained from Toyobo (Osaka, Japan), and the monoclonal antibody to CPD was purchased from Cosmo-Bio Co., Ltd. (Tokyo, Japan). Biotinylated goat anti-mouse immunoglobulin was obtained from Amersham Pharmacia Biotech (Buckinghamshire, England); DNA marker from Promega (Madison, WI, USA); and Trypan blue stain solution (0.5%) from Nacalai Tesque (Kyoto, Japan). Other chemicals were of analytical reagent grade.

#### **4.2.2 Collection and preparation of *T. cordifolia* methanolic extract**

Dry powdered *T. cordifolia* was collected from Bangladesh. After air-drying, 10 gm of the material was extracted by macerating it for 24 to 48 h with continuous stirring in 100 ml of 70% methanol. The macerate was centrifuged at 1,500 rpm for 5 min, and the supernatant filtered using Whatman No. 1 filter paper. After the solvent was evaporated at room temperature, a solid, dark, reddish-brown residue (yield 2.2%) was obtained. It was reconstituted with distilled water at concentrations of 1, 10 and 100 ng/ml and 1, 10 and 100 µg/ml, and these solutions were stored under refrigeration.

#### **4.2.3 Cell culture**

The PC12 cells were maintained in DMEM in a humidified incubator at 37°C under 5% CO<sub>2</sub>. After incubation in 25-cm<sup>2</sup> flasks overnight, the medium was replaced with a serum-rich or serum-deprived medium containing 0–100 µg of *T. cordifolia* methanol extract (TCME). Before transferring cells to the serum-deprived medium,

they were washed twice with serum-free DMEM.

#### **4.2.4 Cell viability**

Trypan blue exclusion assay was used to evaluate cell viability. After incubation with 0-100 µg TCME in serum-rich or serum-deprived medium for 48 h, the PC12 cells were stained with trypan blue 0.25% in phosphate-buffered saline. The total cells and trypan blue-stained cells were counted with using a hemocytometer (TC10™ automated cell counter, Bio-Rad, Hercules, CA). Cell viability was expressed as a percent ratio of total viable cells (unstained) against total cells (stained and unstained), and each experiment was carried out 3 times.

#### **4.2.5 Genomic DNA isolation from PC12 cells**

After treatment with 0-100 µg of TCME in serum-rich and serum-free medium for 48 h, the genomic DNA from PC12 cells was isolated by the method of Yamanoshita et al. (2000) with some modifications. The cells were washed with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and harvested using a scraper. Then, the cells were centrifuged at 1,500 rpm for 5 min, and the supernatant was removed. Subsequently, the cells were incubated with 10 mM Tris-5 mM HCl buffer, 0.1 M EDTA, 0.5% SDS and 20 µg/ml of pancreatic RNase for 15 min at 37°C. Proteinase K was added to the suspension to achieve a concentration of 100 µg/ml, and enzyme reaction was carried out for 3 h at 50°C. The DNA thus obtained was then suspended in 89 mM Tris-HCl buffer (pH 8.1) containing 1 mM EDTA (1× TBE).

#### **4.2.6 Electrophoresis of the genomic DNA isolated from PC12 cells**

The ladder pattern of DNA was analyzed by agarose gel electrophoresis by using 5 µg of the isolated DNA on 1.5% agarose gel. The separated material was visualized with ethidium bromide stain under UV illumination.

#### **4.2.7 Quantification of DNA fragmentation in the PC12 cells by using the TUNEL method**

After the PC12 cells were cultivated for 2 days in a 10% FBS medium containing 5 µg of 6-hydroxy dopamine (6-OHDA) with and without different concentrations of TCME, the genomic DNA was isolated as described above. The DNA so obtained was suspended in 1× TBE buffer, and equal amounts of DNA were added into the wells of a 96-well plate. Two wells were used for one sample. DNA fragmentation in the cells was quantified by the method of Kurasaki et al. (2012). The plate was incubated at 4°C overnight, after which it was washed with 40 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. A 2% blocking reagent dissolved in the same Tris-HCl buffer was added to block the non-specific binding site. After 30 min, the plate was washed twice with the same buffer. The TdT reaction was carried out at 37°C for 1.5 h in a mixture composed of 30 mM Tris-HCl buffer, pH 7.4, containing 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.1 unit TdT/µl and 4 µM biotinylated-dUTP. Thereafter, the plate was washed 3 times with the same buffer and incubated with streptavidin-conjugated peroxidase (1:300 dilution) for 1 h, followed by 5 more washes with the same buffer. After these washes, 0.1% OPD

in 50 mM phosphate-citrate buffer (pH 5.0) was added to each well, and the plate was incubated for 5 to 15 min at 37°C. The enzyme reaction was halted by adding 6M HCl. The absorbance at 495 nm was measured with a Microplate Reader, model 450 (Bio-Rad, Hercules, CA, USA).

#### **4.2.8 UV-irradiation**

PC12 cells from 35-mm tissue culture dishes were cultured for 24 h in serum media containing 0, 10 ng, 100 ng, or 1 µg/ml of TCME. Then, the cells were washed twice with 2 ml of phosphate buffer saline, and the medium was changed by adding 2 ml of the same phosphate buffer solution. A xenon-lamp light source (MAX-301, Asahi Spectra, Tokyo, Japan), equipped with bandpass filters that isolate specific wavelength regions, was used for the UV irradiation step.

Before exposure to each wavelength, the dose of UV-radiation was measured at the position of the target cells by using a radiometer (IL 1400 A, International Light Technologies, Peabody, MA) connected to a silicon-photodiode detector (SEL033, International Light Technologies), and the duration of the exposure was determined by measuring the irradiation dose. The doses were determined according to the following formula: dose (mJ/cm<sup>2</sup>) = exposure time (s) × irradiance (mW/cm<sup>2</sup>). The cells in the dish were exposed to UV radiation in bandwidths of approximately 10 nm at 3 different wavelengths: 250, 270, and 290 nm. Immediately after UV irradiation, the medium was changed with 10% FBS at different concentrations of TCME and incubated for 24 h.

To determine the effect of TCME on cell toxicity and DNA damage, PC12 cells, both alone and with TCME, were irradiated at 250, 270, and 290 nm wavelengths at their corresponding median lethal doses (120, 150, and 300 mJ/cm<sup>2</sup>) before the tests for cell viability and ELISA were carried out. After the radiation step, the cells were cultured for 24 h in a medium containing serum.

#### **4.2.9 Quantification of the formation of CPD by using ELISA**

Genomic DNA was extracted from the UV-irradiated PC12 cell (described above), and then 1× TBE was added to adjust the DNA concentration to 1 µg DNA/µl. DNA was denatured at 100°C for 10 min and then chilled immediately on ice for 15 min. After denaturation, 4 µl of the DNA and 46 µL of 1× TBE were added to each well in a titer plate (2 wells for each sample), and the DNA solutions were dried completely overnight at 37°C.

The CPDs were quantified using ELISA, which was used to detect direct binding of monoclonal antibodies to antigens. After overnight incubation, the titer plate was washed 3 times with a 40 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. To block unbounded areas in the titer plate wells, 100 µl of 2% blocking reagent in the same buffer was added to each well and the solution was incubated for 30 min at 37°C. First, antibodies to the CPDs (diluted 1:1000 in the same buffer) were immobilized by incubating them for 1 h at 37°C. Next, the plate was washed 3 times with buffer. The second antibody, a biotinylated anti-mouse immunoglobulin (diluted 1:500 in the same buffer) was added, and the plate was incubated for 1

h at 37°C. Finally, the plate was washed twice with the buffer, and after addition of streptavidin-conjugated peroxidase (diluted 1:400 with the same buffer), the plate was incubated for 30 min at 37°C. After the wells were washed 5 times with the buffer, 0.1% OPD in 50 mM phosphate-citrate buffer (pH 5.0) containing 0.03% sodium perborate was added. Five to 10 min later, 6N HCl was added to halt the enzyme reaction. The absorbance at 495 nm was measured with a Microplate Reader (model 450; Bio-Rad).

#### **4.2.10 Statistical analysis**

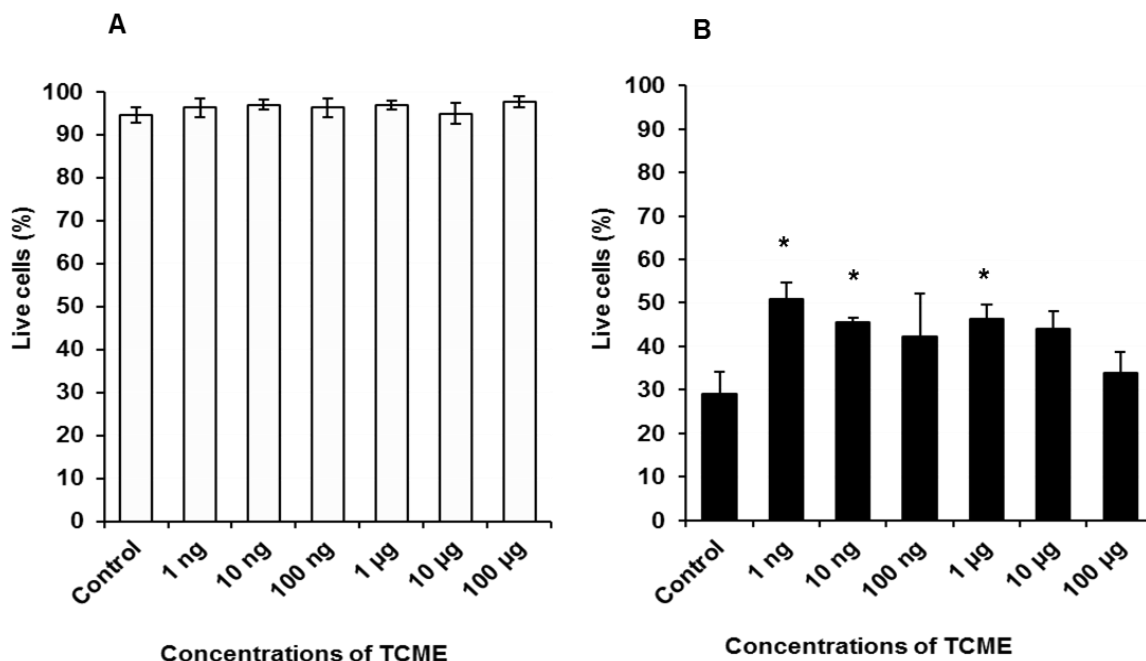
Each value is expressed as mean  $\pm$  SEM. The statistical analysis was performed with the unpaired *t*-test. A difference was considered significant when  $p < 0.05$ . Each experiment was carried out 3-4 times.

### **4.3 Results**

#### **4.3.1 Cell viability count**

To determine whether the TCME itself was toxic, cell viability was measured by staining PC12 cells with trypan blue after exposing them to concentrations from 1 ng to 100  $\mu$ g/ml TCME for 48 h. As shown in Fig. 4.1A, no significant difference was found between the control cells that were cultured in serum-rich medium without TCME and those cultured with TCME. On the other hand, the cell viability of PC12 cells in serum-deprived medium was significantly higher than that of control cells following treatment with 1 ng, 10 ng, and 1  $\mu$ g/ml of TCME (Fig.

