Protective effect of Schizandrin B against damage of UVB irradiated skin cells depend on inhibition of inflammatory pathways

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ABSTRACT
Schizandrin B is extracted from \textit{Schisandra chinensis} (Turcz.) Baill. This study evaluated the photoprotective effect of Schizandrin B on oxidative stress injury of the skin caused by UVB-irradiation and the molecular mechanism of the photoprotective effect of Schizandrin B, and we firstly found that Schizandrin B could block Cox-2, IL-6 and IL-18 signal pathway to protect damage of skin cells given by UVB-irradiation. In the research, we found that Schizandrin B can attenuate the UVB-induced toxicity on keratinocytes and dermal fibroblasts in human body, and can outstandingly eliminated intracellular ROS produced by UVB-irradiation. These results demonstrate that Schizandrin B can regulate the function of decreasing intracellular SOD’s activity and increasing the expression level of MDA in HaCaT cells result from the guidance of UVB, and it markedly reduced the production of inflammatory factors such as Cox-2, IL-6 or IL-18, decreased the expression level of MMP-1, and interdicted degradation process of collagens in UVB-radiated cells. Therefore, skin keratinocytes can be effectively protected from UVB-radiated damage by Schizandrin B, and UVB-irradiation caused inflammatory responses can be inhibited by attenuating process of ROS generating.

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INTRODUCTION
UV radiation, a particular UVB-irradiation of which wavelength was 280~320 nm, can hazarded human health through bring out cyclobutane pyrimidine dimers (CPDs), inflammatory reaction, cellular damage, immunosuppression and ultimate skin cancer.\textsuperscript{1,2} Skin cells secreted reactive oxygen species (ROS) product after being acted on by UVB-irradiation.\textsuperscript{3,4} The high expression level of ROS can effectively oxidate DNA, cellular proteins and lipids, result in activating a number of signaling pathways and release many kinds of inflammatory mediators.\textsuperscript{5} Basal cell layers of epidermis were mostly influenced by UVB-irradiation, thus keratinocytes that was one of typical prime cell populations of basal layer, was primarily affected by UVB-irradiation. Fibroblasts were also primarily affected by UVB-irradiation and can created typical responses of UVB-irradiation photo-damage through releasing and increasing various proinflammatory activators.\textsuperscript{6-11} Inflammation caused by UV radiation activates various matrix-degrading matrix metalloproteases (MMPs), which leads to collagen degradation and cellular apoptosis. Especially, MMP-1 was the main endogenous factor that degraded dermal collagen in the process of human skin senility.\textsuperscript{12-14} Type I collagen was the primary composition of extracellular matrix (ECM). When its expression level was reduced, then had a close relevance with skin senility.

In previous studies, we can see that UV-irradiation had an essence that can take strongly oxidative and photo-oxidative damages on skin.\textsuperscript{15} Therefore, antioxidants that clear up ROS have been proposed as photoprotective agents.\textsuperscript{16,17} More and more skin care products contained botanical agents, which have the potential antioxidant and the properties of anti-inflammatory and anti-carcinogenic, which can avoid damages of UV radiation damage to skin.\textsuperscript{9} Moreover, the extractive of traditional Chinese medicine were proved to effectively avianize oxi-dative cellular damage of UV radiation. Andnzo[a,c] cyclooctadiene lignans were extracted from Schisandra berry and can effectively stop skin cancer from spreading.
Schizandrin B, a Dibenzo (a,c) cyclooctene lignans isolated from Fructus schizandrae, is an anti-hepatotoxic, antiasthmatic, antidiabetic, sedative and tonic agent. It was claimed that unfavorable influence of stress factors can be resisted against increasingly by Schizandrin B besides its adaptogenic activity. In China, the Schizandra berry had been taking into treating patients who were suffering infections arose by chronic virus hepatitis B since the 1970s. The lipid peroxidation of cell membranes could be inhibited and the ability of resisting reactive oxygen species of cells by enhance superoxide dismutase (SOD) with catalase activities could be reinforced both by lots of Schizandra lignans.

