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Polypeptide from *Chlamys farreri* inhibits UVB-induced apoptosis of HaCaT cells via iNOS/NO and HSP90*

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Abstract Polypeptide from *Chlamys farreri* (PCF) is a novel marine bioactive product that was isolated from the gonochoric Chinese scallop *Chlamys farreri,* and was found to be an effective antioxidant in our recent studies. In this study, we investigated the effect of PCF on ultraviolet B (UVB)-induced apoptosis of HaCaT cells and the intracellular signaling pathways involved. Pretreatment with the inducible nitric oxide synthase (iNOS) inhibitor S-methylisothiourea sulfate inhibited UVB-induced apoptosis, indicating that iNOS and NO play important roles in apoptosis. On the other hand, the inhibition of UVB-induced apoptosis in the immortalized keratinocyte (HaCaT) cells by PCF was estimated using a DNA ladder. PCF treatment inhibited UVB-induced iNOS activation, as determined by RT-PCR, NO production, as determined by ESR, and up-regulated heat shock protein (HSP) 90 activation, as determined by Western blotting. Our results indicate that iNOS and NO are involved in UVB-induced apoptosis of HaCaT cells and the protective effect of PCF against UVB irradiation is exerted by suppressing the expression of iNOS, followed by inhibition of NO release and enhanced activation of HSP90.

Keyword: apoptosis; HaCaT cells; HSP; iNOS/NO; Polypeptide from *Chlamys farreri* (PCF); ultraviolet B (UVB)

1 INTRODUCTION

Human beings are exposed to ultraviolet (UV) radiation from the sun every day, and the skin suffers directly from the deleterious effects of UV irradiation. Many researchers have focused on the role of reactive oxygen species (ROS), which participate in UV-induced apoptosis (Afaq et al., 2002; Heck et al., 2004). Studies from our laboratory have revealed that Polypeptide from *Chlamys farreri* (PCF) could inhibit UV-induced apoptosis in immortalized keratinocyte (HaCaT) cells by reducing the production of ROS, inhibiting phospho-P38 mitogen-activated protein kinase (MAPK) and other pathways (Li et al., 2007; Gao et al., 2007; Li et al., 2008; Xing et al., 2008).

With the increasing awareness of the harmfulness of UV to people, researchers are showing further interest in NO and nitric oxide synthase (NOS), which play very important roles in UV-induced oxidative damage and apoptosis in skin (Weller et al., 2003; Cals-Grierson et al., 2004; Jańczyk et al., 2007). Weller demonstrated that the release of NO and melanogenesis is increased in skin cells after UV irradiation (Weller, 2003). Meanwhile, hyper-pigmentation and damage to skin cells can be relieved by topical application of a NOS inhibiter. Cals-Grierson revealed that virtually all skin cells are capable of expressing inducible NOS (iNOS) in response to UV irradiation or other inflammatory skin conditions (Cals-Grierson et al., 2004). The NO liberated by UV irradiation plays an important role in initiating melanogenesis, erythema and immune suppression. Studies from our laboratory, using an orthogonal experimental design, have revealed that the UVB from daylight can be simulated by 20 mJ/cm^2 UVB from UVB lamps, and this could

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induce apoptosis in HaCaT cells (Li et al., 2008). This study imitated the model and detected the release of NO by electron spin resonance (ESR) and we determined the expression of iNOS to investigate their roles in apoptosis of HaCaT cells.

Heat shock proteins (HSPs), also known as chaperones, which may mediate anti-oxidative damage and anti-apoptosis activity, are currently major focuses of research. Low or physiological concentrations of NO were reported to prevent cells from undergoing apoptosis, were mediated by many factors including HSPs and other pathways (Chung et al., 2001). However, whether high concentrations of NO induced by UVB irradiation affect the expression of HSPs is not well known.

2 MATERIALS AND METHODS

2.1 Materials

PCF (Yellow Sea Fishery Research Institute, China) was isolated from *Chlamys farreri*, purified and analyzed by HPLC, dissolved in sterile deionized water, and stored at 4°C. The purity of PCF used in this study is more than 96%. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Co. HSP90 antibody, horseradish peroxidase-conjugated secondary antibodies and the iNOS inhibitor S-methylisothiourea sulfate (SMT) were purchased from Wuhan Boster Biological technology Co. Ltd. (Wuhan, China). β-actin antibody was purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China). iNOS primers and glyceraldehyde phosphate dehydrogenase (GAPDH) were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

