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Protective effect of polypeptide from *Chlamys farreri* on mitochondria in human dermal fibroblasts irradiated by ultraviolet B[1]

WANG Chun-Bo[2], DING Bo-Xiao, GUO Shen-Bo, WANG Yu-Zhen[3], HAN Yan-Tao[3], WANG Yue-Jun[4]

Department of Pharmacology, [3]Department of Physiopathology, Medical College, Qingdao University, Qingdao 266021; [4]Yellow Sea Fishery Research Institute, Qingdao 266071, China

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ABSTRACT

AIM: To study the effect of polypeptide from *Chlamys farreri* (PCF) on mitochondria of human dermal fibroblasts irradiated by ultraviolet B (UVB) *in vitro*. **METHODS**: Malondialdehyde (MDA) and antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) were determined by biochemical methods. Mitochondrial transmembrane potential was measured by flow cytometry. Ultrastructure of fibroblasts was observed with transmission electron microscope. **RESULTS**: UVB (1.176×10^{-4} J· cm^{-2}) induced mitochondria damage in dermal fibroblast and PCF (0.25 %-1 %) reduced the damage in a concentration-dependent manner. Furthermore, PCF also concentration-dependently maintained the stability of mitochondrial transmembrane potential. PCF was able to reduce the MDA formation caused by UVB, meanwhile increased the activities of SOD and GSH-PX. The differences among the PCF groups and UVB model group were significant ($P<0.05$, $P<0.01$). **CONCLUSION**: The UVB-induced mitochondria damage was alleviated by PCF in human dermal fibroblasts.

INTRODUCTION

The recent increase of ultraviolet (UV) rays on Earth due to the increasing size of the ozone hole is suggested to be harmful to life and to accelerate premature photoaging of the skin. The detrimental effects of UV radiation on the skin are associated with the generation of reactive oxygen species (ROS) such as superoxide ion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•) and singlet oxygen ($^1O_2$). Many investigators have detected the generation of ROS in the skin following UV irradiation[1]. Mitochondria can be easily attacked by free radicals[2], meanwhile mitochondria itself can also generate oxygen free radicals[3]. So protection of mitochondria against oxidative damage becomes increasingly important.

Many natural antioxidants have been found and used to inhibit the oxidation. However, these antioxidants mainly come from terrestrial herbs and plants, we seldom read the reports of polypeptides as antioxidants especially for those from marine products. PCF, M_r=800-1000, is a novel marine active material originally isolated from *Chlamys farreri* using bioengineer-

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[2] Correspondence to Prof WANG Chun-Bo.
Phn 86-532-383-8480, ext 3756. Fax 86-532-380-1149.
E-mail liujg666@yahoo.com.cn
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ing technique[4]. Previous studies of our laboratory indicated that PCF exhibited a direct ROS scavenging activity[5] and protective effects on HeLa cells[6] and hairless mouse skin[4] damaged by ultraviolet A. However, relatively little is known about the effect of PCF on ROS-induced mitochondria damage in intact cells, and it is difficult to predict from our previous experiments the actual effects of PCF on the normal skin cell irradiated by UVB *in vitro.*

Solar UV radiation reaching on the earth's surface includes A (320–400 nm) and B (290–320 nm). Although UVB radiation represents 4 % of the total solar UV radiation, UVB can cause more intense skin damage than UVA. UVB is known to generate ROS and result in cell damage[7]. Present study was thus designed to investigate whether PCF was able to reduce the UVB-induced mitochondria damage in cultured human dermal fibroblasts.

MATERIALS AND METHODS

Materials PCF (purity >96 %) was purified and analyzed by HPLC, dissolved in sterile deionized water, and stored at 4 °C. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Co. UVB light source was purchased from Beijing Normal University. The reagent kits for measurement of MDA, SOD, and GSH-PX activities were purchased from Nanjing Institute of Jiancheng Biological Engineering. Rhodamine 123 was obtained from Sigma Chemical Co.