4.1B).



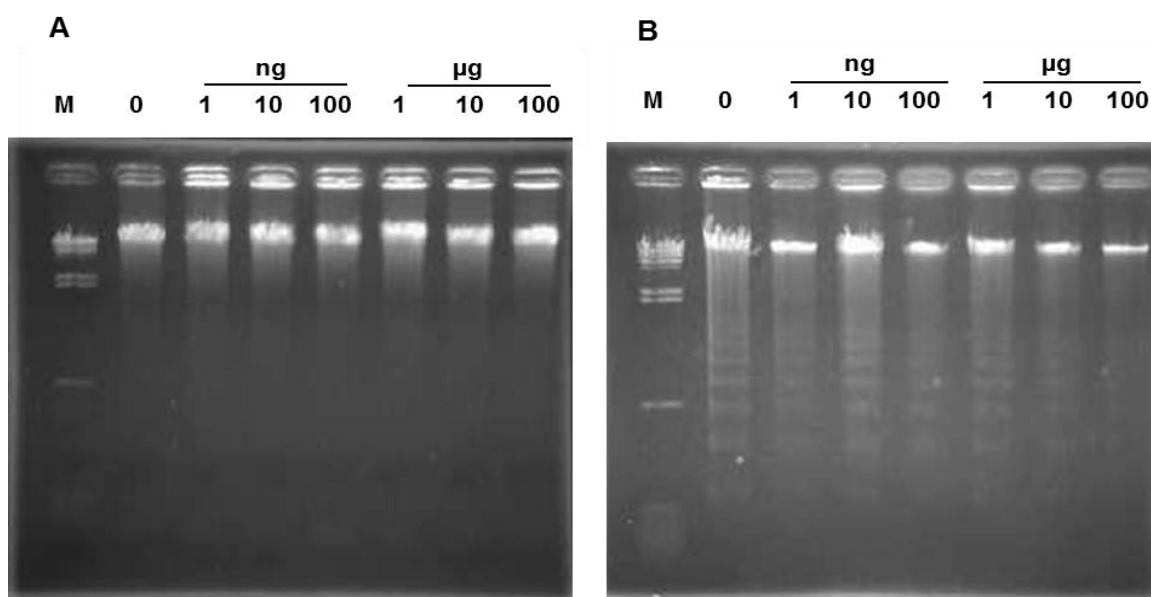
**Fig. 4.1** Viability of PC12 cells treated for 48 h with 0–100 µg/ml *T. cordifolia* methanol extract (TCME) measured by trypan blue staining. PC12 cells cultured in a (A) serum-rich medium and a (B) serum-deprived medium. Error bars indicate SEM (n = 3). Asterisks denote values that are significantly different (\* $p < 0.05$ ) from the control value.

#### **4.3.2 Agarose gel electrophoresis for the detection of DNA fragmentation**

When PC12 cells were cultured for 48 h with or without the different concentrations of TCME in serum-rich or serum-deprived medium, DNA fragmentation was evaluated to determine its effect on apoptosis. Multiple cleavages of DNA into 180–200 base pairs detected in the DNA ladder pattern following agarose gel electrophoresis provided morphological evidence for apoptosis (Woodgate et al., 1999). The ladder pattern was scarcely observed in



PC12 cells treated with 0–100  $\mu\text{g/ml}$  TCME in a serum-rich medium (Fig. 4.2A). In contrast, the DNA ladder pattern disappeared gradually in TCME-treated PC12 cells cultured in the serum-deprived medium, compared to the controls (Fig. 4.2B). TCME in low concentrations also reduced DNA fragmentation in cells cultured in a serum-rich medium containing 6-OHDA (data not shown).

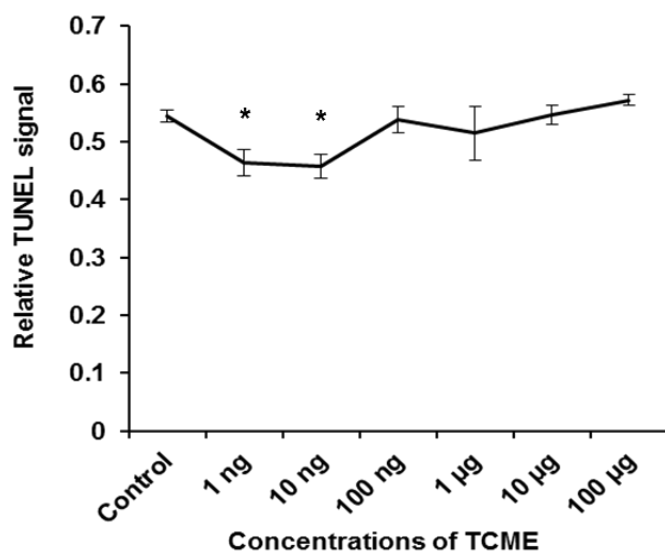


**Fig. 4.2** Agarose gel electrophoresis of DNA extracted from PC12 cells. Cells treated with 0–100  $\mu\text{g/ml}$  *T. cordifolia* methanol extract (TCME) and cultured for 2 days in a (A) serum-rich medium and a (B) serum-deprived medium. M indicates  $\lambda$ DNA digested with *Hind*III as a DNA marker.

### **4.3.3 Quantitative determination of DNA fragmentation by the TUNEL method**

For confirmation of the results of DNA electrophoresis, the TUNEL method was used to determine DNA fragmentation after using 6-OHDA to induce apoptosis in PC12 cells cultured for 48 h in an FBS-medium containing 0–100  $\mu\text{g}$  of TCME. In this method, DNA fragments generated by endonuclease activity are specifically labeled (Gollapudi and Oblinger, 1999). As shown in Fig. 4.3, the TUNEL signal

diminished significantly in TCME-treated PC12 cells, at 1 ng and 10 ng, when compared to the control cells. These results agreed well with those of gel electrophoresis of the cells cultured in a serum-deprived medium with TCME.

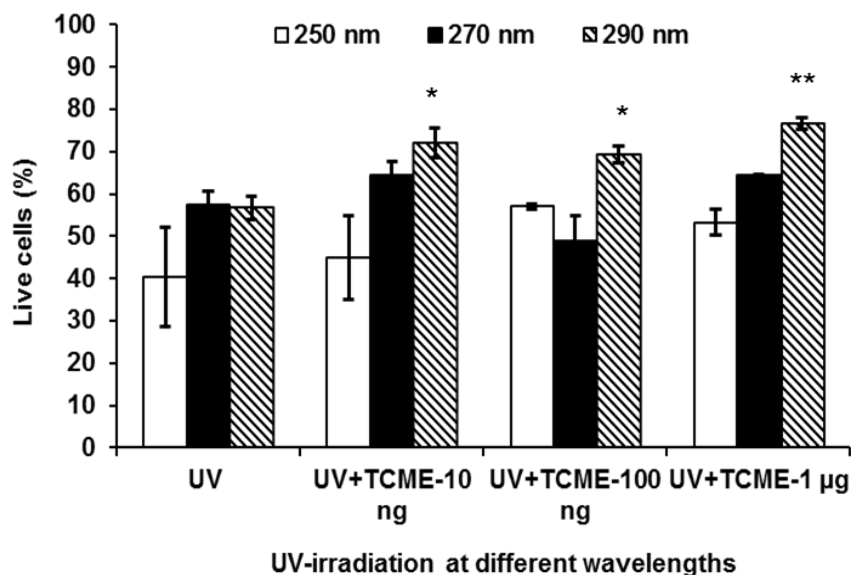


**Fig. 4.3** Fragmented DNA from PC12 cells after treatment with 0–100 µg/ml *T. cordifolia* methanol extract (TCME) measured by TUNEL assay. The PC12 cells were incubated for 48 h in a serum-rich medium containing 5 µg/ml 6-OHDA. The results from 3 separate experiments are expressed as mean ± SEM (\* $p < 0.05$ ,  $n = 3$ ).

#### **4.3.4 Viability of UV-irradiated cells**

To determine the effect of TCME on UV-induced toxicity, PC12 cells alone or treated with 10 ng, 100 ng, or 1 µg/ml TCME were irradiated at 250, 270, and 290 nm, corresponding to 120, 150, and 300 mJ/cm<sup>2</sup>, and the cell viability was evaluated. The irradiation reduced the PC12 cell viability from 40% to 60%. The effect of TCME on PC12 cell survival rate after exposure to 3 different wavelengths of radiation was examined. At every concentration tested, TCME significantly

increased the viability of the cells irradiated at 290 nm (Fig. 4.4).

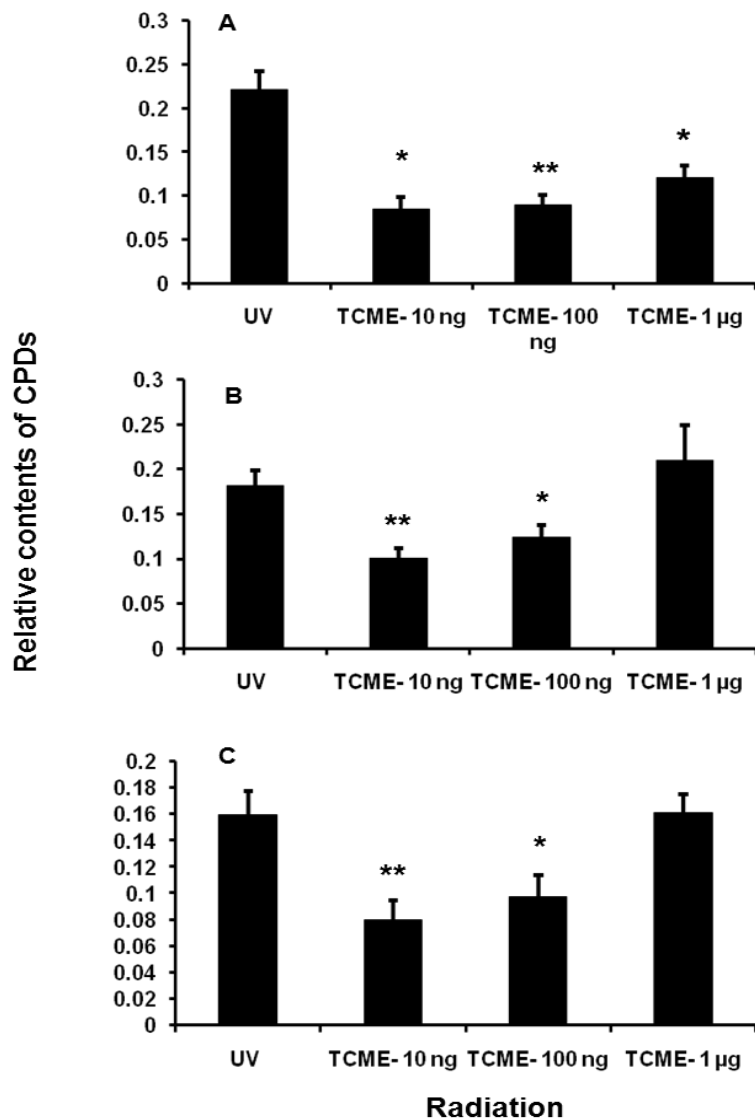


**Fig. 4.4** Effect of *T. cordifolia* methanol extract (TCME; 10 ng, 100 ng, and 1 µg/ml) on viability of PC12 cells after UV irradiation with 250, 270, and 290 nm wavelengths, corresponding to doses of 120, 150, and 300 mJ/cm<sup>2</sup>. Error bars indicate SEM (n = 3). \**p* < 0.05, \*\**p* < 0.01 indicate significant differences from the control PC12 cells irradiated without TCME.