2.2 Cell culture and treatment

The spontaneously immortalized human keratinocyte cell line HaCaT, kindly provided by Dr Ding Boxiao (Yonsei University, Korea), was cultured in DMEM supplemented with 10% fetal bovine serum (Dalian Biological Reagent Factory, Dalian, China) and penicillin (100 U/ml) and streptomycin (100 mg/ml), and maintained at 37°C with 5% CO_2 in a humidified atmosphere. The cells were randomly divided into six groups including a control group, a model group, and experimental groups treated with 1.42 mmol/L PCF (PCF1), 2.84 mmol/L PCF (PCF2) or 5.69 mmol/L PCF (PCF3) or with the iNOS inhibitor SMT (1 mmol/L). PCF and SMT were added to the cell suspension to the final concentrations described above. After incubation for 2 h at 37°C, HaCaT cells were washed twice with PBS. Cells with a very thin layer of PBS were irradiated for 30 minutes under UVB lamps (Beijing Normal University, China). The wavelength range of UVB lamps was 290–320 nm, with a peak wavelength at 297 nm. The intensity of radiation reaching the cells, measured by an IL700 radiometer (International Light Inc.), was 11 mW/cm^2. The dosage irradiated to these cells was 20 mJ/cm^2 (Li et al., 2008). Thirty minutes after the UVB irradiation, the HaCaT cells were harvested for the following experiments.

2.3 DNA fragmentation assay

DNA fragmentation is a marker of cell apoptosis. The pattern of DNA fragmentation was analyzed by agarose gel electrophoresis 18 hours after irradiation. Briefly, 1×10^6 cells were centrifuged at 1 000 ×g for 10 minutes. The cell pellets were resuspended in 500 μl of cell lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris–HCl, pH 7.5, 10 mmol/L EDTA, 0.5% SDS and 500 mg/L proteinase K) and incubated overnight at 50°C. After incubation, the cell lysate was extracted with phenol/chloroform/isopropyl alcohol (25:24:1, v/v). The DNA was precipitated with sodium acetate and ethanol at -20°C overnight and washed with 70% ethanol. The DNA pellets were dissolved in 1× TE buffer and were incubated with RNase A (20 μg/L) for 30 minutes at 37°C. The DNA samples were separated by horizontal electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

2.4 Electron spin resonance technology (ESR)

The release of NO in HaCaT cells was assayed 18 hours after UVB irradiation. Our laboratory has previously shown that the inhibition ratio of 2.84 mmol/L PCF on apoptosis of HaCaT cells induced by 20 mJ/cm^2 UVB is 50% (Li et al., 2008). In the present study, the cells were randomly divided into four groups: a control group, a model group, a group treated with 2.84 mmol/L PCF and a group treated with the iNOS inhibitor SMT (1 mmol/L) group. Each group comprised about 5×10^7 cells. The release of NO was assayed 18 hours after UVB irradiation. The cells harvested were homogenized at 0°C in phospho-Hepes buffer (0.038 mol/L NaH_2PO_4, 0.162 mol/L Na_2HPO_4, 0.01 mmol/L EDTA, 10 mmol/L Hepes, 0.32 mol/L sucrose, 5 mmol/L mercaptoethanol, 10 mmol/L N-tert-Butyl-a-phenylnitrone [PBN], 0.5% Tween-80, and

2 mmol/L diethylenetriamine pentacetic acid [DETAPAC]). The fragments were centrifuged at 12 000 ×g for 10 minutes at 4°C. About 1.4 ml of supernatant was removed and placed in a warm bath for 30 minutes in the presence of 30 μl of a buffer containing 0.5 mol/L $Na_2S_2O_4$, 0.6 mol/L diethyldithiocarbamate (DETC), 10 mmol/L L-Arg, 0.3 mol/L $FeSO_4$. Then, 300 μl of acetic ether was added and the mixture was vortexed for 15 seconds and centrifuged at 12 000 ×g for 8 minutes at 4°C. The organic phase was assayed for ESR (Central Magnetic Field, 3385 G; sweep width, 400 G; power, 20 mW; magnification 4×10^5).

2.5 Reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of iNOS mRNA in HaCaT cells was assayed 12 hours after UVB irradiation. Total RNA was extracted using Beyozol reagent, in accordance with the manufacturer's instructions (Beyozol Beyotime Biotechnology, China). The sequences of the specific oligonucleotide primers were as follows: 5′-CCT GCC AAC GTG GAA TTC AC-3′ and 5′-TTC CCG AAA CCA CTC GTA TTT G-3′ for iNOS (Sangon Biological Engineering Technology, Shanghai, China); 5′-CGT GGA AGG ACT CAT GAC CA-3′ and 5′-TCC AGG GGT CTT ACT CCT TG-3′ for GAPDH (Sangon Biological Engineering Technology, Shanghai, China). DNA was amplified immediately with a single cycle at 95°C for 3 minutes and 35 cycles at 94°C for 45 s and 60°C for 60 s and 72°C for 90 s, with a final extension step at 72°C for 5 minutes. The PCR products were mixed with 2 ml of gel loading buffer, electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining. The intensity of each band was calibrated to the standard molecular marker on the same gel and was normalized against the intensity of GAPDH.

