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The Protective Effects of Sodium Pentaborate Tetrahydrate Against UVB-induced Apoptosis in Human Keratinocytes

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ABSTRACT

Ultraviolet radiation (UV) is an environmental carcinogen causing human skin cancer. Exposure of the skin to UV produces apoptotic keratinocytes called sunburn cells within the epidermis. Boron, an essential element for plants, has several biological properties, such as anti-cancer, anti-microbial, and anti-oxidant. In the present study, the possible protective effects of sodium pentaborate tetrahydrate (SPT) against UVB-induced apoptosis in human keratinocyte cells, HaCaT, were investigated. They were treated with SPT at different concentrations (7.8-125 µg/mL) for 24h after UVB irradiation (20, 30 and 60mJ/cm²). Cell viability, annexin V assay, cell cycle analysis, and apoptosis-related gene levels were measured using RT-PCR. Treatment with SPT (15.6-31.25µg/mL) after 30 mJ/m² UVB exposure significantly increased cell survival. Annexin V apoptosis analysis demonstrated a robust protective effect by treatment with SPT at concentrations of 15.6 and 31.25µg/mL after 30mJ/cm² UVB irradiation. The cell cycle analysis revealed that UVB irradiation elevated the number of cells at the G0/G1 phase while SPT treatment after UVB irradiation increased the number of cells at G2/M phase, suggesting the changes were partially reversed. Furthermore, treatment with 15.6µg/mL SPT after 30 mJ/m² UV irradiation blocked the activation of Caspase 3, Caspase 9, Bax, And P53. These results indicate that treatment with SPT exerts protective effects after UVB irradiation. Thus, treatment with SPT led to strong protection against UVB-induced apoptotic cell death in HaCaT cells.

Keywords:
Sodium pentaborate tetrahydrate; UVB irradiation; Apoptosis; Keratinocyte.

INTRODUCTION

Skin, the largest organ of the body, roles as a barrier to hostile environmental factors. Skin is divided into two main layers; epidermis and dermis [1]. The primary function of the epidermis, the outermost layer of the skin, is to act as a pivotal role against environmental stressors such as Ultraviolet radiation (UV) [2]. Keratinocytes are the most abundant cell type in the epidermis. UV causes damage to these cells. Although the epidermis protects the skin against the harmful effects of UV irradiation, its protective mechanisms are still unknown.

Ultraviolet radiation of sunlight can cause several physiological and biological effects on human cells, such as initiation of skin cancer progression, cellular aging, and apoptosis [3–5]. UV irradiation, a potent inducer of cell death, causes the production of various molecules that lead to apoptosis [5–7]. Apoptosis is a programmed cell death mechanism that eliminates no longer needed or unrepairable damaged cells [8]. Bcl-2 family members and a family of cysteine proteases, caspases, are central regulator of apoptotic cell death. Also, tumor suppressor gene p53 stimulates apoptosis through the induction of several genes that contribute to extrinsic and intrinsic apoptotic pathways [9]. Protective reagents such as antioxidants are required to minimize damage to cells exposed to UV irradiation.

Boron (B) is a natural product and is known as an essential element for plants. Also various human tissues contain boron compounds. Boron presents many biological properties, such as anti-microbial, anti-cancer, and anti-oxidant effects [10]. It is thought that boron increases the antioxidant capacity by decreasing intracellular

ROS level and preventing apoptosis [11–13]. In addition, boron promote tissue repair and regeneration [14]. In addition, recent studies indicated that several boron compounds have the non-toxic potential for healthy cell lines [15,16]. Several studies have shown the anti-cancer activity of sodium perborate tetrahydrate (SPT) which is a boron compounds. SPT has been shown to trigger apoptosis via up-regulating the expression of pro-apoptotic and tumor suppressor genes and down-regulating the expression of anti-apoptotic genes [17,18]. However, the effects of SPT on UV-induced apoptosis has not been demonstrated yet.

In this study, to elucidate the possible role of boron compound sodium pentaborate tetrahydrate SPT in UVB-induced skin damage, we examined the protective effects of SPT on UVB-induced apoptotic cell death using human keratinocyte cell line, HaCaT.

MATERIALS AND METHODS

Cell Culture and Reagents

Human keratinocyte cell line, HaCaT were cultured in DMEM-High Glucose (Gibco, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% Penicillin/Streptomycin/Amphotericin (PSA, Gibco, UK). They were incubated at 5% CO2, 37°C in a humidified incubator and were passaged when they reached 70-80% confluence. Sodium pentaborate tetrahydrate was obtained by Ziegler & Co. GmbH (Wunsiedel, Germany). SPT was prepared in DMEM-High medium at a 1 mg/mL concentration before use.