#### 4.3.5 Quantification of CPD formation by ELISA

The formation of CPD in irradiated PC12 cells, with and without TCME treatment, was analyzed using ELISA. UV irradiation at 250, 270, and 290 nm enhanced CPD formation, compared to control cells that were not irradiated (data not shown). However, when the cells were subjected to TCME treatment before irradiation, the concentration of CPD was reduced significantly. At all 3 radiation wavelengths, and at every concentration tested, TCME treatment significantly diminished the CPD formed at 250 nm (Fig. 4.5A). On the other hand, TCME at concentrations of

10 ng and 100 ng/ml induced a significant reduction in irradiation-induced CPD formation at 270 and 290 nm (Figs. 4.5B and C).



**Fig. 4.5** Effect of *T. cordifolia* methanol extract (TCME) on the formation of CPDs 24 h after UV irradiation. After preincubation for 24 h with 0–1 µg/ml of TCME in 10% FBS containing serum medium, the cells were exposed to UV irradiation at 250, 270, and 290 nm, equivalent to exposures of 120, 150, and 300 mJ/cm<sup>2</sup>. They were incubated for 24 h with TCME. The amount of CPD formed after (A) 250 nm, (B) 270 nm, and (C) 290 nm. Each column shows mean ± SEM (n = 4). Asterisks denote results that are significantly different (\*p < 0.05, \*\*p < 0.01) from those of cells irradiated without TCME.

## **4.4 Discussion**

The present study demonstrated that in PC12 cells cultured in serum-deprived medium, TCME treatment at low concentrations (1 ng-1 µg/ml) enhanced the cell survival rate and was not itself cytotoxic to PC12 cells cultured in a serum medium (Figs. 4.1A and B). *T. cordifolia* treatment is known to ameliorate the leukopenic effects of cyclophosphamide by increasing neutrophils and white blood cells (Thatte et al., 1987). Other studies suggest that treatment with *T. cordifolia* increases red blood cells in anemic conditions (Manjrekar et al., 2000).

In this study, TCME did not induce apoptosis in PC12 cells cultured in a serum-containing medium, and it reduced the degree of DNA fragmentation induced by serum deprivation (Figs. 4.2A and B), a finding supported by the results of the TUNEL assay. The neurotoxin 6-OHDA induced apoptosis in PC12 cells, even in the presence of FBS; the dead cells were morphologically indistinguishable from those in which apoptosis was induced by serum deprivation (Blum et al., 1997). TCME reduced apoptosis induced both by serum deprivation and by 6-OHDA. As shown in Fig. 4.3, TCME treatment at low concentrations (1 ng and 10 ng/ml) significantly reduced the amount of fragmented DNA from apoptosis induced by 6-OHDA. However, higher doses of a hexane extract of *T. cordifolia* induce apoptosis in Ehrlich ascites tumor cells (Thippeswamy and Salimath, 2007). The reason for the discrepancy is not clear.

Plants that exhibit anti-inflammatory, antimicrobial, immunomodulatory, anti-stress, lipid peroxidation inhibitory, and free-radical scavenging activity may also be radioprotectors (Jagetia, 2007). Premanath and Lakshmidevi (2010) report that leaf extracts of *T. cordifolia* inhibit lipid peroxidation. Methanol extracts of *T. cordifolia* exhibit antibacterial properties against *Staphylococcus aureus*, as reported by Rose *et al.* (Rose *et al.*, 2010). On the other hand, (1,4)- $\alpha$ -D-glucan isolated from *T. cordifolia* activates the immune system by activating macrophages (Nair *et al.*, 2006). While a number of different therapeutic properties of TCME are already known, this study, for the first time, demonstrates its effects on UV-induced cytotoxicity and DNA damage in the PC12 cell system. TCME increased the cell survival rate at all the UV wavelengths tested, although significant protection against cytotoxicity was seen at 290 nm (Fig. 4.4). Cell death from UV light is caused by high amounts DNA damage, damage that is greatest at shorter wavelengths (Borges *et al.*, 2008). *T. cordifolia* has been shown to extend the life span of mice with Ehrlich ascites carcinoma exposed to 6 Gy of hemi-body gamma radiation. It reduced the mortality of Swiss albino mice from  $^{60}\text{Co}$ -gamma radiation (Rao *et al.*, 2008). Another study demonstrated that in gamma-irradiated control mice, pre-irradiation treatment with a stem extract of *T. cordifolia* reduced mortality to 76.3% compared to the 100% mortality normally seen (Goel *et al.*, 2004).

In this study, TCME treatment significantly reduced CPD formation (Fig. 4.5). A previous report proposes that wavelengths shorter than 310 nm induce DNA damage through dimer formation (Mitchell, 1988). Plant extracts rich in

antioxidants evidently protect against the effects of UVB-induced DNA damage (Huang et al., 2013). An earlier study by Russo et al. (2001) affirms that methanol extracts of *Picrorhiza kurroa*, *Celastrus paniculatus*, and *Withania somnifera* exert a protective potential against DNA cleavage induced by H<sub>2</sub>O<sub>2</sub>-UV photolysis by scavenging free radicals. *Rhodiola imbricata*, a high-altitude plant also rich in antioxidants, exhibits both in vitro and in vivo radioprotection via a superoxide ion scavenging process (Arora et al., 2005). Recently, Huang et al. (2013) found that extracts of the leaves of *Nelumbo nucifera* Gaertn, because of its antioxidant effects, protects animal models against UVB-induced phototoxicity. Polyphenols (mainly phenols and flavonoids) are the major components of plants that showed antioxidant activity (Demiray et al., 2009). Chandrashekhar et al. (2010) suggested that methanol extracts of *T. cordifolia*, found to contain copious amounts of polyphenol, protected human peripheral lymphocytes from oxidatively generated DNA damage and offered a 64% protection against Fe- and As-induced DNA fragmentation.

In conclusion, low doses of TCME significantly reduced the cytotoxicity and apoptosis induced by serum deprivation; furthermore, they were found to attenuate the DNA fragmentation enhanced by 6-OHDA. In addition, TCME exerts significant protection against UVB- and UVC-mediated DNA damage. This study demonstrates that different wavelengths of UV radiation induce cytotoxicity. DNA damage due to CPD formation was minimized by TCME treatment. These results suggest the possibility of using *T. cordifolia* plant extract as a photoprotective agent in clinical settings. However, to verify this probability, the exact molecular

mechanism underlying the protective effect of *T. cordifolia* should be investigated further.

## **References**

- J. Aguilera, M.V. de Galvez, C. Sanchez, E. Herrera-Ceballos, Changes in photoinduced cutaneous erythema with topical application of a combination of vitamins C and E before and after UV exposure, *J. Dermatol. Sci.* 66 (2012) 216-220.
- L. Aravind, V.M. Dixit, E.V. Koonin, The domains of death: evolution of the apoptosis machinery, *Trends Biochem. Sci.* 24 (1999) 47-53.
- R. Arora, R. Chawla, R. Sagar, J. Prasad, S. Singh, R. Kumar, A. Sharma, S. Singh, R.K. Sharma, Evaluation of radioprotective activities of *Rhodiola imbrica* Edgew-A high altitude plant, *Mol. Cell Biochem.* 273 (2005) 209-223.
- V.A. Badar, V.R. Thawani, P.T. Wakode, M.P. Shrivastava, K.J. Gharpure, L.L. Hingorani, R.M. Khiyani, Efficacy of *Tinospora cordifolia* in allergic rhinitis, *J. Ethnopharmacol.* 96 (2005) 445-449.
- M.S. Baliga, G.C. Jagetia, P. Venkatesh, R. Reddy, J.N. Ulloor, Radioprotective effect of abana, a polyherbal drug following total body irradiation, *Br. J. Radiol.* 77 (2004) 1027-1035.
- S. Beissert, R.D. Granstein, UV-Induced cutaneous photobiology, *Crit. Rev. Biochem. Mol. Biol.* 31 (1995) 381-404.
- D. Blum, Y. Wu, M.F. Nissou, S. Arnaud, A.L. Bebabid, J.M. Verna, p53 and Bax activation in 6-hydroxydopamine-induced apoptosis in PC12 cells, *Brain Res*



751 (1997) 139-142.

H.L. Borges, R. Linden, J.Y.J. Wang, DNA damage-induced cell death: lessons from the central nervous system, *Cell Res.* 18 (2008) 17-26.

S. Chandrashekar, S. Umesha, K.M. Chethan, S. Chandan, Inhibition of pro-oxidant induced DNA damage in isolated human peripheral lymphocytes by methanolic extract of *Guduchi* (*Tinospora cordifolia*) leaves, *Intern. J. Pharm. Sci. Res.* 1 (2010) 445-450.

S. Choi, S.K. Lee, J.E. Kim, M.H. Chung, Y.I. Park, Aloesin inhibits hyperpigmentation induced by UV radiation, *Clin. Exp. Dermatol.* 27 (2002) 513-515.

S. Demiray, M.E. Pintado, P.M.L. Castro, Evaluation of phenolic profiles and antioxidant activities of Turkish medicinal plants: *Tilia argentea*, *Crataegi folium* leaves and *Polygonum bistorta* roots, *World Acad. Sci. Eng. Tech.* 54 (2009) 312-317.

S.E. Freeman, H. Hacham, R.W. Gange, D.J. Maytum, J.C. Sutherland, Wavelength dependence of pyrimidine dimer formation in DNA of human skin irradiated *in situ* with ultraviolet light, *Proc. Natl. Acad. Sci. USA* 86 (1989) 5605-5609.

S. Fulda, K.M. Debatin, Apoptosis signaling in tumor therapy, *Ann. NY. Acad. Sci.* 1028 (2004) 150-156.

H.C. Goel, J. Prasad, S. Singh, R.K. Sagar, P.K. Agarwala, M. Bala, A.K. Sinha, R. Dogra, Radioprotective potential of an herbal extract of *Tinospora cordifolia*, *J. Radiat. Res.* 45 (2004) 61-68.