The activity of succedaneums were comparably familiar with that of vitamin E, particularly with liver microsomes. Schisandrin B was used to be a hepatoprotectant to depress or eliminate toxicity of xenobiotic agents to diabetic animals' cells. The free radical scavenging activity (FRSA), which acted on hydroxyl radical and superoxides in schisandrol A and B, was functionally familiar with that of vitamin E and C, and its eliminating efficiency of hydroxyl radical was twice as high as that of superoxide anion in 2 vitamins. However, inhibitory actions of Schizandrin B in photoaging have yet to be studied. In our study, we have evaluated the agent for its antioxidant and cell membrane stability properties in order to prevent and treat photo-damage and photoageing. Our research result shows that Schizandrin B has a potential protective effect on skin cell damage by UVB-irradiation.

In this paper, the activities of Schizandrin B which inhibited against collagen degradation and skin cells' UVB-irradiation inflammatory response were elaborated. It was investigated if the synthesis and secretion of collagenolytic MMP, inflammatory interleukin 6 and 18 (IL-6 and IL-18) in the UVB-exposed human keratinocytes (HaCaT) and dermal fibroblasts (FB) could be regulated by Schizandrin B.

**Experimental**

**Cell culture**

Human dermal fibroblasts and melanoma cell were obtained from the Blood Institute of the China Union Medical University. The human keratinocyte HaCaT cell line was purchased from KeyGEN Biotechnology (Changchun, China). All cells were cultured in Dulbecco’s modified Eagle’s media (DMEM, Sigma-Aldrich Co., Ltd, St. Louis, MO) containing 10% FBS (Life Technologies, Grand Island, NY), 2 mmol/L of glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin with an experimental environment of 37°C and 5% CO2.

**UVB-irradiation and treatment**

**UVB-irradiation**

In the UVB-irradiation experiments, the medium was detached from culture plates and cells were washed twice by phosphate buffered saline (PBS) in order to avoid radiation procedure being influenced by medium components. The cells were covered by a thin covering of PBS without or with test compounds, and then acted on by UVB-irradiation chamber which had a set of 5 UVB lamps on the 15 cm above cells. The doses of UVB-irradiation were calculated with a radiometer. Control samples were given a non-irradiated dealt with.

Healthy dermal fibroblasts and keratinocyte HaCaT were propagated in the 70% DMEM according to 1×10^4 cells per well in 96-wells plates. Cells were seeded in serum-free DMEM for 24 h, then washed by PBS and exposed under 312 nm of UVB-irradiation at the successive doses of 0–36 MJ/cm². The doses of lamps irradiation were metered with a UV-meter. The optimum irradiation dose was estimated at 30 mJ/cm², therefore this dose of UV-B was used in further experiments.

**Cell viability assay**

The UVB-irradiated cell viabilities were assayed by thiazolyl blue tetrazolium blue (MTT, Sigma Co., Ltd, St. Louis, MO) on the conditions of Schizandrin B or no Schizandrin B. Culture medium was cleared up and the 1 mg/mL of MTT was added to react for 4 h at 37°C. The liquid supernatant was detruded and then dimethylsulfoxide (DMSO Sigma Co., Ltd, St. Louis, MO) was added to decomposed the rest formazan product. The cell intensity was detected colorimetrically at 550 nm.

**Detection of intracellular ROS**

Cells were taken a UVB-irradiated pretreatment and then dyed for avoiding the direct fluorescent-dyed photooxidation. HaCaT cells were irradiated under 30 ml/cm² of UVB with or without Schizandrin B, then acted on by 5 µm of DCFH-DA. The intensity of
DCF fluorescence was metered by a Shimadza UV-260 spectrofluorophotometer. Samples were observed under a confocal laser-scanning microscope.

