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Sulphide Donor Exhibits Cytoprotective and Antioxidative Activity in UV-induced HaCaT Cell Lines

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Oxidative stress due to excessive of reactive oxygen species (ROS) in the body is commonly associated as one of the underlying mechanisms of ultraviolet radiation (UV)-induced damage in the skin. Reactive sulphur species (RSS) has been implicated with potent antioxidant properties and involves in modulating cellular signalling transduction pathway. In this current study, the potential involvement of the RSS in alleviating UV-induced damage in HaCaT keratinocytes was explored. We first established the suitable dose of UVA and UVB radiation that can depressed HaCaT cells population, 80 mJ/cm^2 and 10 mJ/cm^2, respectively. The sulphide donor, NaHS, was also tested for its cytotoxicity profile, and 200 µM was considered non-toxic concentration for the cells. Our data showed that pre-treating the cells with 200 µM of NaHS decreased the detrimental effects of UV radiation especially UVA on HaCaT cells as demonstrated by the MTT assay. In this work, we used 2',7'-dichlorofluorescin-diacetate (DCFH-DA), a useful indicator of ROS, with regard to the determining of antioxidant potential of NaHS. Our data demonstrate that 200 µM of NaHS significantly suppressed the intracellular ROS production induced by both UVA and UVB irradiation in comparison with cells absence of NaHS (p<0.05). In conclusion, supplementation of sulphide in biological condition particularly NaHS provides protective effect for skin against UV irradiation by diminishing the formation of ROS. Nonetheless, this study provides a preliminary insight on the potential role of RSS in modulating UV-induced damages in *in vitro* skin model which can be further expand with precision technique in the future.

Keywords: keratinocytes; reactive sulphur species; reactive oxygen species; ultraviolet radiation; antioxidant

I. INTRODUCTION

Optimal exposure of ultraviolet (UV) radiation has been scientifically proven to have beneficial health effects (Alfredsson *et al.*, 2020). Nonetheless, prolonged exposure towards UV through several anthropogenic activities such as sunbathing, tanning and farming has been implicated with skin pathological conditions including skin cancer and the development of accelerated skin ageing (Krutmann *et al.,* 2017). Cellular injury due to exposure to UV-radiation can occur in reactive oxygen species (ROS)-dependent and independent pathway (Ziegler *et al.,* 1994). It has been reported that UVA that has longer wavelength, photosensitizes biomolecules and produces toxicity primarily through ROS generation. On the other hand, UVB which has shorter wavelength properties can directly photoreact with the chromophores such as DNA (Ikehata & Yamamoto, 2018). Nevertheless, regardless of the types of UV radiation, the overexposure of UV is noted to cause disturbance in the balance of ROS level in the skin leading to damaging effects such as protein modifications, lipid peroxidation, and DNA mutation which is commonly associated as one of the underlying mechanisms (De Jager *et. al.,* 2017; Krutmann *et al.,* 2021).

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Our study embarked on the fact that reactive sulphur species (RSS) such as endogenous persulphides (RSSH) and polysulphides (RSS_nH) have been, notably, proficient antioxidants (Zhang *et al.,* 2021). Moreover, the RSS posses high nucleophilicity and good flexibility rendering them as vital components in an interplay between other gasotransmitters such as the ROS and reactive nitrogen species (RNS) for cellular signalling pathway (Cortese-Krott *et. al.,* 2017; Ihara *et al.,* 2017). The involvement of RSS in the electrophile detoxification process has been observed (Ihara *et al.,* 2017). In plants, endogenous or exogenous presence of RSS such as hydrogen sulphide (H_2S) has been implicated with beneficial outcomes against the environmental stresses including climate change (Corpas & Palma, 2020). In this study, by using sulphide donor, NaHS, we attempted to establish the role of low molecular weight RSS and to evaluate its cytoprotective and ROS production/antioxidant effects in skin cells exposed to UV.

II. MATERIALS AND METHOD

A. Cell Culture

The Human keratinocyte HaCaT cells were a gift from The Pharmacology-Toxicology Research Laboratory, Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Puncak Alam Campus, Selangor, Malaysia. The cells were cultured in a flask (Nunc, Kamstrup, Denmark) containing Dulbecco's Modified Eagle Medium (DMEM) (Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin (Gibco, Rockville, MD, USA) at 37°C in a humidified incubator with 5% CO_2. All works were conducted under a sterilised environment.