L. Gollapaudi, M.M. Oblinger, Stable transfection of PC12 cells with estrogen

- receptor (ERalpha): protective effects of estrogen on cell survival after serum deprivation, J. Neurosci. Res. 56 (1999) 99-108.
- B. Huang, L. Zhu, S. Liu, D. Li, Y. Chen, B. Ma, Y. Wang, *In vitro* and *in vivo* evaluation of inhibition of lotus (*Nelumbo nucifera* Gaertn.) leaves against ultraviolet B-induced phototoxicity, J. Photochem. Photobiol. B 121 (2013) 1-5.
- G.C. Jagetia, Radioprotective potential of plants and herbs against the effects of ionizing radiation, J. Clin. Biochem. Nutr. 40 (2007) 74-81.
- A. Kapil, S. Sharma, Immunopotentiating compounds from *Tinospora cordifolia*, J. Ethnopharmacol. 58 (1997) 89-95.
- Y.R. Karkal, L.K. Bairy, Safety of aqueous of *Tinospora cordifolia* (Tc) in healthy volunteers: A double blind randomized placebo controlled study, Iranian J. Pharmacol. Therap. 6 (2007) 59-61.
- M. Kurasaki, Y. Sun, M. Komori, M. Miyajima, T. Hosokawa, T. Saito, Measurement of DNA damage by terminal deoxynucleotidyl transferase reaction, Adv. Biol. Chem. 2 (2012) 243-247.
- P.N. Manjrekar, C.I. Jolly, S. Narayanan, Comparative studies of the immunomodulatory activity of *Tinospora cordifolia* and *Tinospora sinensis*, Fitoterapia 71 (2000) 254-257.
- Y. Matsumura, H.N. Ananthaswamy. Molecular mechanisms of photocarcinogenesis, Front. Biosci. 7 (2002) D765-D783.
- Y. Matsumura, H.N. Ananthaswamy, Toxic effects of ultraviolet radiation on the skin, Toxicol. Appl. Pharmacol. 195 (2004) 298-308.
- D.L. Mitchell, The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells, Photochem. Photobiol. 48 (1988) 51-57.

- P. More, K. Pai, Immunomodulatory effects of *Tinospora cordifolia* (guduchi) on macrophage activation, Biol. Med. 3 (2011) 134-140.
- P.K.R. Nair, S.J. Melnick, R. Ramachandran, E. Escalon, C. Ramachandran, Mechanism of macrophage activation by (1,4)- $\beta$ -D-glucan isolated from *Tinospora cordifolia*, Int. Immunopharmacol. 6 (2006) 1815-1824.
- R. Pallela, Y.N. Young, S.K. Kim, Anti-photoaging and photoprotective compounds derived from marine organisms, Mar. Drugs. 8 (2010) 1189-1202.
- R. Premanath, N. Lakshmidhevi, Studies on anti-oxidant activity of *Tinospora cordifolia* (Miers.) leaves using in vitro models, J. Am. Sci. 6 (2010) 736-743.
- M.J. Pygmalion, L. Ruiz, E. Popovic, J. Gizard, P. Portes, X. Marat, K. Lucet-Levannier, B. Muller, J.B. Galey, Skin cell protection against UVA by Sideroxyl, a new antioxidant complementary to sunscreens, Free Radic. Biol. Med. 49 (2010) 1629-1637.
- S.K. Rao, P.S. Rao, B.N. Rao, Preliminary investigation of the radiosensitizing activity of guduchi (*Tinospora cordifolia*) in tumor-bearing mice, Phytother. Res. 22 (2008) 1482-1489.
- M.F. Rose, K.M. Noorulla, M. Asma, R. Kalaichelvi, K. Vadivel, B. Thangabalan, B.N. Sinha, In vitro antibacterial activity of methanolic root extract of *Tinospora cordifolia* (Willd), Internation. J. Pharma. Res. Dev. 2 (2010) 1-5.
- T.M. Runger, U.P. Kappes, Mechanisms of mutation formation with long-wave ultraviolet light (UVA), Photodermatol. Photoimmunol. Photomed. 24 (2008) 2-10.
- A. Russo, A.A. Izzo, V. Cardile, F. Borrelli, A. Vanella, Indian medicinal plants as antiradicals and DNA cleavage protectors. Phytomedicine 8 (2001)125-132.

- S. Sengupta, A. Mukherjee, R. Goswami, S. Basu, Hypoglycemic activity of the antioxidant saponarin, characterized as  $\alpha$ -glucosidase inhibitor present in *Tinospora cordifolia*, J. of Enm. Inh. Med. Chem. 24 (2009) 684-690.
- S.M. Singh, N. Singh, P. Shrivastava, Effect of alcoholic extract of Ayurvedic herb *Tinospora cordifolia* on the proliferation and myeloid differentiation of bone marrow precursor cells in a tumor-bearing host, Fitoterapia 77 (2006) 1-11.
- Y. Sun, K. Takahashi, T. Hosokawa, T. Saito, M. Kurasaki, Diethyl phthalate enhances apoptosis induced by serum deprivation in PC12 cells, Basic Clin. Pharmacol. Toxicol. 111 (2012) 113-119.
- U.M. Thatte, S.N. Chabria, S.M. Karandikar, S.A. Dahanukar, Protective effects of Indian medicinal plants against cyclophosphamide neutropenia, J. Postgrad. Med. 33 (1987) 185-188.
- G. Thippeswamy, B.P. Salimath, Induction of caspase-3 activated DNase mediated apoptosis by hexane fraction of *Tinospora cordifolia* in EAT cells, Environ. Toxicol. Pharmacol. 23 (2007) 212-220.
- L. Tripathi, N.J. Tripathi, Role of biotechnology in medicinal plants, Trop. J. Pharmaceu. Res. 2 (2003) 243-253.
- J.J. Wesley, A.J. Christina, N. Chidambaranathan, Effect of alcoholic extract of *Tinospora cordifolia* on acute and subacute inflammation, Pharmacologyonline 3 (2008) 683-687.
- A. Woodgate, G. MacGibbon, M. Walton, M. Dragunow, The toxicity of 6-hydroxydopamine on PC12 and P19 cells, Brain Res. Mol. Brain Res. 69 (1999) 84-92.
- O. Yamanoshita, M. Kurasaki, T. Saito, K. Takahashi, H. Sasaki, T. Hosokawa, M.

***Chapter IV Effect of Tinospora cordifolia on the reduction of ultraviolet radiation-induced cytotoxicity and DNA damage in PC12 Cells***

Okabe, J. Mochida, T. Iwakuma, Diverse Effect of Tributyltin on apoptosis in PC12 cells, Biochem. Biophys. Res. Commun. 272 (2000) 557-562.

## **Chapter V**

# **Discussion about cell sensitivity and mechanism of cell toxicity of UV radiation**

### **5.1 Introduction**

Solar UV (Ultraviolet) radiation is the prime cause of skin cancer and other cutaneous pathologies in human. Solar UV radiation elicits many adverse biological effects in the skin including hyperpigmentation, erythema, photo aging, immunosuppression and cancer (Goihman-Yahr, 1996; Ananthaswamy et al., 1997; Afaq and Mukhtar, 2001). UV radiation alters cellular function via DNA damage. DNA damage mediated through cyclobutane dimer formation (CPD) (Eller et al., 1997), generation of reactive oxygen species (ROS), formation of 8-oxo-7,8-dihydro-2' deoxyguanosine (8-oxo-dG), alteration of a variety of signaling events and activation of mitochondrial apoptotic pathways (Gilchrest et al., 1996; Matsumura and Ananthaswamy, 2002). Such damage is strongly implicated in both cell death and malignant transformation (Ridley et al., 2009).

UV induced DNA damage varies and depends on different types of cell. Cell sensitivity on UV radiation differs due to distinct characteristics and sources of cells. PC12 cell, a cell line of rat pheochromocytoma, a tumor arising from chromaffin cells of the adrenal medulla. PC12 cells considered as a useful model for studying toxicity of environmental pollutants. The toxic effects of trace amount of chemical substances detects easily by using PC12 cell system (Yamanoshita et

al., 2001; Aoki et al., 2004; Kawakami et al., 2008; Sun et al., 2012). Rapid dividing capacity and distinct responses to differentiation, proliferation makes PC12 cells ideal for different biochemical and toxicological study (Dumen et al., 2008; Vaudry et al., 2002). Chinese hamster ovary (CHO), another kind of cell lines derived from the ovary of Chinese hamster. CHO cells are useful in studying genetic, toxicity, nutrition and gene expression in medical and biological research field (Jayapal et al., 2007). This cell provide an excellent model for determining various epidermal growth factor receptor (EGFR) mutation (Ahsan et al., 2009). Presence of very low chromosome number ( $2n=22$ ) for a mammal considered CHO ideal for studying radiation, cytogenetic and tissue culture (Tjio and Puck, 1958). On the other hand normal human epidermal keratinocytes (NHEK), another cell line, originate in the stratum basale, isolated from the epidermis of juvenile foreskin or adult skin from different locations like face, breasts, abdomen and thighs. NHEK is the major cell type of epidermis, making up about 90% of the cells (McGrath et al., 2004; James et al., 2005). Special characteristics like production of variety of cytokines, growth factors, interleukins and good immune responses make the cell line special for biochemical research field. NHEK used as an excellent model for detection of UV radiated DNA damage and induction of apoptosis (Ahmed et al., 1997; Santosh et al., 2011). Human umbilical vein endothelial cells (HUVEC) derived from the endothelium of veins from the umbilical cord. HUVEC is used as a laboratory model system for the study of the function and pathology of endothelial cells. HUVEC are responsible for cytokines stimulation and used for physiological investigation of macromolecule transport, blood coagulation and fibrolysis (Furie et al., 1988; Wojta, 1993). HUVEC is also

used as special model for cytotoxicity assay and rapid diagnosis of pertussis (Halperin et al., 1990).

The purpose of the present study is to assess the cell sensitivity to UV radiation. CPD formation and cytotoxicity assay for the detection of DNA damage have become standard laboratory tools. For studying cell sensitivity to UV radiation via quantification of DNA damage, 4 types of cell lines (like PC12, CHO, NHEK and HUVEC) were used. These cells were irradiated at 250, 270 and 290 nm wavelengths at 2, 5, 10, 20, 100 and 200 mJ/cm<sup>2</sup> exposure doses and after that cell viability and CPD formation were measured through trypan blue exclusion assay and enzyme linked immune sorbent assay (ELISA). The future plan and purpose of this study is to determine the reason behind the different cell sensitivity to UV radiation and identify the molecular mechanism of UV radiation mediated cell toxicity of 4 types of cells.

## **5.2 Materials and Methods**

### **5.2.1 Materials**

PC12 (rat pheochromocytoma) and Chinese hamster ovary (CHO) cell were purchased from the American Type Culture Collection (USA and Canada). Normal human keratinocytes (NHEK), Human umbilical vein endothelial (HUVEC) cells, Keratinocytes Cell Basal Medium (KBM-Gold), Endothelial Cell Basal Medium (EBM-2), Hepes buffered saline solution and Trypsin Neutralizing Solution (TNS) were collected from Lonza Walkersville, MD, USA). 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 and Polyclonal antibody against mTOR were purchased from Cosmo-Bio Co., Ltd. (Japan). Biotinylated goat anti-mouse immunoglobulin was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Proteinase K from Roche Diagnostica (Mannheim, Germany) and Trypan blue stain solution (0.5%) was purchased from Nacalai Tesque (Kyoto, Japan). All the other chemicals were of analytical reagent grade.