UVB irradiation

UVB irradiation was carried out using a Vilber Lourmat UV cross-linker (Marne-la-Vallée, France). HaCaT cells were cultured for 24h in a humidified incubator and then were rinsed with 1X phosphate buffered saline (1X PBS) and exposed to increasing UVB doses (0, 20, 30, and 60 mJ/cm²). Then, they were treated with or without different SPT doses (7.8-125 µg/mL) for 24h.

Cell Viability Assay

Cell viability was assessed by 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium salt (MTS) assay. Briefly, cells were cultured into 96-well plates (5x10^3 cells/well). After 24h, they were exposed to various doses (20, 30, and 60 mJ/cm²) of UVB irradiation. Then, SPT (7.8, 15.6, 31.25, 62.5, and 125 µg/mL) was added to cells for 24h. At the end of the incubation periods, 10 µl MTS solution and 100 µl DPBS containing 4.5 g/L D-glucose were added to the each well and incubated 1h at 37 ˚C in the dark. The absorbance values were read at 495 nm by using ELISA plate reader (BioTek, Winooski, VT).

Apoptosis Assay

Annexin V analysis was revealed using Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, USA). Briefly, cells were cultured into 6-well plates (15x10^4 cells/well). After 24h, cells were exposed to 30mJ/cm^2 UVB irradiation. 15.6 and 31.25µg/mL SPT was added to cells exposed to UVB irradiation. After 24h, they were harvested and resuspended in 100µl Annexin V binding buffer. Then, 5µl Annexin V-FITC, and 5µl PI staining solution were added and incubated (RT, 20 min) in the dark. After the incubation, they were analyzed under BD FACS Calibur Cell Sorting System (BD Biosciences Pharmingen).

Cell Cycle Analysis

The cell cycle was performed using flow cytometry. Briefly, the HaCaT were cultured into 6-well plates (15x10^4 cells/well). After 24h, cells were exposed to 30mJ/cm² UVB irradiation and then were treated with 15.6 and 31.25µg/mL SPT for 24h. After the incubation, they were collected, centrifuged at 1300 rpm (5min), and the pellet was rinsed with 1X PBS. Cells were fixed in 95% ethanol at -20°C for 2 h and stained with PI staining solution for 5 min in the dark. After the incubation, they were analyzed by BD FACS Calibur Cell Sorting System (BD Biosciences Pharmingen).

Real-Time PCR Analysis

The mRNA expression of apoptosis-related genes was determined by qRT-PCR analysis. Briefly, the HaCaT were cultured into 6-well plates (15x10^4 cells/well). After 24h, 15.6µg/mL SPT was added to cells exposed to 30mJ/cm^2 UVB irradiation. After 24h, total RNA was isolated as recommended by the manufacturer's instructions using High Pure Roche RNA Isolation Kit (Roche, USA). After the isolation, cDNA was synthesized using the High Fidelity cDNA synthesis kit (Roche, USA) as recommended by the manufacturer. Then, qRT-PCR was performed by using 5 µl SYBR Green Master mix (Applied BiosystemsTM), 2µL PCR grade distilled water, primers (forward (0.5 µl), and reverse (0.5 µl)) with a 10 µl final volume for each well. Caspase 3, caspase 9, bax and p53 primer sequences are shown in Table 1. The reaction was carried out by the iCycler qRT-PCR system (CFX96 RT-PCR system, Bio-Rad, Hercules, CA). 18S was used as a housekeeping gene.

Table 1. The primer sequences used in the qRT-PCR analysis

Gene	Forward (5'- 3') Sequence	Reverse (5'- 3') Sequence
Caspase 3	ACTGGACTGTGGCATT-GAGA	GCACAAAGCGACT-GGATGAA
Caspase 9	GTGA-ACTTCTGCCGTGAGTC	CTGACAGCCGTGAGA-GAGAA
Bax	TGCAGAGGAT-GATTGCCGCCG	ACCCAAC-CACCCTGGTGTTGG
P53	GCCCAACAACAC-CAGCTCCT	CCTGGGCATCC-TTGAGTTCC
18S	GTAACCCGTTGAACC-CCATT	CCATCCAATCGGTAG-TAGCG

Statistical Analysis

Data were presented as mean ± SD of the three independent experiments. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used to analyzed. $p<0.05$ was considered statistically significant.

RESULTS

The effects of SPT on UV-induced inhibition of HaCaT cell viability

Cells were exposed to different UV irradiation (20, 30 and 60 mJ/cm²) followed by various SPT concentrations (7.8, 15.6, 31.25, 62.5, and 125 µg/mL) for 24h. 20, 30, and 60 mJ/cm² UVB exposure marked decrease in the viability of keratinocytes.