**Measurement of SOD, MDA release**

HaCaT cells were pretreated individually with and without of Schizandrin B for 2~4 h. Then they were irradiated with 30 mJ/cm² of UVB-irradiation for 24 h. After all, expression levels of SOD and MDA of supernatant and cells were measured by assay kit (Beyotime Co., Ltd, Jiangsu, China).

**Western blot**

Conditioned medium containing FB cell lysates were gathered. Total protein concentration was measured by Bradford assay. Protein equal amounts were transferred onto PVDF membrane which was blocked by 5% skim milk mixed with TBS/T for 90 min and incubated by first antibodies, and then analyzed by 10% SDS-PAGE. Monoclonal anti-α procollagen aminoterminal extension peptide antibody was used at a 1:250 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) under 4°C in an overnight response. Membranes were then washed with TBST and incubated for 1 h with a secondary antibody before being visualized by DAB. Images were taken by western blots for quantitative analysis. Band intensities images were scanned to quantify by Gel-Pro Analyzer 3.1 Automated Digitizing System. Each experiment was repeated 3 times.

**RT-PCR**

Total RNA of HaCaT was extracted. The reverse transcription of RNA-to-cDNA was implemented with a synthesis kit. The sequences of primers and cycling conditions were shown in Table 9.

Reacted products were electrophoresed with 1.5% agarose gel and dyed with ethidium bromide. Strengths of signals were quantified with a densitometric program and its software kit of quantity one analysis, which were produced by Bio-Rad Laboratories. Increased or decreased ratios of each gene were respectively detected after the intensity of β-actin was normalized.

**Statistical analysis**

All of experimental data were gathered and statistically analyzed with student’s t-test Data. All of Statistical analysis were handled with SPSS for windows (SPSS Inc., Chicago, IL). Results were expressed in the way of means ± SEM(×±s). While P value < 0.05, stands for statistically significant difference.

**Results and discussion**

**Cell viability of Schizandrin B after UVB-irradiation acting on HaCaT**

The cytotoxic effect of Schizandrin B HaCaT cells acted on by UVB-irradiation was detected by MTT assay. The result showed that cell viability can not be reduced by different Schizandrin B concentrations that were from 0.1 μmol/L to 10 μmol/L for 24 h (Fig. 1, Table 1). Cell viability of HaCaT was decreased to nearly 80% survival at the dose of 30 mj·cm⁻² after UVB treating. Along with the dose of UVB increase, cell viability of HaCaT was further reducing. The optimum irradiation dose was found to be 30 mj·cm², therefore this dosage of UV-B were used in further experiments (Table 2).

The effect of that Schizandrin B protected cell viability of HaCaT from reduction induced by UVB was detected by the MTT analysis. In the Table 2 show, the survival rate of irradiated non-Schizandrin-B cells

| Schizandrin B (μmol/L) | Cell survival rate |
|-----------------------|--------------------|
| Normal                | 100.00 ± 0.00      |
| 0.1                   | 103.21 ± 2.05      |
| 1                     | 102.89 ± 4.24      |
| 10                    | 100.13 ± 2.81      |

Note: p>0.1; compared with Normal.
The generation of intracellular ROS can be determined by DCFH-DA, because it could pass through the cell membrane freely. The DCFH-DA could be oxidized by peroxides and then constituted fluorescent 2’, 7’-dichlorofluorescin, which can be used during experiment that needed spectrophotometer and confocal laser scanning microscopy. The effect of that Schizandrin B cleared up intracellular ROS induced by UVB-irradiation was detected to show in Table 3 and Fig. 2. The level of ROS of UVB-irradiation cells was 177.47% as much as that of non-UVB-irradiation control cells. However, the cells were acted on by Schizandrin B undering UVB-irradiation, and the levels of intracellular ROS were reduced to 128.57% at 0.1 μmol/L, 106.64% at 1 μmol/L, and 89.01% at 10 μmol/L, respectively. Figure 2 shows highlighted green fluorescence in UVB-exposed HaCaT cells, and that Schizandrin B could reduce green fluorescence in UVB-exposed HaCaT cells. The inhibition was enhanced in a dose-dependent fashion.

