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Kinetics of Sulforaphane Supplementation in an Immortalizedhuman Keratinocyte (HaCaT) Cell Line Exposed to UVB Irradiation

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ABSTRACT

Introduction: *Exposure of skin to UVB irradiation leads to adverse effects on health including skin aging, cancer, etc. Sulforaphane is an antioxidant compound derived naturally from cruciferous vegetables and also known for its chemo-preventive properties. This research project aims to investigate the effect of sulforaphane supplementation in human keratinocyte (HaCaT) cells exposed to UVB irradiation.*

Methods and Results: *Briefly, exposure to UVB has a negative impact in HaCaT cell viability in a manner where the higher the intensity of the exposure the greater the reduction in cell viability. In addition, it was observed that the greatest degree of sulforaphane-induced protection was when the cells were pre-treated (for 24 hrs) and 48 hrs after exposure to the highest dose of UVB irradiation (200mJ/cm^2). In fact, at this time point (48 hrs), sulforaphane was found to cause an increased rate of cell proliferation observed at every concentration tested: 0.5µM (122.0%), 1.0µM (130.0%), 2.5µM (136.0%), 5.0µM (141.0%) and 10.0µM (111.0%).*

Conclusion: *Our data suggest that sulforaphane significantly protected HaCaT cells from exposure to UVB. In addition, we have also demonstrated that when added post-exposure, sulforaphane was less effective in providing protection against UVB irradiation, in HaCaT cells, and was even cytotoxic at concentrations of 10.0µM and higher.*

Keywords

Antioxidant, Cancer Prevention, Isothiocyanates, Sulfurophane, Vegetables, Fruits.

Introduction

Cruciferous vegetables such as cauliflower, cress, cabbage, chines cabbage and broccoli contain numerous sulfur compounds known as glucosinalates [1] in addition to fibre, float, chlorophyll and carotenoids [2]. Moreover, glucosinolates are water soluble compounds with up to 50% of them being potentially lost after 10 min of boiling or steaming [3]. Sulforaphane is an isothiocyanate with rich anti-oxidant properties obtained from chewed or crushed broccoli [3,4]. Besides its anti-inflammatory and anti-oxidant effects [5], it has other properties as well including its chemo-preventive action [6,7]. First identified in 1992 [8]. There is a strong correlation between consumption of a diet rich in isothiocyanates and a decreased rate of skin ageing and cancer [9,10]. In particular, many studies have documented the effects of sulforaphane by using in vitro cell culture models [11,12].

Ultraviolet (UV) irradiation is divided into three categories: UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm) (1). Its main source is from sunlight or from other artificial sources (e.g. sunbeds) and can be the cause of burning of the retina of the eye, skin disease, etc. UV irradiation is the most detrimental component of solar radiation [13] and thus can be considered an environmental carcinogenic agent [14]. To this end, people whose skin burns easily (with exposure to sunlight) and do not tan, run

the risk of developing skin cancer [15]. In addition, UV irradiation decreases the production of collagen and inhibits the growth factor hormone [16]. UVC as well as the majority of UVB radiation, are absorbed by the ozone, however, UVB can be absorbed by DNA which in turn can lead to cell death [14,17]. Artificial UVB and UVA irradiation are used in the context of treating skin diseases, such as vitiligo and psoriasis. However, although such treatment is considered beneficial it can have adverse health effects [18]. In addition, studies have shown that exposure to UV leads to skin aging and the development of skin cancer [17-19]. Overall, UV irradiation is a known shareholder to skin aging and skin cancer with UVB having a greater effect on DNA and skin damage [20,21].