### **5.2.2 Cell culture**

#### **PC12 and CHO cells**

PC12 or CHO cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator with 5% CO<sub>2</sub>. The cells were pre-incubated overnight in 35-mm tissue culture dishes, after which the medium was replaced with DMEM containing 10% FBS.

#### **NHEK**

NHEK were maintained in KBM-Gold medium in a humidified incubator at 37°C with 5% CO<sub>2</sub>. In that case, at first adding KBM-Gold to NHEK and kept in incubator for 2 days. After that, discarding the medium and washing with 3 ml of hepes buffered saline solution. Then 2 ml of trypsin was added and put in the incubator for 4-7 minutes. Transferred the solution to 15 ml tube after adding and mixing

with 2 ml of TNS, then centrifuged for 2 minutes. After discarding the TNS, 5 ml of KBM-Gold were added by washing with 2 ml of same KBM-Gold medium. After mixing well, 0.5 ml of the solution containing NHEK were distributed to dishes. The final volume of the dishes was adjusted by adding 2 ml of KBM-gold medium and kept in the incubator for 24-48 hrs.

### **HUVEC cell**

HUVEC cells were maintained in EBM-2 medium in humidified incubator at the same 37°C with 5% CO<sub>2</sub>. EBM-2 medium were added to HUVEC cell and after incubation (2 days), wash with 1 ml of trypsin buffer. Same concentration (2 ml) of trypsin and TNS were then added, waited for 3-5 minutes and then observed under microscope. If all elongated cells become round, then transferred to the 15 ml tube. Centrifuged for 5 minutes at 1500 rpm and discarded the solution. After that, 5 ml of EBM-2 was added to the tube and mixed well. Finally distributed the cell to the dishes and volume was adjusted by adding extra 2 ml of EBM-2 medium and kept in the incubator for 24-48 hrs.

### **5.2.3 UV-irradiation**

Cells 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 and 290) of UV radiation at 2, 5, 10, 20, 100, and 200 mJ/cm<sup>2</sup> exposure doses.

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/cm<sup>2</sup>) = exposure time (s) × irradiance (mW/cm<sup>2</sup>).

To compare the cell sensitivity based on cytotoxicity and DNA damage, induced by UV irradiated 4 different types of cells; we quantified cell viability and the CPDs formation following UV irradiation with the 3 wavelengths after culturing for 24 h.

To detect the mechanism of cell toxicity about UV irradiation, western blot analysis will be done by using Anti mTOR antibody

#### **5.2.4 Cell viability**

The trypan blue exclusion assay was used to evaluate cell viability. After UV irradiation, the cells were incubated for 24 h and then 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 times. Cell

viability was calculated using the following formula:

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

### **5.2.5 Genomic DNA isolation and quantification of CPD formation by ELISA**

Following UV irradiation of cells, genomic DNA was isolated using the procedure described by Yamanoshita et al. (2000) with some modifications. 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 1:1000 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 antimouse 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, 6 N HCl was added 5–10 min later. The absorbance at 495 nm was measured using a Microplate Reader (model 450; Bio-Rad).

#### **5.2.6. Statistical analysis**

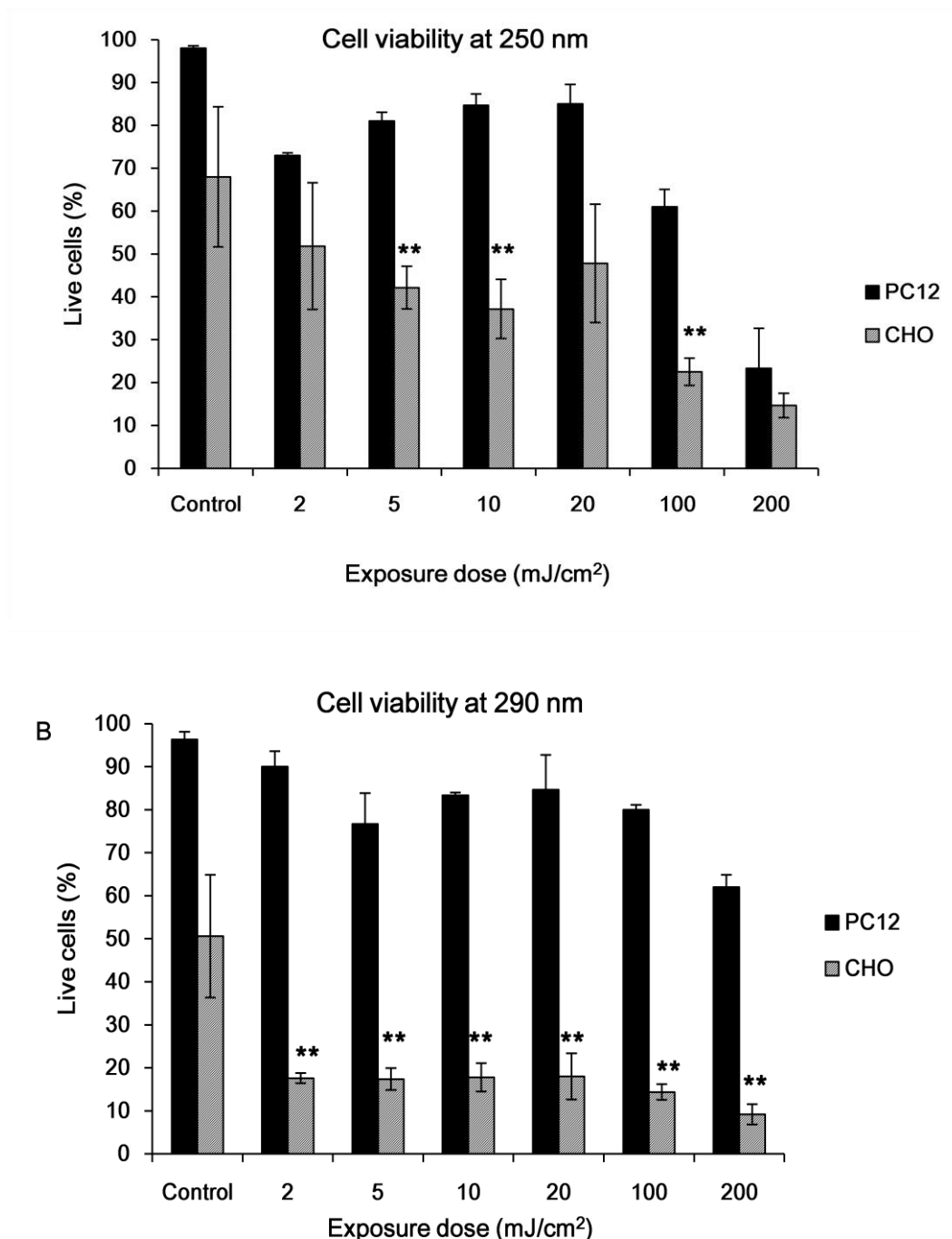
Each value is expressed as the mean $\pm$ SEM. Statistical analysis was performed using the unpaired t-test.

### **5.3 Results**

#### **5.3.1 Determination of cell viability**

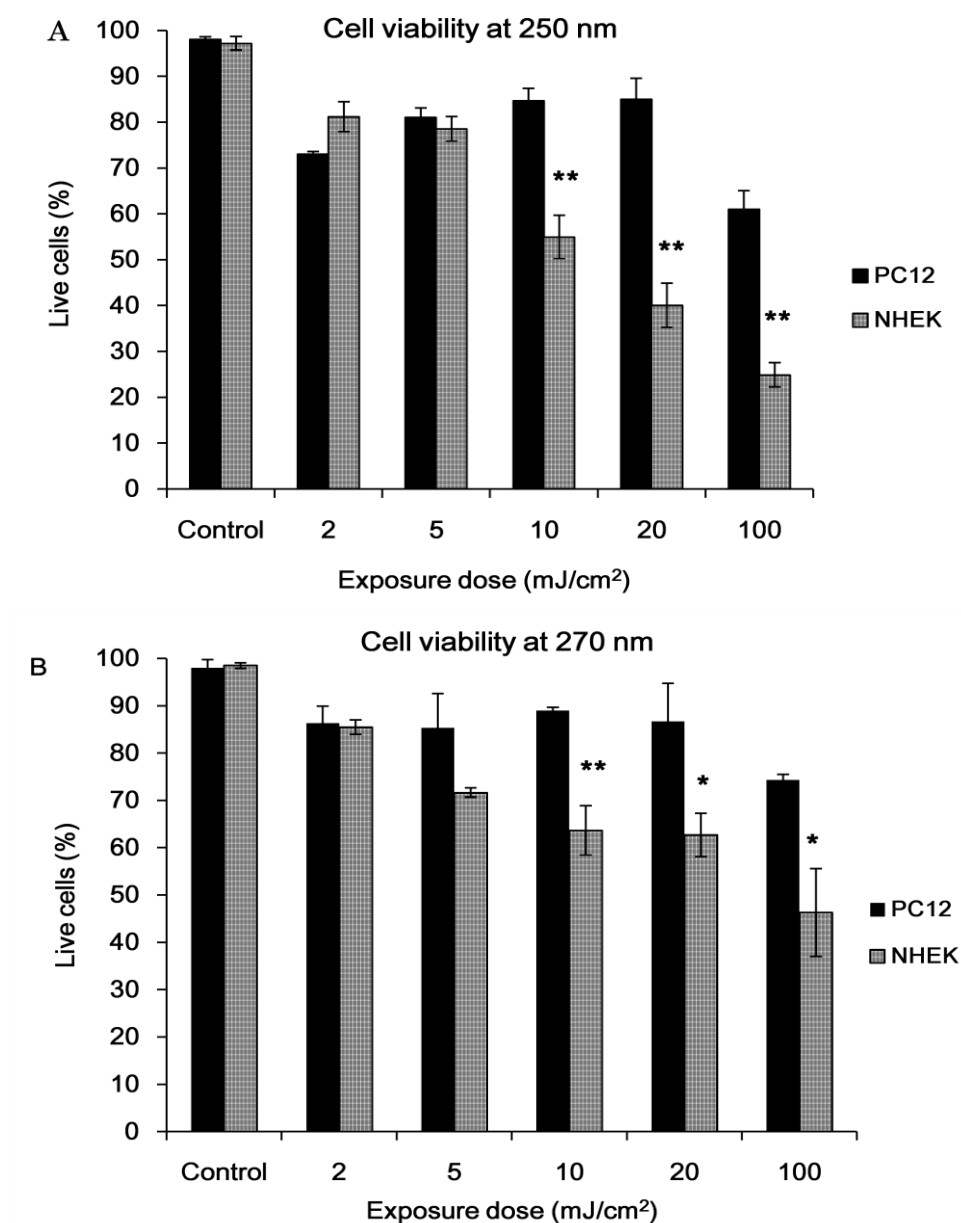
To observe the cell sensitivity to UV irradiation, cytotoxicity of PC12, CHO, NHEK and HUVEC cells were determined by counting cell viability of UV irradiated cell. For this, CHO cells were irradiated to 250 and 290 nm wavelengths at 2–200 mJ/cm<sup>2</sup> exposure doses whereas NHEK and HUVEC to 250 and 270 nm at 2–100 mJ/cm<sup>2</sup> doses and compared the result with UV irradiated PC12 cells. As shown in Figs. 5.1, 5.2 and 5.3, cell viability of all types of cell was decreased at all exposure doses compared to control cells that were not irradiated. The cell survival rate of CHO were significantly decreased compared to PC12 cell at 5, 10 and 100 mJ/cm<sup>2</sup> exposure doses when irradiated to 250 nm wavelengths (Fig. 5.1

A). On the other hand, 290 nm irradiated CHO cells exhibited significant reduction of live cells at all exposure doses in contrast to PC12 cells. (Fig. 5.1 B).