**Schizandrin B can decrease activity of intracellular SOD and increase level of MDA of UVB-induced HaCaT**

SOD could purge oxyradicals and reduce oxidative stress, and MDA was thought as experimental marker that could precisely show the level of lipid peroxidation by oxyradicals inducement. Thus, the activity decreasing of SOD and the level increasing of MDA were used to indicate to show the oxidative damage degree. To evaluate antioxidant activity of Schizandrin B and SOD, an MDA assay was performed in this experiment. HaCaT cells were pretreated with Schizandrin B after a 2 h and exposure to 30 mJ/cm² of UVB-irradiation. Cells were incubated for 24 h to measure SOD and MDA secretion. The result revealed expression levels of intracellular SOD and MDA in HaCaT could be regulated by Schizandrin B treatment. The result showed that Schizandrin B treatment can effectively increase the level of intracellular SOD activity and decrease the level of MDA at 10 μmol/L (Figs. 3, 4). So, we can conclude that Schizandrin B had a strong antioxidant effect.

**Effects of Schizandrin B on expression levels of COX-2, IL-6, IL-18 mRNA in UVB-irradiation-induced HaCaT**

In order to analyze if Schizandrin B could apply an anti-inflammation to UVB-damaged HaCaT, expression levels of COX-2, IL-6 and IL-18 were detected. Extra-expressed level of COX-2 protein can evaluate the degree of inflammatory after being catalyzedly bio-synthetized PGE₂. The results showed that COX-2 expression was markedly enhanced in HaCaT cells after 8 h or 12 h of UVB-irradiation. This enhancement was gradually cut down with increasing dosages of Schizandrin B. At 10 μmol/L, the COX-2 expression was nearly abolished at 8 h and 12h after UVB-irradiation. (Fig. 5, Table 4)

IL-6 can precisely evaluate the degree of inflammatory reaction coming from body stress response to injurious stimulus, such as UVB lights causing a severe sunburn reaction. Some reports

| Group | Fluorescence intensity(%) |
|-------|------------------------|
| UVB (-) | 100 ± 0 |
| UVB (+) | 177.47 ± 23 |
| 0.1 μmol/L SchB + UVB (+) | 128.57 ± 33 |
| 1 μmol/L SchB + UVB (+) | 106.04 ± 21 |
| 10 μmol/L SchB + UVB (+) | 89.01 ± 22 |

Note: *p < 0.05; compared with Normal.
showed that the level of IL-6 of human keratinocyte cells were significantly increase in exposure under UVB-irradiation. IL-18 was a kind of functional proteins containing IFN-γ factor, and was secreted by lipopolysaccharide activated macrophages or Kupffer cells, its molecular weight was 18 kDa. Much more IFN-γ can be produced by th1 cells and NK cells of immune system with the action effect of IL-18. The cytotoxicity of NK cells was strengthened and the proliferation of th1 cells was activate by IL-18. In recent papers, it assumed that IL-18 can easily bring out malignant skin tumors and was a main product of UVB-irradiation. Expression levels of IL-6 and IL-18 of HaCaT can be significantly enhanced at 8h and 12h of UVB-irradiation. Schizandrin B can significantly reduce the IL-6, IL-18 expression in HaCat cells after UVB-irradiation. (Figs. 6, 7; Table 5, 6)

Figure 2. Morphological appearance of HaCaT cells treated with different doses of Schizandrin B by Confocal microscopy detection (×400): A HaCaT cells treated with 0.1% DMSO; B HaCaT cells treated with 0.1%DMSO + UVB; C HaCaT cells treated with 0.1 mol/L SchB + UVB; D HaCaT cells treated with 1 μmol/L SchB + UVB; E HaCaT cells treated with 10 μmol/L SchB + UVB.
Effect of Schizandrin B on expression levels of MMP-1 mRNA in FB cells induced by UVB

MMP-1, -3 and -9 of heath human epidermis can be induced by UVB-irradiation. MMP-1 can directly contribute to photoaging through degrading collagen. So, UVB-induced photoaging can effectively depressed by inhibiting MMP-1 expression of FB cells. The result revealed that the effect of that Schizandrin B depressed expression level of MMP-1 of FB cells was dose-dependent in this paper (Fig. 8, Table 7).