Human skin is the largest organ of the body and consists of three layers: epidermis, dermis and subcutaneous tissue. The epidermis is the layer that contains keratinocytes and melanocytes whereas the subcutaneous tissue contains mainly fat cells [16]. The adverse effects of long-term exposure to sunlight on skin are evident when compared to unexposed skin. To this end, both UVA and UVB irradiation can contribute to skin aging [22]. However, the sunlight is not the only contributor to the skin aging process but also other environmental (e.g. excessive consumption of alcohol, lack of nutrition and smoking) [23] and internal factors (genetic involvement in various metabolic processes, hormonal changes, etc.) [16] Can contribute as well [24,25]. Overall, exposure to the sunlight leads to decreased collagen production, skin wrinkles, changes in skin pigmentation, etc. [8,16,26]

This study aims to investigate the effect of sulforaphane in an immortalized human keratinocyte (HaCaT) cell line in an attempt to identify optimum supplementation conditions against UVB exposure. Finally, the choice of cell line used in our study was due to the high capacity of these cells to proliferate, in vitro, while still maintaining a "physiological" keratinocyte phenotype as indicated in the bibliography [27,28].

Materials and Methods

Chemicals and Equipment

R,S-Sulforaphane was obtained from Abcam, USA. High Glucose-Dulbecco's Modified Eagle's Medium (DMEM), Trypsin, Dulbecco's Phosphate Buffered Saline (PBS), Foetal Bovine Serum (FBS), L-Glutamine and Penicillin/Streptomycin were obtained from Labtech, UK. Dimethyl Sulfoxide (DMSO) was supplied by Sigma, USA. Trypan Blue solution was purchased from Hyclone, USA. Cell culture flasks (75cm^2) and 96 well plates were supplied by Corning, USA. Cell culture incubators (370C, 5% CO_2) and a class II biosafety hood were obtained from Triplered, UK. A UVB irradiation oven was purchased from Crosslinker UVP, Upland, USA. A multi-mode ELISA plate reader (Spectramax M5) was purchased from Molecular Devices, UK.

Cell Culture and Treatments

Human keratinocyte (HaCaT) cells were a kind gift from Dr. Sharon Broby from the Dermal Toxicology and Effects Group; Centre for Radiation, Chemical and Environmental Hazards; Public Health England, UK. Briefly, HaCaT cells were cultured at (37ºC, 5% CO_2, 95% O_2) in 75cm^2 flasks using DMEM medium containing 10% FBS, 2mM L-Glutamine and 1% Penicillin/Streptomycin (100U/ml Penicillin and 100µg/ml Streptomycin). Cells were trypsinized, suspended in 100µl of medium per well and kept in the incubator (37ºC, 5% CO_2, 95% O_2) overnight in order to attach. Finally, both DMSO and sulforaphane were diluted with DMEM medium to obtain the desired concentrations used throughout the study.

UVB Irradiation Protocol

After cells attached, they were washed once with DMEM (without FBS). Then, fresh DMEM (without FBS) medium was added and cells were subjected to UVB irradiation at 200, 100, 50 and 25mJ/cm^2. After exposure, the medium was replaced with DMEM (with FBS) and cells were transferred to 96 well plates (37°C, 5% CO_2, 95% O_2) for 24 hrs.

Measurement of Cell Viability

Ten (10) µl of resazurin reagent was added into each well of the 96-well plates and then the plates were kept at 37ºC for 4 hrs. The supernatant from each sample was transferred to an opaque 96-well plate and fluorescence readings were observed, at 560Ex/590Em nm, with the use of a multi-mode ELISA plate reader (Spectramax M5).

Exposure Protocols

Protocol 1: Time course and dose response kinetics of UVB irradiation

Ten (10) plates were used each of which was set up as follows: 5 wells were filled with medium only whereas 10 were filled with 1×10^4 cells per well and then kept in the incubator overnight. Next day, 8 of the plates were irradiated with UVB (i.e. 2 plates each at 200, 100, 50 and 25mJ/cm^2) with the remaining 2 plates serving as control (non-irradiated). Ten (10) µl of resazurin reagent was added, in each of the wells, immediately after (0h) or 24 hrs after UVB exposure and levels of cell viability were measured. A total of two independent experiments were performed (Figure 1).