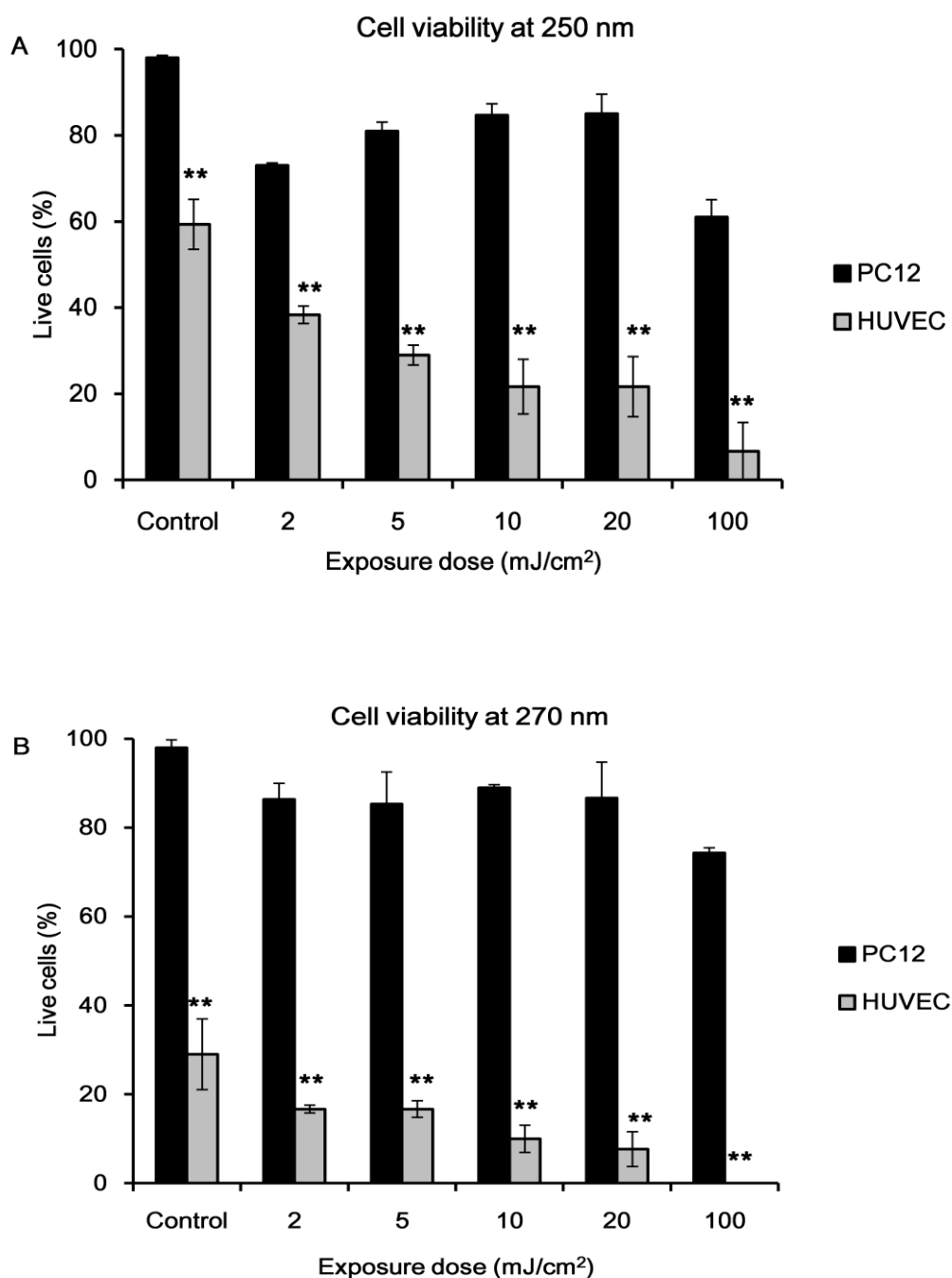
**Fig. 5.1** Comparison between the cell viability of UV irradiated PC12 and CHO cells at **A)** 250 nm **B)** 290 nm wavelengths at 2-200 mJ/cm<sup>2</sup> exposure doses. Error bars indicate SEM (n=3-6). \*\*p < 0.01 indicates significant differences from PC12 cells.

After analyzing the viability of PC12 and NHEK, it was found that cell viability of irradiated NHEK was significantly lowered following exposure to high irradiated doses (10, 20 and 100 mJ/cm<sup>2</sup>) at both 250 and 270 nm wavelengths (Figs. 5.2. A and B).



**Fig. 5.2** Comparison between the cell viability of UV irradiated PC12 and NHEK cells at **A)** 250 nm **B)** 270 nm wavelengths at 2, 5, 10, 20 and 100 mJ/cm<sup>2</sup> exposure doses. Error bars indicate SEM (n=3). \*p < 0.05, \*\*p < 0.01 indicate significant differences from PC12 cells.

As shown in Fig. 5.3 A and B, live cells were significantly reduced with both 250 and 270 nm UV irradiated HUVEC compared to PC12 cells at all exposure doses

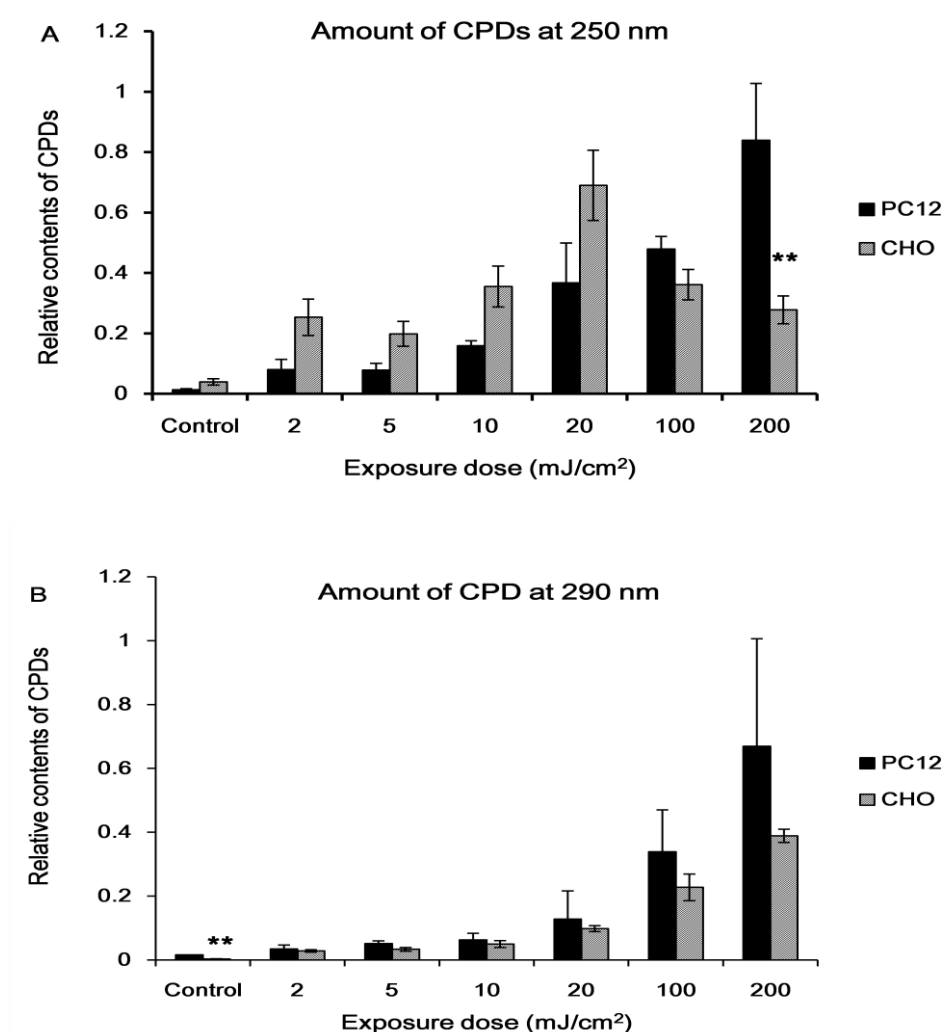


**Fig. 5.3** Comparison between the cell viability of UV irradiated PC12 and HUVEC cells at **A**) 250 nm **B**) 270 nm wavelengths at 2, 5, 10, 20 and 100 mJ/cm<sup>2</sup> exposure doses. Error bars indicate SEM (n=3). \*\*p < 0.01 indicates significant differences from PC12 cells.