Effect of Schizandrin B on expression level of type 1 pro-collagen mRNA

Collagen can majorly maintain the elasticity of skin connective tissues consisting of multiple structures. Fibrillar collagen and elastin were damaged and decomposed by collagenolytic MMP enzymes, and then dermal strength and resiliency
were lost.\textsuperscript{30} MMP inhibition can eliminate UV-triggered photo-damage. To examine whether the expression level of pro-collagen were enhanced by Schizandrin B and dermal fibroblast was well treated by 2 h of 0.1 \textasciitilde10 \mu \text{mol/L of Schizandrin B and then} 30 \text{mj/cm}^2 of UVB-irradiation. The amount of synthesis of pro-collagen was detected with western blot. Result revealed that 60% of synthesis of pro-collagen were cut down. However, the reduced amount of synthesis of pro-collagen caused by UVB-irradiation can be restored by Schizandrin B, and the result of restoration depend on the doses of UVB-irradiation. Taken together, the expression level of type 1 pro-collagen can be effectively increased by Schizandrin B, so the result depend on the dose of Schizandrin B.

Table 5. Effect of Schizandrin B on IL-6 expression in HaCaT Cells after UVB-irradiation (\(\bar{x} \pm s, n \geq 3\)).

| Group                  | 4 h       | 8 h       | 12 h      |
|------------------------|-----------|-----------|-----------|
| Normal                 | 0.65 ± 0.11* | 0.53 ± 0.15* | 0.23 ± 0.06* |
| 0 \mu mol/L SchB       | 0.97 ± 0.12 | 0.99 ± 0.16 | 0.88 ± 0.19 |
| 0.1 \mu mol/L SchB + UVB| 0.69 ± 0.13* | 0.53 ± 0.11* | 0.38 ± 0.09* |
| 1 \mu mol/L SchB + UVB | 0.83 ± 0.14 | 0.35 ± 0.15 | 0.37 ± 0.08* |
| 10 \mu mol/L SchB + UVB | 0.58 ± 0.09 | 0.47 ± 0.13 | 0.32 ± 0.11 |

Note: * \(p < 0.05\); compared with Normal.

Table 6. Effect of Schizandrin B on IL-18 expression in HaCaT Cells after UVB-irradiation (\(\bar{x} \pm s, n \geq 3\)).

| Group                  | 4 h       | 8 h       | 12 h      |
|------------------------|-----------|-----------|-----------|
| Normal                 | 0.507 ± 0.124* | 0.439 ± 0.075* | 0.562 ± 0.063* |
| 0 \mu mol/L SchB       | 0.724 ± 0.131 | 0.668 ± 0.064 | 0.902 ± 0.084 |
| 0.1 \mu mol/L SchB + UVB| 0.465 ± 0.119 | 0.550 ± 0.076 | 0.495 ± 0.075 |
| 1 \mu mol/L SchB + UVB | 0.575 ± 0.087* | 0.491 ± 0.059 | 0.666 ± 0.072 |
| 10 \mu mol/L SchB + UVB | 0.435 ± 0.019 | 0.503 ± 0.088 | 0.426 ± 0.063 |

Note: * \(p < 0.05\); compared with Normal.