B. Pre-treatment with Sulphide Donour and UV Irradiation

HaCaT cells were plated onto a 96-well microplate at a concentration of 1 x 10^6 cells/well, and allowed to grow for 24 h. Cells were washed with phosphate buffered saline (PBS) and then were incubated with various concentration of sodium hydrosulphide (NaHS; 0, 50, 200 µM) for 3 h. Prior to UV exposure from a bank of 15 Watt tube lamps with a wavelength of 312 nm (Lab Logistic Group, Germany), the cells were washed with PBS and covered with a thin layer of PBS to ensure that the results would not be modified by the colour of the media. The cells were initially exposed to several doses of UVA (0, 40, 60 80 and 100 mJ/ cm^2) and UVB (0, 0.2, 0.5, 1 and 10 mJ/ cm^2) irradiation. The irradiance of the lamps was calculated by a calibrated photometer (UV-340B, Sampo Scientific Instrument Co. Ltd., Shenzen). The works were performed in dark to avoid interference from ambient atmosphere. Thereafter, PBS was replaced with fresh medium, and the cells were incubated for 24 h for 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and ROS assay. Control without any treatment was also included.

C. Cell Viability Assay

Cell viability was determined by MTT colourimetric method. At the end of the incubation, 50 µl (5 mg/ml) of the MTT reagent (Sigma, St. Louis, MO, USA) was added to each well and cells were incubated in the incubator in the dark for 2 h. The media was removed and 200 µl of DMSO was added to solubilise the purple formazan. The absorbance was measured at 490 nm by using Tecan M200 Infinite® Pro Microplate Reader.

D. Evaluation of UV-induced ROS Formation using Dichloro-Dihydro-Fluorescein Diacetate (DCFH-DA) Assay

The intracellular ROS generated by both UVA and UVB was evaluated by dichloro-dihydro-fluoresceine deacetate (DCFH-DA) assay. HaCaT cells were cultured into a dark 96-well plate. Briefly, in a dark environment, the cells were incubated with the diluted DCFH-DA solution (100 µl/well) for 30 min in the incubator at 37°C. After that, the solution was discarded, and the cells were washed 2 times with PBS. Then, in the presence of buffer or media, the fluorescence of the 2',7'-dichlorofluorescein product was detected and quantified using a plate reader.

E. Statistical Analysis

All the experiments were carried out in triplicates. Statistical analysis was performed using SPSS and the results were analysed using Student's t test to determine the statistical significance between the groups. A *p* value less than 0.05 was

regarded as statistically significant. All data were expressed as the mean ± standard error mean (SEM).

III. RESULTS AND DISCUSSION

A. Effect of UV Doses on Cell Viability

We established a baseline study for UV radiation in order to determine UV-induced toxicity to the cells at different doses as depicted in Figure 1. Evidently, there was a marked decrease of cell viability in cells exposed towards 80 mJ/ cm² of UVA and 10 mJ/ cm² of UVB. These doses were chosen as dose of interest for this study to analyse the maximum range of effects and response to UV exposure.

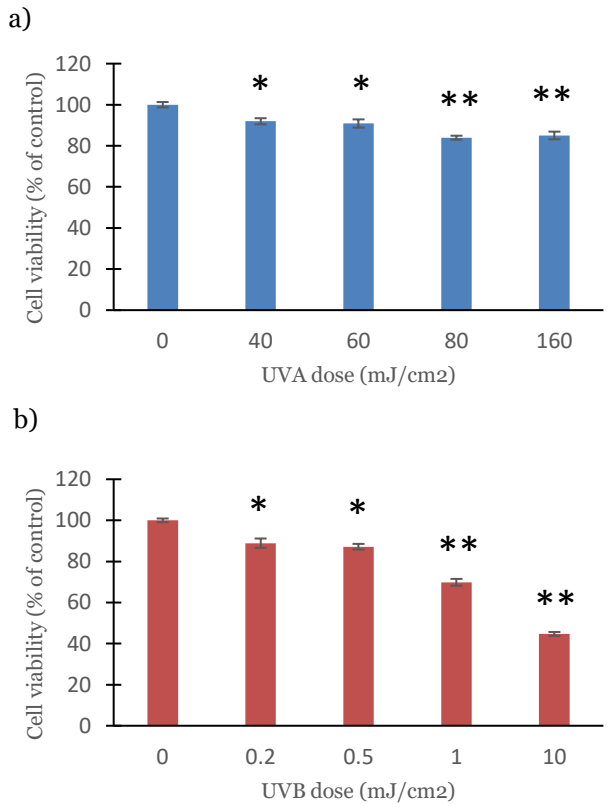

Figure 1. Effect of different UV doses (A) UVA (40, 60, 80 and 160 mJ/cm²) (B) UVB (0.2, 0.5, 1 and 10 mJ/cm²) on cell viability determined by MTT assay. Samples unexposed to the UV irradiation served as control. Results are expressed as percentage of cells compared to the control group (mean±SEM). * p<0.05 vs control; ** p<0.01 vs control