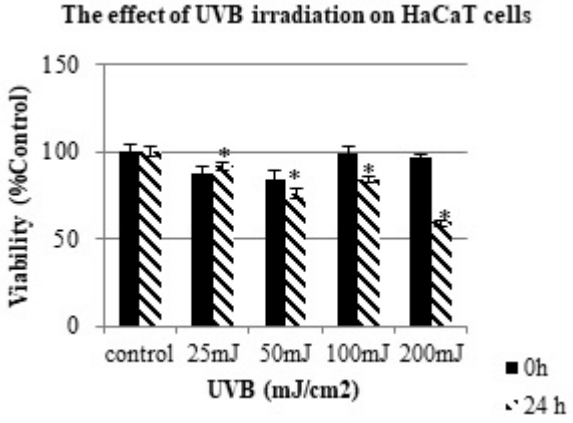

Figure 1: Experimental conditions are described under "Exposure Protocol 1 in Materials & Methods". Data is representative of two independent experiments. An asterisk (*) indicates statistical significance when compared to their respective controls ($p<0.05$).

Protocol 2: Treatment with sulforaphane post irradiation to UVB)

Two (2) plates were used each of which was set up as follows: 5 wells were filled with media only whereas 50 were filled with 1×10^4 cells/well and then kept in the incubator overnight. Next day, one of the plates was irradiated with UVB (200mJ/cm^2) whereas the other one served as control (non-irradiated). After UVB exposure, 10 wells (of the total of 50) had DMSO only added to the medium (0.1%) whereas the remaining 40 wells had sulforaphane added as follows: 10 wells each with 1.0µM, 2.5µM, 5.0µM, and 10.0µM respectively and then placed in the incubator for a further 24 hrs. Next day, the plates were removed and 10µl of resazurin reagent was added, in each of the wells, and levels of cell viability were measured. A total of two independent experiments were performed (Table 1).

The effect of sulforaphane on HaCaT cells as a post-exposure treatment to UVB irradiation.		
Sulforaphane (µM)	Viability (% Control)	
	Control	UVB
0.0	100	100
DMSO (0.01%)	85.3	94.9
1.0	103.9	104.1
2.5	84.2	91.6
5.0	85.1	73.2*
10.0	76.9*	59.1*

Table 1: Experimental conditions are described under "Exposure Protocol 2 in Materials & Methods". Data is representative of two independent experiments. An asterisk (*) indicates statistical significance when compared to their respective controls ($p<0.05$).

Protocol 3: Treatment with sulforaphane prior to UVB irradiation and while cells were seeded)

Two (2) plates were used each of which was set up as follows: 5 wells were filled with media only whereas 10 and 50 were filled with 1×10^4 cells/well and DMSO (0.1%) only or various sulforaphane concentrations (10 wells each with 1.0µM, 2.5µM, 5.0µM, and 10.0µM) respectively. Then all plates were kept in the incubator overnight. Next day, one of the plates was irradiated with UVB (200mJ/cm^2) whereas the other one served as control (non-irradiated) and 24 hrs after, the plates were removed and 10µl of resazurin reagent was added, in each of the wells, and levels of cell viability were measured. A total of two independent experiments were performed (Table 2).

The effect of sulforaphane on HaCaT cells prior to UVB irradiation and while cells were seeded		
Sulforaphane (µM)	Viability (% Control)	
	Control	UVB
0.0	100	100
DMSO (0.01%)	87.0	100.7
0.5	112.8	101.9
1.0	118.1	91.8
2.5	95.5	83.4
5.0	100.6	94.3
10.0	78.1*	67.6*

Table 2: Experimental conditions are described under "Exposure Protocol 3 in Materials & Methods". Data is representative from two independent experiments. An asterisk (*) indicates statistical significance when compared to their respective controls ($p<0.05$).