Cell culture and treatment[8] Human dermal fibroblasts were isolated from adolescent foreskin. Briefly, foreskin was treated with 0.25 % dispase for 16 to 18 h. The dermis was separated from the epidermis with forceps. The dermis sheet was then trypsinized for 5 min, and the cells were seeded into 50-cm^2 tissue culture in DMEM supplemented with penicillin G (100 kU/L), streptomycin (100 mg/L), and fetal bovine serum (10 %). The cells were incubated at 37 °C in a humidified CO_2 (5 %) incubator and split twice per week with alternate 1/3 and 1/4 dilutions. Cells used were passages 4 to 10. Fibroblasts were randomly divided into five groups: control group, model group, 0.25 % PCF group, 0.5 % PCF group, and 1 % PCF group. When they were in exponential growth phase, the cells were harvested and resuspended at 3×10^7/L, then transferred to 6-well plates. At the time of experiment, PCF were added to culture medium at concentrations of 0.25 %, 0.5 %, and 1 % respectively. After 30-min incubation, the cells were exposed to UVB radiation at a dose of 1.176×10^{-4} J·cm^{-2} and then incubated for 2 h.

Assay of MDA and antioxidant enzyme activities[9,10] Cells were washed twice with PBS and harvested. First, cells in cold sterile deionized water were resuspended at a concentration of 1×10^{10}/L and then the homogenate of the cells was prepared in ice path through cell homogenizer. Second, the homogenate was centrifuged for biochemical tests. After determining the amount of total protein in the supernatants, the enzymes including SOD, GSH-PX as well as MDA were detected by biochemical method using the supernatants following the instruction of reagent kits.

Measurements of mitochondrial transmembrane potential[11] After irradiation by UVB, the cells were incubated for 2 h and then trypsinized. Cells were washed twice with PBS and resuspended in PBS at a concentration of 1×10^9/L. The solution 500 μL was transferred to a 5-mL culture tube and rhodamine 123 was added at a final concentration of 1 μmol/L. After incubation at 37 °C for 30 min, cells were washed twice with PBS and then analyzed using Becton Dickinson FACS Vantage flow cytometer. Fluoresecent signal intensity was examined with Cellguest software. For each sample 10000 events were collected.

Preparation for transmission electron microscopy[12] The cultured fibroblasts were trypsinized and collected into Eppendorff tube after washing. They were fixed by 2.5 % glutaraldehyde at 0-4 °C and washed by PBS, fixed by osmic acid, then washed by distilled water, and dehydrated by dimethylketone. After embedment in Epon-812, the sample was cut into ultrathin sections (70 nm). The ultrathin sections were dyed with uranium acetate and plumbum citrate. They were examined with JEM-1200EX electron microscopy.

Statistical analysis Data were expressed as mean±SD. One-way ANOVA with Student-Newman-Keuls procedure was used to evaluate difference. A level of 0.05 was used to determine the significance.

RESULTS

PCF attenuated UVB-induced increase of lipid peroxidation Generation of lipid peroxidation in fibroblasts was stimulated by UVB (Tab 1). An obvious concentration-dependent inhibitory effect of PCF on UVB-induced lipid peroxidation was noted in fibroblasts. Pretreatment of cells with 0.25 %-1 % PCF reduced the MDA concentration (Tab 1).

PCF increased activities of antioxidant enzymes After fibroblasts were irradiated by UVB for 2

Tab 1. Effect of PCF on UVB-induced increase in lipid peroxidation in fibroblasts. *n*=9. Mean±SD. $^{c}P<0.01$ *vs* control group. $^{f}P<0.01$ *vs* UVB model group.

Groups	MDA/μmol· L^{-1}· g^{-1} (protein)
Control	1.6±0.8
UVB model	2.8±0.4^{c}
UVB+0.25 % PCF	2.61±0.06^{f}
UVB+0.5 % PCF	2.46±0.29^{f}
UVB+1 % PCF	2.2±0.4^{f}

h, obvious decrease of antioxidant enzymes, SOD and GSH-PX was observed ($P<0.01$ compared with corresponding control groups). PCF dose-dependently attenuated the decrease of SOD and GSH-PX (Tab 2).

Tab 2. Effect of PCF on antioxidant enzyme activities in fibroblasts. *n*=9. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* UVB model group.