When the cells were treated with 7.8, 15.6, 31.25, and 62.5 µg/mL SPT for 24h, the cell survival rates were recovered to 101.2%, 101.1%, 104.7%, and 101.9% respectively, for 20 mJ/cm² (Figure 1A); 100.4%, 132.8%, 116.7%, and 96% respectively, for 30 mJ/cm² (Figure 1B); 100.4%, 109.5%, 103.3%, and 86.9% respectively, for 60 mJ/cm² (Figure 1C). On the other hand, 125 µg/mL SPT had cytotoxic effects on HaCaT cells.

After treatment with SPT doses, cell viability was markedly increased compared to UVB irradiation group, suggesting that SPT significantly reduced UV-induced apoptosis and affected cell recovery in HaCaT. The most effective UVB dose on cell viability was 30mJ/cm² and SPT doses of 15.6 and 31.25 µg/mL were selected for further testing.

The effects of SPT on the apoptosis of UV-exposed HaCaT cells

The cells were treated with SPT (31.25 and 15.6µg/mL) for 24 followed by 30 mJ/cm² UVB irradiation. The number of apoptotic cells increased after UV irradiation (~45%). 31.25 and 15.6µg/mL SPT doses were significantly decreased apoptotic cell ratio (~5%) compared to

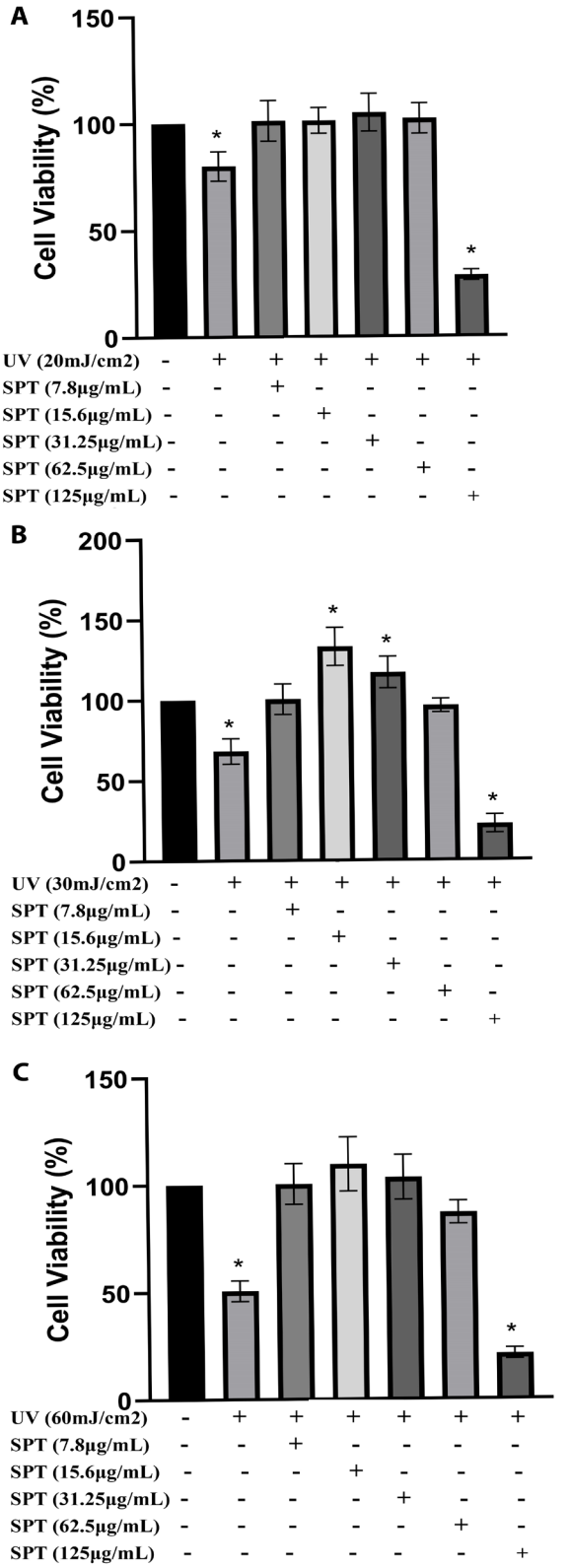

Figure 1. Effect of SPT on the viability of UVB-irradiated HaCaT. Cells were treated with SPT concentrations (7.8, 15.6, 31.25, 62.5, and 125 µg/mL) for 24 after 20 (A), 30 (B) and 60 mJ/cm² (C) UVB irradiation. Data represent the mean values ± S.D of three independent experiments. *P < 0.05 vs. control group. SPT: Sodium pentaborate tetrahydrate, Control: growth medium-treated HaCaT.