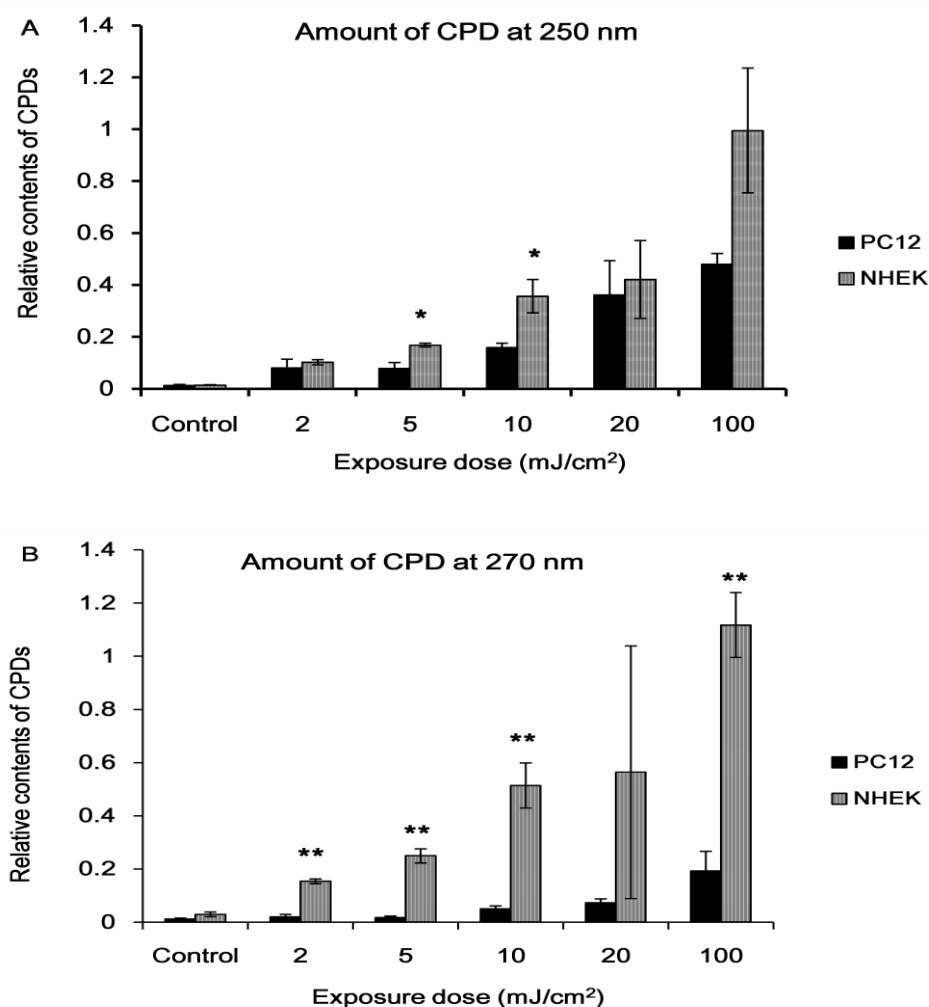
### 5.3.2 Quantification of CPD formation

CPD formation after UV irradiation considered as a marker of induction of DNA damage. To observe the cell sensitivity to UV irradiation, CPD formation at UV irradiated PC12, CHO, NHEK and HUVEC cells were measured and compared. As shown in Figs 5.4, 5.5 and 5.6, CPD formation was gradually increased in 4 types of cells compared to control at all wavelengths.



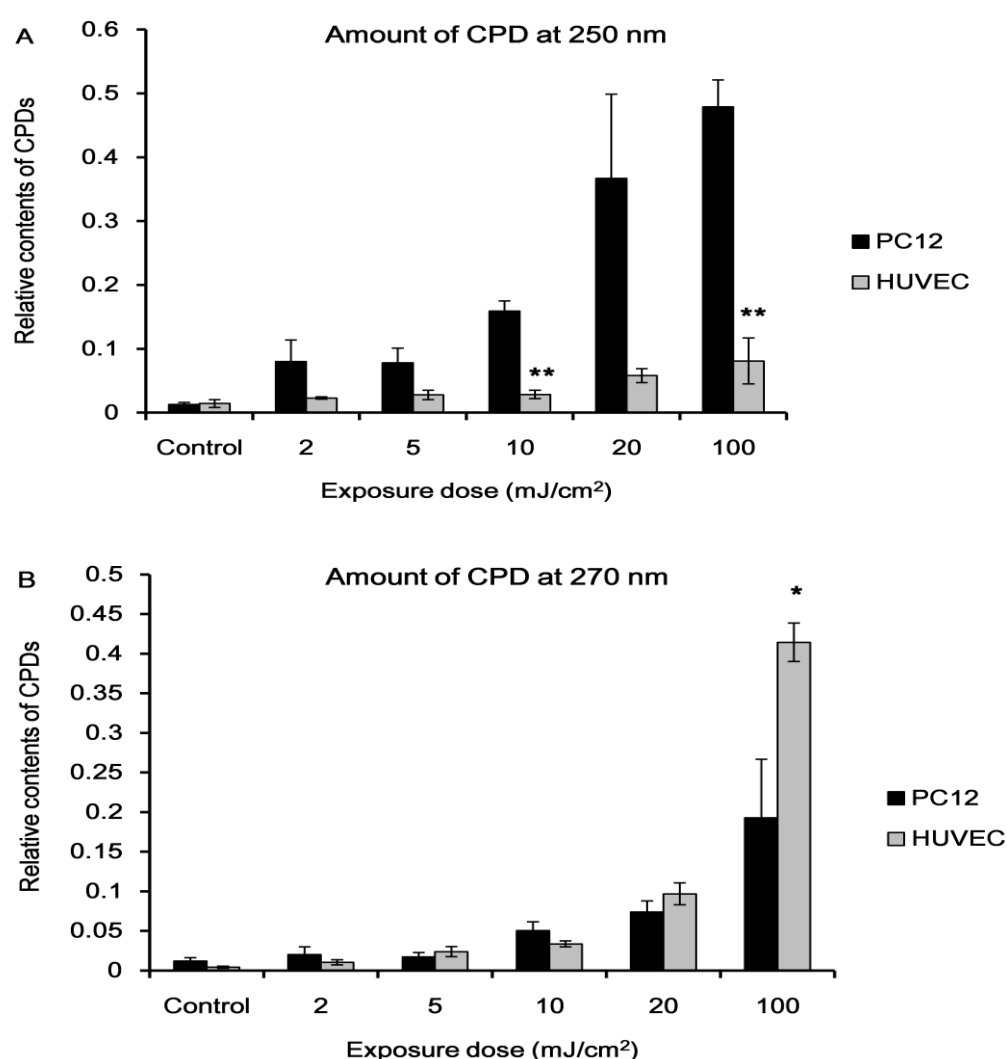
**Fig. 5.4** Comparison between the CPDs formation of UV irradiated PC12 and CHO cells at **A)** 250 nm **B)** 290 nm wavelengths at 2, 5, 10, 20, 100 and 200 mJ/cm<sup>2</sup> exposure doses. Error bars indicate SEM (n=3-6). Asterisk denote value that is significantly different (\*\*p < 0.01) from the irradiated PC12 cells value.

The relative contents of CPDs was higher in CHO compared to PC12 cells when irradiated at 250 nm while around similar upto 100 mJ/cm<sup>2</sup> exposure doses at 290 nm wavelength (Figs 5.4 A and B). In case of PC12 and NHEK cells, the magnitude of CPD formation was found to be higher in NHEK cells (Fig 5.5 A and B). 250 nm irradiated NHEK showed significant production of CPDs at low exposure doses (5 and 10 mJ/cm<sup>2</sup>), since 270 nm significantly increased the CPD formation at all exposure doses (except 20 mJ/cm<sup>2</sup>) compared to PC12 cell.



**Fig. 5.5** Comparison between the CPDs formation of UV irradiated PC12 and NHEK cells at **A)** 250 nm **B)** 270 nm wavelengths at 2, 5, 10, 20 and 100 mJ/cm<sup>2</sup> exposure doses. Each column shows Mean ± SEM (n=3). Asterisks denote values are significantly different (\*p < 0.05, \*\*p < 0.01) from the irradiated PC12 cells value.

After observing the results of CPDs formation of PC12 cells and HUVEC, it was found that the relative contents of CPDs was lowered at 250 nm UV irradiated HUVEC cells. The results were found to be significant at 20 and 100 mJ/cm<sup>2</sup> exposure doses. 270 nm UV irradiated HUVEC cells revealed the similar CPDs formation as PC12 cells upto 20 mJ/cm<sup>2</sup> exposure doses whereas 100 mJ/cm<sup>2</sup> doses abruptly increased the CPDs formation at significant amount compared to PC12 cell (Figs. 5.6 A and B).



**Fig. 5.6** Comparison between the CPDs formation of UV irradiated PC12 and HUVEC cells at **A)** 250 nm **B)** 270 nm wavelengths at 2, 5, 10, 20 and 100 mJ/cm<sup>2</sup> exposure doses. Each column shows Mean±SEM (n=3). Asterisks denote indicate significantly different (\*p < 0.05, \*\*p < 0.01) from the irradiated PC12 cells value.

## **5.4 Discussion**

Cell sensitivity to UV radiation varied with cell types. Among these four cells, NHEK found to be more sensitive to UV radiation. Santosh et al. (2011) suggested that exposure to UVB radiation induce apoptotic cell death of keratinocytes. Previous studies revealed that NHEKs has minimum LD<sub>50</sub> value and porcine lens epithelial cells (LECs), the minimum LD<sub>50</sub> values for 269-nm and 261.7-nm radiations were 1.64 and 1.77 mJ/cm<sup>2</sup>, respectively, and for human LECs the minimum LD<sub>50</sub> for the 267.5-nm radiation was 8.86 mJ/cm<sup>2</sup> (Aoki et al., 2011; Okuno et al., 2012; Okuno, 2007). In contrast to these cells, PC12 cell tolerate high LD<sub>50</sub> (ranging from 200-6000 mJ/cm<sup>2</sup>) of 250-310 nm wavelengths of UV radiation and was 50 times that for NHEKs and LECs (Masuma et al., 2013).

CPD formation also exhibited the same pattern. NHEK showed highest DNA damage via CPD formation among these 4 cells. CHO cells found to be more sensitive compared to PC12 cell in case of both cytotoxicity and DNA damage induced by UV radiation. HUVEC also exhibited strong sensitivity to cytotoxicity assay but have similar pattern of cell sensitivity to DNA damage compared to PC12 cells. The cell sensitivity sequences according to cell viability and CPD formation were NHEK >> HUVEC ≥ CHO ≥ PC12. Different cells were derived from specific location of the body and also there characteristics are dissimilar. Determination of prime causes of this discrepancy is great concern. To resolve this problem, molecular mechanism of UV irradiated cells should be elucidated. Detection of cell sensitivity and molecular mechanism behind the reasons of cell

sensitivity to UV radiation will help in photobiology sectors and have become standard laboratory tools in research field.

## **Reference**

- F. Afaq, H. Mukhtar, Inhibition of ultraviolet B-mediated activation of nuclear factor  $\kappa$ B in normal human epidermal keratinocytes by green tea Constituent (-)-epigallocatechin-3-gallate, *J. Photochem. Photobiol. B* 63 (2001) 61-69
- N. Ahmed, D.K. Feyes, R. Agarwal, H. Mukhter, Green tea constituent epigallocatechin-3 gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells, *J. Nation. Canc. Inst.* 89 (1997) 1881-1886.
- A. Ahsan, S. M. Hiniker, M.A. Davis, T.S. Lawrence, M.K. Nyati, Role of cell cycle in epidermal growth factor receptor inhibitor-mediated radiosensitization, *Canc. Res.* 69 (2009) 5108-5114.
- H.N. Ananthaswamy, S. M, Loughlin, P. Cox, R. L. Evans, S.E. Ullrich, M.L. Kripke, Sunlight and skin cancer: inhibition of p53 mutations in UV-irradiated mouse skin by sunscreens, *Nat. Med.* 3 (1997) 510-514.
- M. Aoki, M. Kurasaki, T. Saito, S. Seki, T. Hosokawa, Y. Tahakahashi, H. Fujita, T. Iwakuma, Nonylphenol enhances apoptosis induced by serum deprivation in PC12 cells, *Life Sci.* 74 (2004) 2301-2312.
- K. Aoki, T. Nakanishi-Ueda, M. Tsuji, T. Okuno, Y. Saito, H. Yasuhara, Ultraviolet action spectrum and effect of EPC-K1 on ultraviolet radiation-induced injury in cultured normal human epidermal keratinocytes, *Showa Univ. J. Med. Sci.* 23 (2011) 1-10.