Protocol 4: Treatment with sulforaphane prior to UVB irradiation and 24 hrs after cells were seeded)

Eight (8) plates were used each of which was set up as follows: 5 wells were filled with media only and 30 were filled with 0.5×10^4 cells/well. Next day, of the 30 wells, 5 were added with DMSO (0.1%) whereas the other 25 were added with various sulforaphane concentrations (5 wells each with 0.5µM, 1.0µM, 2.5µM, 5.0µM, 10.0µM) respectively. Then all plates were kept in the incubator overnight. Next day, 4 of the plates were irradiated with UVB (200mJ/cm^2) whereas the other 4 served as control (non-irradiated). At 2, 4, 24 and 48 hrs after UVB exposure, two plates were removed from the incubator (i.e. one irradiated and one control) and 10µl of resazurin reagent was added, in each of the wells, in order to measure the levels of cell viability. A total of two independent experiments were performed (Figure 2A-D).

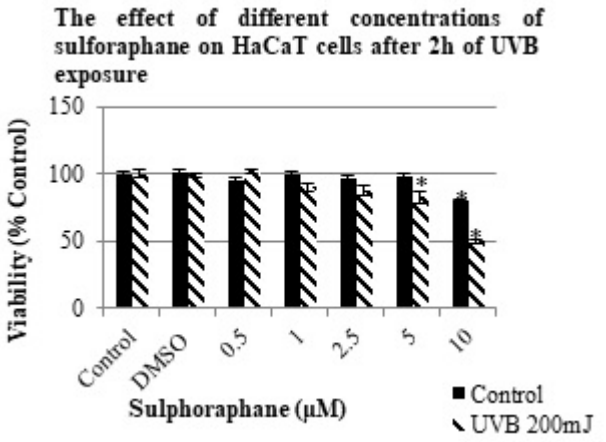

Figure 2A: Experimental conditions are described under "Exposure Protocol 4 in Materials & Methods". Data is representative from two independent experiments. An asterisk (*) indicates statistical significance when compared to their respective controls ($p<0.05$).

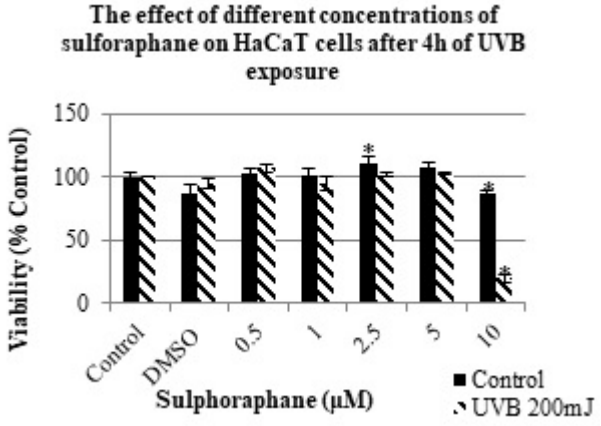

Figure 2B: Experimental conditions are described under "Exposure Protocol 4 in Materials & Methods". Data is representative from two independent experiments. An asterisk (*) indicates statistical significance

when compared to their respective controls ($p<0.05$)

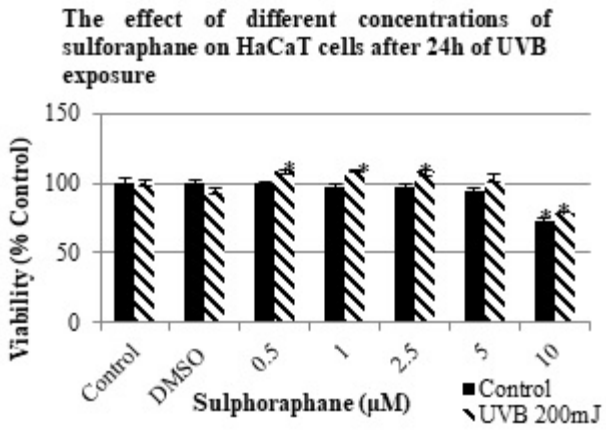

Figure 2C: Experimental conditions are described under "Exposure Protocol 4 in Materials & Methods". Data is representative from two independent experiments. An asterisk (*) indicates statistical significance when compared to their respective controls ($p<0.05$).