UVB irradiation. As shown in Figure 2A, after SPT treatment, the apoptotic cell ratio was similar to the negative control group.

The effects of SPT on UV-induced changes in cell cycle of HaCaT cells

To determine whether SPT alters the cell cycle phase distribution, cells were treated with SPT (31.25 and 15.6μg/mL) for 24 followed by 30 mJ/cm² UVB irradiation. As shown in Figure 2B, 30 J/m² UVB markedly increased G0/G1 phase population to 71%. The SPT treatment of 15.6μg/mL and 31.25 decreased G0/G1 population to 41% and 39% while increased G2/M population to 49% and 50%. This result indicated that SPT protected cells against UVB-induced cell death.

The effects of SPT on apoptosis-related genes in UV-exposed HaCaT

To investigate the effects of SPT on apoptosis-related gene (Caspase 3, Caspase 9, Bax, and P53) levels were examined by qRT-PCR. As shown in Figure 3, the qRT-PCR analysis demonstrated that UVB-irradiation induced increased the level of Caspase 3, Caspase 9, Bax, and P53 gene to 1.6, 1.5, 1.7 and 2.5 fold, respectively (Figure 3). Treatment of HaCaT cells with 15.6μg/mL SPT inhibited the activation of apoptosis-related genes, suggesting that SPT could protect UV-induced apoptotic cell death.

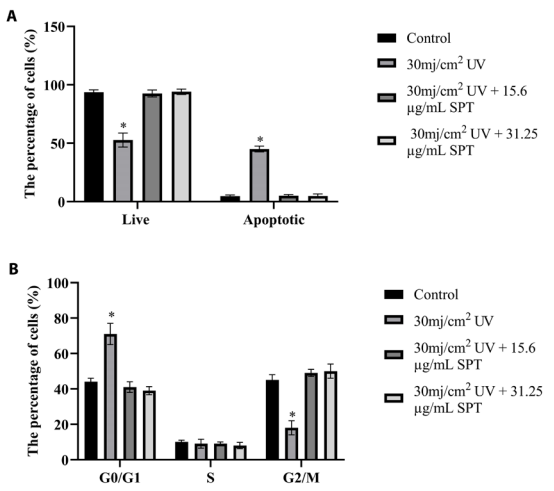

Figure 2. The effects of SPT on apoptotic cell ratio (A) and cell cycle phases (B) of UVB-exposed HaCaT. Cells were treated with 15.6, 31.25μg/mL SPT for 24 after 30mJ/cm² UVB irradiation. Data represent the mean values ± S.D of three independent experiments. *P < 0.05 vs. control group. SPT: Sodium pentaborate tetrahydrate, Control: growth medium-treated HaCaT cells.

DISCUSSION

Ultraviolet radiation plays a vital role in skin aging, inflammation and apoptosis [19]. It is known that ROS is a key factor in most of the UV-induced biological effects, including apoptosis. Several studies have shown that natural compounds with anti-oxidant properties have the ability to scavenge free radicals [8,20,21]. Boron, an essential element for plants, plays a crucial role in many biological processes. Boron compounds showed many biological properties such as antibacterial [22], antiviral [23], anti-carcinogenic [24], anti-angiogenic [25] , anti-inflammatory [26], and anti-oxidative [27]. Boron elevates antioxidant activity by decreasing the levels of ROS, Ca+2 ion and inflammatory cytokines (TNFα and IL-6) and thus prevents apoptosis [12,13]. Moreover, boron stimulate tissue repair and regeneration [14]. Boron may be beneficial in preventing or reducing the progression of various oxidative stress-induced diseases [28]. Recent studies indicated that boron compounds has non-toxic potential for healthy cell lines [15,16]. Sodium perborate tetrahydrate, one of the boron compounds, has been shown to anti-cancer activity [18]. However, the protective effects of SPT against UV-induced apoptosis has not been demonstrated yet. In this study, we investigated the effects of SPT on cell survival in HaCaT cells that are irradiated with UVB. Together with our reults, we provide evidence that SPT has a protective effect against UVB exposure.