Groups	SOD/kNU· g^{-1} (protein)	GSH-PX/ kU· g^{-1} (protein)
Control	48.7±2.4	79±2.1
UVB model	43.9±1.2^{b}	51.8±2.5^{c}
UVB+0.25 % PCF	46.9±0.7^{e}	85.7±2.4^{f}
UVB+0.5 % PCF	50.1±1.8^{f}	111.7±4.5^{f}
UVB+1 % PCF	58.5±0.4^{f}	123.5±1.7^{f}

PCF inhibited UVB-induced decline of mitochondrial transmembrane potential Mitochondrial transmembrane potential was evaluated by the changes in rhodamine 123 fluorescence intensity. PCF concentration-dependently increased mitochondrial transmembrane potential in UVB-irradiated cells. Rhodamine 123 fluorescences intensity increased from 112.89±0.01 in cells treated with UVB to values between 113.48±0.09 and 159.18±0.11 in cells treated with UVB and 0.25 %-1 % PCF (Tab 3).

PCF protected mitochondria against UVB-induced damage Cell ultrastructure in control group was normal. Mitochondrial structure was clear. The mitochondrial membrane was integrated. After exposure to UVB, mitochondrial structure was not clear, the density of its crista was diminished, vacuolizations were seen, and mitochondrial membrane was broken. PCF (1 %) diminished damage at mitochondrial crista and matrix induced by UVB. The mitochondrial membrane was integrated, almost normal (Fig 1).

Tab 3. Effect of PCF on UVB-induced decline of mitochondrial transmembrane potential. *n*=3. Mean±SD. $^{c}P<0.01$ *vs* control group. $^{f}P<0.01$ *vs* UVB model group.

Groups	Rhodamine 123 fluorescence intensity
Control	235.67±0.01
UVB model	112.89±0.01^{c}
UVB+0.25 % PCF	113.48±0.09^{f}
UVB+0.5 % PCF	150.96±0.10^{f}
UVB+1 % PCF	159.18±0.11^{f}

DISCUSSION

The UVB radiations have been postulated to be a major cause of skin cell damage and shown to induce lipid peroxidation in fibroblasts or keratinocytes. In this work, we successfully established the unitary UVB oxidative damage model of dermal fibroblasts. Exposure of cultured dermal fibroblasts to UVB, which produces ROS, resulted in accumulation of lipid peroxidation. Compared with control group, UVB model group showed a 1.7-fold increase in MDA formation. Our results clearly demonstrated that the supplement of PCF to cultures resulted in a decrease of the MDA formation of lipid peroxidation products by UVB. This effect was proportional to PCF concentration. It could therefore be concluded that the UVB-induced lipid peroxidation of cellular lipids could be inhibited by PCF in a concentration-dependent manner. Furthermore, this result indicated that PCF could protect fibroblasts against the ROS insult.

The lipid peroxidation products alter the activities of numerous enzymes controlling the cellular intermediary metabolism and ion transporters. SOD and GSH-PX were thought to be the major enzymes that protected mitochondria against damage caused by potentially cytotoxic reactivities related to the aging of organism. Some findings even suggested a causal relationship between antioxidant enzyme activities and life span of animal species[13]. SOD can dismutate two O_2^- into H_2O_2 and O_2 and, in the presence of GSH-PX, can protect mitochondria from the damage induced by O_2^-[14]. Our studies showed that UVB could markedly decrease SOD and GSH-PX activities in cultured der-