B. Cytoprotective Role of Sulphide Donor Towards UV-induced HaCaT Cells

Prior to pre-treating the cells with NaHS, we first identified that the cytotoxicity profile of NaHS. HaCaT cells treated with NaHS at concentration ranging from 2.5 to 200 µM for 24 h have nearly the same proliferation rate as control cells (Figure 2) which are in agreement with Xin *et al.* (2016). At 200 µM, NaHS alone did not affect the viability of HaCaT cells (Figure 2) hence it may not influence the cell viability and proliferation in the cells which will be used in further experiments.

Next, we studied the cytoprotective impact of NaHS in cells subjected to 80 mJ/ cm² of UVA or UVB 10 mJ/ cm² of UVB. Prior exposure to the UV, cells were incubated with 50 and 200 µM of NaHS for 3 h. Untreated cells were included as control. Evidently, without NaHS pre-treatment, UVA and UVB significantly decrease the cell viability. Our finding showed that pre-treatment of HaCaT cells with NaHS significantly attenuated UVA damaging effect. Conversely, the pre-treatment of NaHS in the cells exposed to UVB did not show significance difference with the NaHS-untreated samples (Figure 3). On the other hand, the ROS production in UVB-exposed cells was significantly reduced in cell pre-treated with high concentrations of NaHS. UVB is known to cause cellular damage via ROS-independent pathway, nonetheless, cells exposed with UVB commonly shown to have high ROS level. A study using *Arabidopsis* plants showed that several components of cells can produce ROS in response to the UVB irradiation (Soheila *et al.,* 2001).

These findings are in-line with previous work that indicated UVB cause cellular damage mainly through ROS-independent pathway (Chang *et al.,* 2003). We were not able to significantly prove the cytoprotective effect of RSS against UVB in this study. However, a recent study suggested that supplementation of NaHS has been associated with the promotion on cell proliferation and melanin synthesis in primary human epidermal melanocytes (Ying *et al.,* 2020). Interestingly, a study conducted by Malaga and team (1999) showed that N-acetylcysteine (NAC) exhibit protective mechanism against UVB irradiation in algae and soybean leaves. NAC is somewhat considered as sulphide or RSS donor as well. Though the fact that the difference between NaHS and NAC is in the presence of cysteine amino acid

skeleton in the latter. As a matter of fact, another amino acid, histidine, can produce a by-product known as urocanic acid, which is a chromophore or a major ultraviolet-absorbing component of the stratum corneum. This compound can suppress UV-B-induced inflammation and cellular damage (Gibbs *et al.*, 2008). Whether compounds with amino acid skeletal structure, particularly cysteine can influence the damaging effect of UV requires further investigation.

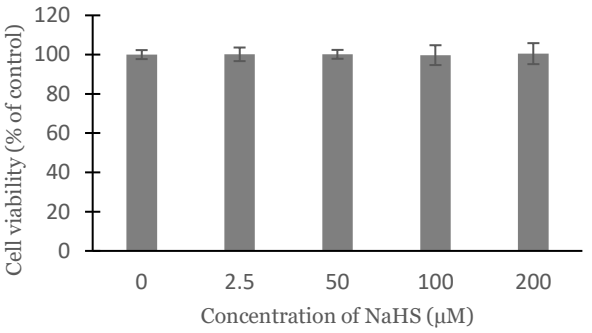

Figure 2. Cytotoxicity profile of HaCaT cells towards NaHS. Data is shown as mean ± SEM. The experiment was performed in triplicate