Table 7. Effect of Schizandrin B on MMP-1 expression in FB Cells after UVB-irradiation (\(\bar{x} \pm s, n \geq 3\)).

| Group                  | 24 h      |
|------------------------|-----------|
| Normal                 | 0.46 ± 0.06 |
| 0 \mu mol/L SchB       | 0.60 ± 0.08 |
| 0.1 \mu mol/L SchB + UVB| 0.43 ± 0.07 |
| 1 \mu mol/L SchB + UVB | 0.37 ± 0.05 |
| 10 \mu mol/L SchB + UVB| 0.33 ± 0.04 |

Note: * \(p < 0.05\); compared with Normal.

Table 8. Effect of Schizandrin B on Type I pro-collage expression in FB Cells. (\(\bar{x} \pm s, n = 3\)).

| Group                  | Ratio of IOD (type I pro collage/GAPDH) |
|------------------------|-----------------------------------------|
| Normal                 | 1.263 ± 0.122* |
| 0 \mu mol/L SchB       | 0.787 ± 0.131 |
| 0.1 \mu mol/L SchB + UVB| 1.322 ± 0.167* |
| 1 \mu mol/L SchB + UVB | 1.229 ± 0.134* |
| 10 \mu mol/L SchB + UVB| 0.887 ± 0.133 |

Note: * \(p < 0.05\); compared with Normal.
Thus, Schizandrin B can be an important additive to produce anti-aging cosmetics (Fig. 9, Table 8).

In the experimental results of this paper, it showed that anti-oxidant UVB-irradiation damages of Skin fibroblast and epidermal keratinocytes can be obviously reduced by Schizandrin B. The functional mechanism of that Schizandrin B depressed the UVB-irradiation-caused oxidative cellular damage may be as follows: a scavenging effect on the intracellular ROS generated by UVB-irradiation; reduced expression level of MMP-1; reduced expression levels of COX-2, IL-6 and IL-18; reduced expression level of MMP-1/3 expressions and secretions by suppressing UV-induced apoptosis in skin cells: new implication to produce anti-aging cosmetics (Fig. 9, Table 8).

**Table 9. The sequences of primers and cycling conditions.**

| Gene  | Primers   | DNA sequence                | cycling condition |
|-------|-----------|-----------------------------|-------------------|
| IL-18 | sense     | 5’-AGGAATTAAGATGCTGCTGAAC-3’| 95°C 50s, 60°C 1min, 72°C 45s, for 32cycles |
|       | antisense | 5’-GCTACACCCACACTTACCTCC-3’|                   |
| IL-6  | sense     | 5’-CTCAGTTTGGCAAGGCTCCT-3’ | 95°C 30s,58°C 30s, 72°C 1min, for 35 cycles |
|       | antisense | 5’-TTCAAATGAGATTGTGGGAAAATTGCT-3’|                  |
| COX-2 | sense     | 5’-AGATCATCTCCGCTGAGTATCT-3’| 94°C 56s, 50°C 90s,72°C 2 min, for 30 cycles |
|       | antisense | 5’-AGGATACCCCAAGGACATCT-3’|                   |
| MMP-1 | sense     | 5’-CTCAGGAAAGACAGCAGCTATG-3’| 95°C 30s,55°C 30s,72°C 1 min, for 35 cycles |
|       | antisense | 5’-GGCCATCTCTTGCTCGAAGT-3’|                   |
| β-actin| sense     | 5’-GCCCAGAGGCAAAGAGGCAAT-3’| 94°C 30s, 56°C 30s, 72°C 1min, for 30 cycles |
|       | antisense | 5’-AGATCATCTCTGGCTGAGTATCT-3’|                  |

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No potential conflicts of interest were disclosed.

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**Notes on contributors**

Researches on molecular genetic were carried out, and related sequence alignments were assisted to complete, and research manuscripts were recorded and drafted by CB and GCG. Immunoassays were carried out by HJ. The task of sequence alignment was participated in by CH. Whole structure of researches were assistedly designed and main statistical analysis were carried out by HJ. Many significant assistances and coordinations were given by LT on the concept and design of research and drafting of manuscript. The final manuscript was agreed on by all authors.

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