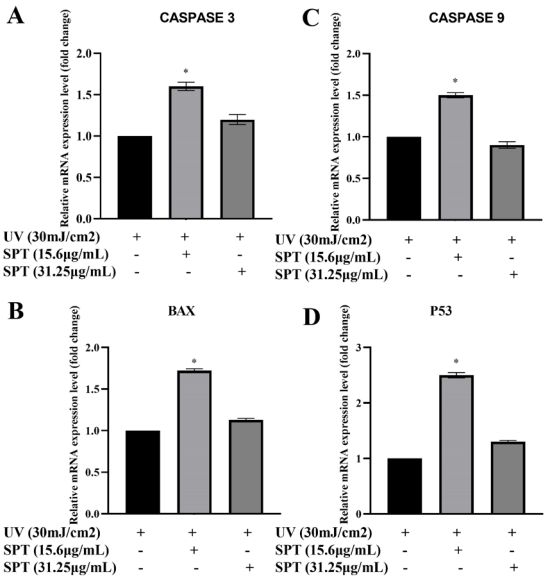

Figure 3. The effects of SPT on apoptosis-related genes, Caspase 3 (A), Bax (B), Caspase 9 (C), and P53 (D), in UVB-exposed HaCaT. Cells were treated with 15.6μg/mL SPT for 24 after 30mJ/cm² UVB irradiation. Data represent the mean values ± S.D of three independent experiments. *P < 0.05 vs. control group. SPT: Sodium pentaborate tetrahydrate, Control: growth medium-treated HaCaT cells.

These results showed that 20, 30 and 60 mJ/cm² UVB irradiation significantly reduced the viability of HaCaT. However, the addition of SPT doses to the medium significantly increased the viability of cells after UV exposure. The viability of cells in 15.6 and 31.25μg/mL SPT groups exposed

to 30 mJ/cm² UVB was significantly increased compared to other treatment groups. The results of the cell viability assay were confirmed with apoptosis analysis. Apoptotic keratinocytes were assessed by the Annexin V-FITC analysis. 31.25 and 15.6µg/mL SPT doses markedly decreased apoptotic cell rate after 30 mJ/cm² UVB exposure. In addition, 30 J/m² UVB irradiation elevated G0/G1 phase population, while 15.6 and 31.25 µg/mL SPT treatment increased G2/M phase population. Cell cycle results also supported cell viability and apoptosis analysis. Similar to these results, a study showed that low doses of boron application increase cell viability and G2 phase cell accumulation in primary rat cells [29]. It has been reported that a different natural product, propolis protected HaCaT cells against apoptosis induced by UV-irradiation [8,30]. In another study, melatonin was shown to increase the cell viability of keratinocytes exposed to UV [31].

Ultraviolet irradiation induces apoptosis, leading to sunburn cells. Blockage of apoptosis is considered to be protective against UV-induced effects [31–33]. Keratinocytes become sensitive to apoptotic stimuli, including UV irradiation, as they lack of survival factors. So, keratinocytes, an abundant cell type in the epidermis, undergo apoptosis at low UV-doses. Moreover, keratinocytes express bcl-2 and bcl-xL anti-apoptotic proteins [34,35]. UV irradiation upregulates Bax and caspases and downregulates bcl-2 and bcl-xL [36]. Bcl-2 family proteins act as a crucial role in the apoptotic process. The balance between pro-apoptotic protein Bax/Bcl-2 is important for apoptosis [37–39].

Caspases act as a key mediator in apoptosis. Initiator caspases, caspase-8, 9 and 10, activate caspase 3, 6 and 7 executioner caspases [40]. In addition, p53 is a major tumor suppressor protein activated by many environmental stress factors, including UV radiation. p53 gene normally induces apoptosis of cells with unrepairable DNA damage [41–43]. p53 is important for the skin's protective responses to UV, and loss of its function induces UV-induced carcinogenesis [42].

In the present study, qRT-PCR analysis revealed that UVB-irradiation upregulated Caspase 3, Caspase 9, Bax and P53 gene expressions. UVB-induced alterations in the Bax, Caspase 9, Caspase 3, and P53 levels were suppressed in the presence of 15.6µg/mL SPT. This study demonstrated that UVB induces apoptosis via Bax, Caspase 9 and Caspase 3. Consistent with our results, it has been shown that Astaxanthin treatment significantly reduced the UV-induced caspase-3 and -9 activity [37]. Additionaly, boron treatment induced anti-oxidant activity and decreased caspase 3 level [44].

Taken together, SPT treatment prevents the deleterious effects of UVB irradiation by reducing apoptosis in keratinocytes. SPT has been shown to play an anti-apoptotic substance in UVB- exposed cells, suggesting that the natural product may be useful to skin protection from UV irradiation. However, the protective effects of SPT against UV-induced apoptosis warrants further investigation.

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CONFLICT OF INTEREST

The author deny any conflict of interest.

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