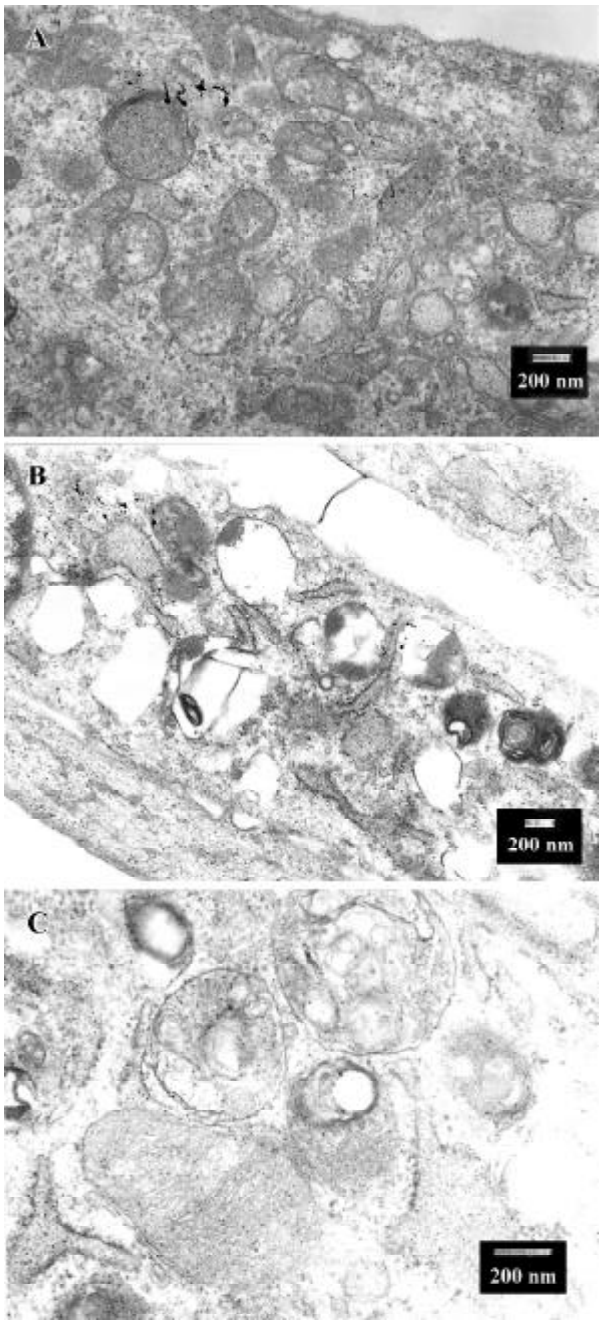

Fig 1. Electron micrographs of cultured fibroblasts. A: Control group. B: UVB model group. C: UVB+1 % PCF group.

mal fibroblasts. The inhibitory effect of UVB on antioxidant enzymes could be attenuated by PCF, suggesting that the PCF abilities might contribute to its mitochondria-protective effect.

Taken together, it was also of note that, under our experimental conditions, the activity of SOD in 1 % PCF group was slightly higher than that in control group, while the activity of GSH-PX in 1 % PCF group was markedly higher than that in control group. Such a phenomenon has also been observed in our previous experimental model: HeLa cells exposed to UVA[6]. Up to now, information on this topic was lacking. Thus the modulation of the activity of GSH-PX to UV radiation by PCF appears to be an interesting way of research.

The process that mitochondria uptake rhodamine 123 is dependent on mitochondrial transmembrane potential, so detection of rhodamine 123 fluorescence intensity can indirectly indicate mitochondrial transmembrane potential[11]. The decline of mitochondrial transmembrane potential can cause serious damage of mitochondria[15]. In this study, mitochondrial transmembrane potential was greatly decreased by UVB in fibroblasts. After treatment with PCF, mitochondrial transmembrane potential was increased correspondently, compared with UVB model group. Our results suggested that PCF could inhibit the decline of mitochondrial transmembrane potential in fibroblasts caused by UVB, thus protect mitochondria against the UVB-induced damage.

To further confirm the protective effect of PCF on mitochondria, we employed transmission electron microscopy to detect ultrastructure of mitochondria. Our experiments showed that: (1) the prominent pathological changes induced by UVB, such as broken-membrane, crista diminution, and vacuolizations formation; (2) PCF (0.25 %-1 %) concentration-dependently alleviates UVB-induced mitochondrial damage, especially at the concentration of 1 %. Mitochondria structure in cells treated with UVB and 1 % PCF was almost normal.

In conclusion, PCF protected mitochondria against UVB-induced injury. The protective action of PCF may be attributed to its antiperoxidative effect, suggesting that PCF could prevent oxygen free radical-induced disorders.

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