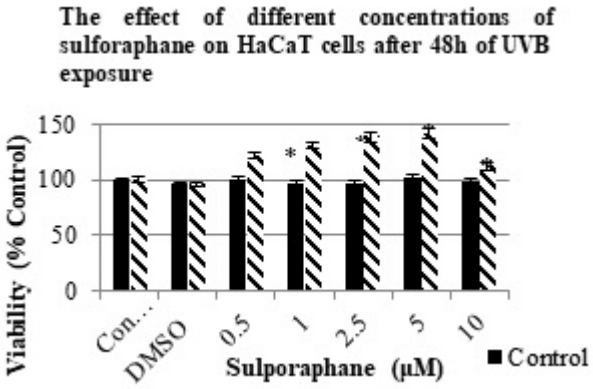

Figure 2D: Experimental conditions are described under "Exposure Protocol 4 in Materials & Methods". Data is representative from two independent experiments. An asterisk (*) indicates statistical significance when compared to their respective controls ($p<0.05$).

Statistical analysis

All measurements were performed in quintuplicate (n=5) and all values are expressed as mean ± standard error. Statistical analysis was performed by means of a Student t Test. A value of $p<0.05$ was considered statistically significant.

Results

First, we investigated the optimum experimental conditions by which different doses of UVB irradiation had an effect on HaCaT cells as determined by the resazurin-based cell viability assay. In particular, Figure 1 shows that cell viability levels of HaCaT cells decreased significantly when exposed to various doses of UVB after 24 hrs of exposure but not when assesses immediately after irradiation (0h). In fact, UVB exposure at 200mJ/cm^2 reduced cell viability to 58.8%, 100mJ/cm^2 to 84.0%, 50mJ/cm^2 to 75.8% and 25mJ/cm^2 to 91.9%. Consequently, we decided to utilize 200mJ/cm^2 of UVB exposure, after 24 hrs, as our in vitro exposure protocol in all of our experiments.

Next, we investigated the effects of the addition of various concentrations of sulforaphane to HaCaT cells post-exposure to UVB. Previous research in our lab has found that sulforaphane concentrations greater than 10.0μM are toxic to HaCaT cells and thus we did not use concentrations greater than that. However, a toxicity effect was associated even with a sulforaphane concentration of 10.0μM for both control (76.9%) and UVB-exposed (59.1%) cells as can be seen in Table 1. Similarly, for control and UVB groups, cell viability was highest (103.9% in control and 104.1% in UVB exposed cells) at the lowest concentration of sulforaphane (1.0μM) indicating no cytotoxicity. In addition, a reduction in cell viability was also observed with sulforaphane concentrations at 2.5μM and 5.0μM in both experimental groups (84.2% and 85.1% in control group vs 91.6% and 73.2% in UVB group). For the highest concentrations (5.0μM and 10.0μM) the cell viability in the control group was greater than in the UVB group. Finally, the addition of DMSO (0.1%) alone resulted in some cytotoxicity in the control group (85.3%) as opposed to the UVB one (94.9%). Overall, the lowest concentration of sulforaphane (1.0μM) provided for some degree of protection to HaCaT cells, although minimal, when added post UVB exposure.

Then, we investigated the supplementation effect of sulforaphane when added pre-exposure to UVB but while HaCaT cells were seeded overnight. In accordance to other studies, we have included a 0.5μM of sulforaphane as well since this concentration was previously shown to provide the highest degree of protection against UVB irradiation [29].

Table 2 shows that at all concentrations of sulforaphane, the cell viability was greater in the control group than in the UVB one. This indicates that sulforaphane added pre-exposed to UVB does not provide significant protection to HaCaT cells. Once again, cytotoxicity was observed at the highest concentration of sulforaphane (10.0μM) in both control (78.1%) and UVB (67.6%) groups.