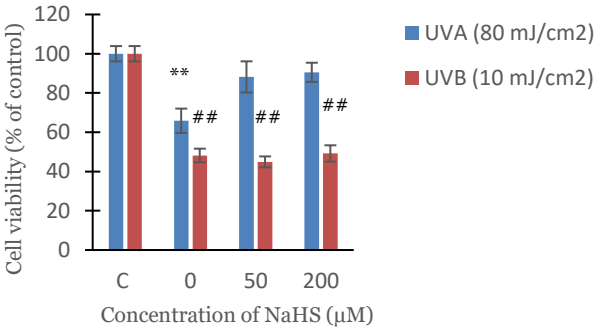

Figure 3. Pretreatment of NaHS in UVA and UVB-induced cytotoxic effect in HaCaT cells. Cells were exposed to UVA and UVB radiation at dose 80 mJ/cm² and 10 mJ/cm², respectively. Data is shown as mean ± SEM. ** $p<0.01$ (UVA), ## $p<0.01$ (UVB) compared with the untreated samples. The experiments were carried out in triplicate

C. Sulphide Donour Attenuates ROS Production in UV-exposed Cells

DCFH-DA is a useful indicator of ROS and oxidative stress. A series of pre-treated samples with various concentration of NaHS (0, 50 and 200 μM) was tested in this experiment. The samples were exposed with either UVA or UVB. Alpha tocopherol was included as positive control. The results shown in Figure 5 and Figure 6 indicate that the cells pre-treated with 200 μM of NaHS significantly reduced the formation of ROS induced by both UVA and UVB irradiation as compared with cells absence of NaHS ($p<0.05$). However, the data on the production ROS shown in Figure 4 is not consistent with Figure 3. It is assumed that UVB irradiation causes more production of ROS in *in vitro* model compared to the UVA. Hence, the amount of NaSH and alpha-tocopherol applied herein is insufficient to effectively prevent the oxidation of non-fluorescent dichlorodihydrofluorescein (DCFH) into fluorescent derivative dichlorofluorescein (DCF). Such an occurrence was also observed in a previous study (Bajgar *et al.*, 2021) whereas keratinocytes exposed to UVB exhibit higher production of ROS compared to UVA. Nonetheless, after the data were converted to effective dose, following minimal erythma dose, the actual level of ROS produced by UVB is lower compared to UVA. Hence, primary mode of damages of UVA is based on ROS-dependent pathway.

It is currently an established fact that RSS plays an important role in scavenging ROS. In addition, the persulphides (for example cysteine hydropersulphides (CysSSH), glutathione persulphides (GSSH), was said to have higher nucleophilicity compared to the conservative thiol are gaining more attention as of late, particularly in redox modulation and electrophiles detoxification (Kasamatsu & Ihara, 2021; Shinkai & Kumagai, 2019). The crosstalk between the ROS and RSS have been implicated in various cellular signalling (Heppner *et. al.*, 2018; Sawa *et al.*, 2021). By using the persulphides or perhaps the polysulphides that contains much longer sulphur chain, better cytoprotective properties against UV-exposed cell can be provided. Indeed, different elements of RSS effects towards UV exposure warrants further investigations.

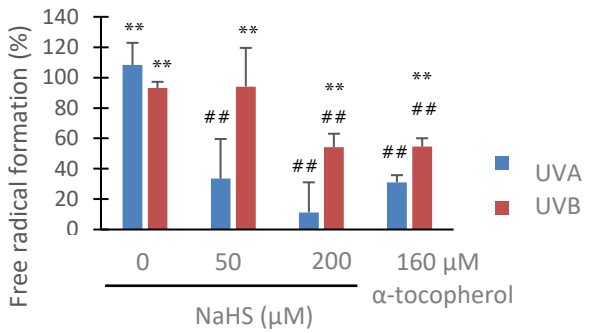

Figure 4. Quantification of oxidative stress in pre-treated NaHS HaCaT cells exposed with UVA or UVB. Tha data represent the mean ± SEM of triplicate experiment. ** represents $p<0.01$ compared to negative control, ## represents $p<0.01$ compared to 0 µM NaHS

IV. CONCLUSION

Excessive exposure to UV radiation can produce many damaging effects on health. Our study revealed that supplementation of sulphide donor, NaHS alleviated oxidative stress in cells exposed to UV radiation and the protective effect was observed in the samples exposed towards UVA but not UVB. These findings suggested that the NaHS or generally RSS as a potential cytoprotective element against UV radiation particularly via ROS-dependent pathway. Nonetheless, the involvement of RSS in regulating UV-induced damages regardless of different UV type should be considered and warrant further investigations. In short, the role of RSS can potentially, in part, or fully, amplify antioxidant cascade of the cellular system which can further expand our understanding on the physiological role of sulphur biology.

V. ACKNOWLEDGEMENT

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