- J. G. Duman, L. Chen, B. Hille, Calcium transport mechanisms of PC12 cells, *J. Gen. Physiol.* 131 (2008) 307-323.
- M.S. Eller, T. Maeda, C. Magnoni, D. Atwal, B.A. Gilchrest, Enhancement of DNA repair in human skin cells by thymidine dinucleotides: evidence for a p53-mediated mammalian SOS response, *Proc. Natl. Acad. Sci. USA* 94 (1997) 12627-12632.
- B. Furie, B.C. Furie, The molecular basis of blood coagulation, *Cell* 53 (1988) 505-518.
- B.A. Gilchrest, H.Y. Park, M.S. Eller, M. Yaar, Mechanisms of ultraviolet light-induced pigmentation, *Photochem. Photobiol.* 63 (1996) 1-10.
- M. Goihman-Yahr, Skin aging and photoaging: An outlook, *Clin. Dermatol.* 14 (1996) 153-160.
- S.A. Halperin, R. Bortolussi, A. Kasina, A.J. Wort, Use of Chinese Hamster Ovary cell cytotoxicity assay for the rapid diagnosis of Pertussis, 28 (1990) 3238.
- W. James, T. Berger, D. Elston, Andrew's diseases of the skin, *Clinical Dermatology* (10<sup>th</sup> eds.) Saunders pp 5-6.
- K.P. Jayapal, K.F. Wlaschin, M.G.S. Yap, W-S. Hu, Recombinant protein therapeutics from CHO cells- 20 years and counting, *Chem. Eng. prog.* 103 (2007) 40-47.
- M. Kawakami, R. Inagawa, T. Hosokawa, T. Saito, M. Kurasaki, Mechanism of apoptosis induced by copper in PC12 cells, *Food Chem. Toxicol.* 46 (2008) 2157-2164.
- R. Masuma, S. Kashima, M. Kurasaki, T. Okuno, Effects of UV wavelengths on cell damages caused by UV irradiation in PC12 cells, *J. Photochem. Photobiol. B:*

- Biol. 125 (2013) 202-208.
- Y. Matsumura, H.N. Ananthaswamy. Molecular mechanisms of photocarcinogenesis, Front. Biosci. 7 (2002) D765-D783.
- J.A. McGrath, R.A.J. Eady, F.M Pop, Anatomy and organization of human skin, In T. Burns, S. Breathnach, N. Cox, C. Griffiths Rook's Textbook of dermatology (7<sup>th</sup> ed.), Blackwell publishing p 4190.
- T. Okuno, Ultraviolet action spectrum for cell killing in a human lens epithelial cell line, Ind. Health 45 (2007) 137-142.
- T. Okuno, T. Nakanishi-Ueda, T. Ueda, H. Yasuhara, R. Koide, Ultraviolet action spectrum for cell killing of primary porcine lens epithelial cells, J. Occ. Health 54 (2012) 181-186.
- A.J. Ridley, J.R. Whiteside, T.J. McMillan, S.L. Allinson, Cellular and sub-cellular responses to UVA in relation to carcinogenesis, Int. J. Radiant. Biol. 85 (2009) 177-195.
- K.K. Santosh, S.K. Mantena, S.M. Meeran, Silymarin protects epidermal keratinocytes from ultraviolet radiation induced apoptosis and DNA damage by Nucleotide Excision Repair mechanism, Plos-one 6 (2011) e21410.
- Y. Sun, K. Takahashi, T. Hosokawa, T. Saito, M. Kurasaki, Diethyl phthalate enhances apoptosis induced by serum deprivation in PC12 cells, Basic Clin. Pharmacol. Toxicol. 111 (2012) 113-119.
- J.H. Tjio, T.T. Puck, Genetics of somatic mammalian cells. II. chromosomal constitution of cells in tissue culture, J. Exp. Med. 108 (1958) 259-271.
- D. Vaudry, P. J. S. Stork, .P. Lazarovici, L.E. Eiden. Signaling pathways for PC12 cell differentiation: making the right connection, Science 296 (2002)

1648-1649.

- J. Wojta, M. Gallicchio, H. Zoellner, F.L. Filonzi, J.A. Hamilton, K. McGrath, Interleukin-4 stimulates expression of urokinase-type-plasminogen activator in cultured human foreskin microvascular endothelial cells, *Blood* 81 (1993) 3283-3292.
- O. Yamanoshita, M. Kurasaki, T. Saito, K. Takahashi, H. Sasaki, T. Hosokawa, M. Okabe, J. Mochida, T. Iwakuma, Diverse effect of tributyltin on apoptosis in PC12 cells, *Biochem. Biophys. Res. Commun.* 272 (2000) 557-562
- O. Yamanoshita, T. Saito, K. Takahashi, T. Hosokawa, M. Okabe, K. Ito, M. Kurasaki, 2,4,5-Trichlorophenoxyacetic acid inhibits apoptosis in PC12 cells, *Life Sci.* 69 (2001) 403-408.



## **Chapter VI**

### **General Discussion and Conclusion**

This study was performed to determine the toxic effects of UV radiation in PC12 cells. Special emphasis was given on cytotoxicity and DNA damage induced by cyclobutane dimer formation through different wavelengths of UV radiation. Ongoing destruction of ozone layer, exposure to artificial sources of UVC and few researches on UVC radiation, made concern to perform research on UVC radiation. Determination of toxicity actuated by different wavelengths of UVB and UVC may provide information for the betterment of photobiology, oncology, dermatology and cosmetology field.

In chapter III, I quantified cell survival rate and CPD formation at PC12 cell exposed to different wavelengths of UV radiation at several irradiation doses and tried to delineate the relationship within cell viability, DNA damage and different UV wavelengths with respect to irradiation dose and culture time. In this study, cell viability increased with increasing wavelength and 250 nm exhibited the highest cytotoxicity among 4- wavelengths. In this study, it was observed that each wavelength has different LD<sub>50</sub> and 310 nm has 50, 40 and 20 times higher LD<sub>50</sub> than that of 250, 270 and 290 nm wavelengths. The LD<sub>50</sub> of UV irradiated PC12 cells depicted that this cell line has higher UV resistant capacity than other cell lines like NHEKs and LEC. DNA damage after UV-irradiation was measured through CPD formation. The proportion of CPD formation was the highest at 250

nm and the lowest at 310 nm wavelengths. This study confirmed that UV radiation mediated DNA damage was wavelength dependent and among 4-wavelengths of UVB and UVC radiation, the most effective, toxic one was 250 nm. To find out the DNA repair ability of 4 wavelengths, cells were irradiated at LD<sub>50</sub> doses of their corresponding wavelengths. 290 nm wavelengths at its LD<sub>50</sub> dose showed maximum DNA repair through recovery of CPD formation. On the other hand, cells irradiated at 310 nm did not show any repair ability at their LD<sub>50</sub> dose. In conclusion, the cytotoxicity and DNA damage induction varied with wavelengths and exposure doses. Among 4 wavelengths, 250 nm showed highest toxicity in all experiment related to damage induction. Though 310 nm (longer wavelength) was not lethal for cell but longer exposure causes severe damage to cell.

In chapter IV, the safety of *Tinospora cordifolia* and its potential to protect against UV-radiation induced cytotoxicity and DNA damage in PC12 cells were investigated. *Tinospora cordifolia* methanolic extract increased cell viability in serum deprived medium at lower doses. The extract did not induce apoptosis observed by agarose gel electrophoresis and lessen the DNA ladder pattern induced by serum deprivation or 6-OHDA. These results also supported by TUNEL assay where fragmented DNA amount obtained from PC12 cells (treated with 1 and 10 ng of *Tinospora cordifolia*) were significantly lowered compared to control cell. All of these results represent *Tinospora cordifolia* as a safe medicinal plant. Protective potentials of *Tinospora cordifolia* against UV induced cytotoxicity and DNA damage manifested a better, positive results. *Tinospora cordifolia* significantly reduced the CPD formation at all wavelengths (250, 270 and 290 nm).

This study suggested that *Tinospora cordifolia* has aptitude to alleviate the DNA damage that is mediated through toxic, lowered wavelengths of UV radiation and open a possibility of using *Tinospora cordifolia* as photo-protective agent in medical sectors.

In chapter V, sensitivity to UV radiation of PC12, CHO, NHEK and HUVEC cells were determined and observed that among these 4 types of cells, NHEK is more sensitive to UV radiation. Cytotoxicity via cell death occurred in all 4- types of UV irradiated cell but PC12 cell reveal strongest one. According to CPD formation results, the trends of toxicity are similar with the result of cytotoxicity where PC12 cells formed lower amount of CPD compared to UV irradiated other 3 type of cells. Depending on the results of cytotoxicity and CPD formation, NHEK found to be worst and most UV sensitive cell (Figs. 5.2 and 5.4). The discrepancy of cell sensitivity to UV radiation is still unclear. Identification of exact reasons of different cell sensitivity will provide new era in photobiology field and further helps in the research of oncology, biology and medical field.

## **Acknowledgements**

First of all, I like to express my heartiest, sincerest gratitude and profound thanks to my supervisor Dr. Masaaki KURASAKI for his patient guidance, overall supervision, valuable suggestions, throughout the research period. His friendly behavior, positive attitude, editing skills makes my research and dissertation writing easier. His continuous encouragement and confidence inspired me at every steps of my research.

I am thankful to Mrs. Miyako KOMORI for her technical support and assistance, which made my laboratory work easier.

I am grateful to Dr Tsutomu OKUNO for providing instrument, his valuable advice and guidance on radiation research.

I must express my gratitude to the faculty members of Environmental Adaptation Science. Prof. Shunitz TANAKA, Prof. Bunshi FUGETSU, Prof. Tatsufumi OKINO, Prof Tadashi NIIOKA and Prof. Kazuhiro TOYODA help me by expressing their valuable suggestion and truthful criticisms in my study period.

I like to thanks Emeritus Prof. Yoshinori KUBOKI for his valuable suggestion.

I acknowledge Japan Student Services Organization (JASSO) and Global COE

(Centers of Excellence) program of JSPS for providing scholarship and funding during my study period. I also like to express my heartiest thanks to Hokkaido University to give me the chance for higher study.

I am also thankful to my Lab mates for their cheerful and helpful attitude. I never feel alone due to their friendly behavior. I am grateful to Department of Pharmacy, Jahangirnagar University for granting my study leave to pursue higher study in Hokkaido University.

I am extremely grateful to my parent, parents-in-law, brothers and sisters for their continuous moral support. I must thank to my husband Dr. A.K.M. Rashidul Alam and beloved daughter R.R. Rashed for their unconditional love, support and encouragements. Without their support, it was impossible to finish my Ph.D. program.