Finally, we investigated the supplementation effect of sulforaphane when added pre-exposure to UVB and 24 hrs after cells were seeded. More specifically, after 2h, it was observed that there was a slight improvement in cell viability at 0.5μM (102.1%) with concomitant reductions at 1.0μM (90.2%), 2.5μM (87.8%), 5.0μM (82.6%) and 10.0μM (49.4%) of sulforaphane (Figure 2A).

In addition, after 4 hrs, it was also observed a slight increase in cell viability at 0.5μM (106.5%), 2.5μM (101.6%) and 5.0μM (102.6%) but with further reductions at 1.0μM (95.0%) and 10.0μM (19.5%) respectively (Figure 2B). Thus, it was concluded that there was no protection due to sulforaphane supplementation on HaCaT cells exposed to UVB under these experimental conditions. The same pattern was also observed, after 24 hrs, with improved cell viability levels at 0.5μM (108.5%), 1.0μM (108.0%), 2.5μM (108.5%) and 5.0μM (102.9%) but with reduced levels at 10.0μM (77.5%) (Figure 2C). Finally, after 48 hrs, it was observed that there was a significant increase in cell viability at 0.5μM (121.8%), 1.0μM (130.3%), 2.5μM (138.6%), 5.0μM (141.3%)

and 10.0μM (111.4%) of sulforaphane (Figure 2D). Consequently, it was concluded that there was significant protection induced by sulforaphane supplementation on HaCaT cells exposed to UVB under these experimental conditions.

Discussion

Overall, our results have demonstrated that exposure to UVB irradiation has a negative impact on HaCaT cell viability in a way that the greater the dose of the exposure, even greater the reduction in HaCaT cell viability (Figure 1). It was also demonstrated that when added post-exposure, sulforaphane is less effective in providing protection to HaCaT cells with cytotoxicity observed at concentrations over 10.0μM (Table 1).

Furthermore, it was observed that sulforaphane supplementation on HaCaT cells pre-exposed to UVB (for 24 hrs while cells were seeded) did not account for significant protection when compared to adding it post-exposure (Table 2). However, pre-exposure conditions at 48 hrs after cells were seeded resulted in significant protection of cells at every concentration of sulforaphane tested (Figure 2D). To these ends, the findings of other research studies have shown that 10.0μM of sulforaphane promoted HaCaT cell proliferation, an effect which was abolished at 25.0μM [30]. Also, in the same study, it was found that sulforaphane had the capacity to reduce UVB-induced skin inflammation as well. Furthermore, in another study, the authors have demonstrated that when human colon (non-synchronized HT29) carcinoma cells were incubated for 48 hrs with various sulforaphane concentrations (ranging from 5.0 to 50.0μM) resulted in decreased cell viability [3].

In general, UVB irradiation leads to temporal changes in the cutaneous cytokine micromilieu with keratinocytes considered to be the main source of chemokines, growth factors and cytokines. The production of these factors by keratinocytes is low but, nevertheless, can be enhanced by UVB irradiation [31,32]. Thus, UVB-induced release of proinflammatory mediators, from keratinocytes, could be responsible for the onset of an inflammatory response and the induction of chemotaxis of neutrophils and macrophages into the skin [33,34]. In addition, Shibata et al. [30] shown that sulforaphane had the ability to attenuate UVB-induced skin inflammation by suppressing MAPK activation. In addition, sulforaphane is known to protect skin cells exposed to UVB irradiation by various means including (i) the inhibition of the activity of Activator Protein-1 (AP-1) both in vitro [43] and in vivo [20], (ii) the activation of the transcription factor Nrf2 and (iii) the induction of phase-2 as well as other antioxidant enzymes in vitro [35]. Moreover, in another study, it was found that 48 hrs after supplementation with sulforaphane, there was a reduction in cell viability of malignant melanoma cells [29].

Sulforaphane was also found to inhibit tumor development and reduce tumor size in an in vivo animal model as well [36]. Finally, Fimognari et al. [37] also found that sulforaphane was able to suppress the proliferation of tumor cells, while glucose-regulated protein did not. These results indicate a protective role of sulforaphane against UVB-induced cellular damage.

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