2.6 Western blotting analysis

The expression of HSP90 in HaCaT cells was assayed 24 hours after UVB irradiation. Total cellular protein was extracted in lysis buffer (20 mmol/L Tris–HC1, pH 7.5, 150 mmol/L NaC1, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 mg/L leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride). The cells were scraped and centrifuged at 10 000 ×g for 10 minutes at 4°C. The protein levels were quantified using a bicinchoninic acid assay (Beyotime Biotechnology, China). For Western blotting analysis, 40 μg of protein was resolved on 12% SDS-polyacrylamide gel. The protein was then transferred to a nitrocellulose membrane. The membrane was then blocked with 5% BSA in TBST (25 mmol/L Tris, 140 mmol/L NaCl, 3 mmol/L KCl, 0.05% Tween-20, pH 8.0) for 1 hour at room temperature and subsequently incubated with rabbit antibodies (1:200) overnight at 4°C. Goat anti-rabbit secondary antibodies were diluted at 1:500 in 5% BSA/TBST and were incubated with membranes for 1 hour at room temperature. The membranes were washed three times for 5 minutes each in TBST between antibody incubations. The protein bands were visualized using a diamino-benzidine detection kit (Boster Biotechnology, Wuhan, China). The densities of the bands were analyzed with Quantity One analysis software.

2.7 Statistical analysis

Data are expressed as means±SD. Statistical significance was determined by one-way ANOVA. A difference was considered to be statistically significant at $P<0.05$.

3 RESULTS

3.1 Inhibitory effects of PCF and iNOS inhibitor SMT on apoptosis of HaCaT cells induced by UVB irradiation

DNA fragmentation, a hallmark of apoptosis, was confirmed using agarose gel electrophoresis. A typical ladder pattern of inter-nucleosomal DNA fragmentation was observed in the UVB model group compared with the control group (Fig.1). DNA fragmentation was not obvious in any of the PCF groups (1.42–5.69 mm), confirming that UVB directly induces DNA damage and DNA fragmentation was reduced by PCF pretreatment (Fig.1). The iNOS inhibitor SMT also demonstrated anti-apoptotic activity (Fig.1), suggesting that the UVB-induced apoptosis of HaCaT cells may be mediated by iNOS.

3.2 Effect of PCF on the release of NO induced by UVB irradiation in HaCaT cells

We assayed the release of NO after UVB irradiation in HaCaT cells by ESR. The results in Fig.2 indicate that NO production increased after UVB irradiation ($P<0.01$) and was decreased by the iNOS inhibitor SMT ($P<0.01$). Furthermore, pretreatment with 2.84 mmol/L PCF inhibited NO production ($P<0.01$).

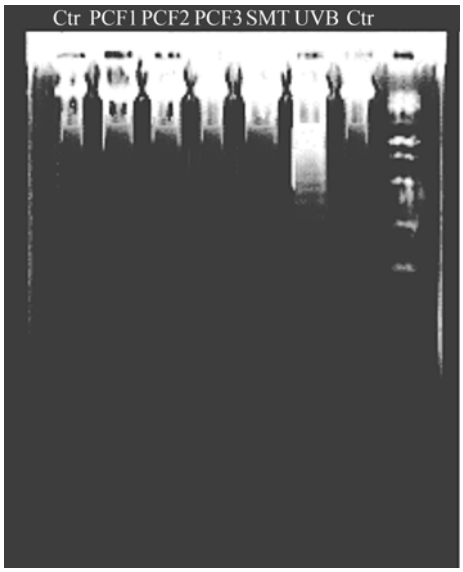

Fig.1 Effects of PCF and SMT on UVB-induced DNA fragmentation in HaCaT cells

Lane 1: control group; Lane 2: 1.42 mmol/L PCF group (PCF1); Lane 3: 2.84 mmol/L PCF group (PCF2); Lane 4: 5.69 mmol/L PCF group (PCF3); Lane 5: SMT (1 mmol/L) group; Lane 6: UVB group; Lane 7: control group

3.3 Effect of PCF on the expression of iNOS induced by UVB irradiation in HaCaT cells

Our RT-PCR (Fig.3) results show that UVB irradiation increased the expression of iNOS compared with the control group (P<0.01). Pretreatment with PCF dose-dependently (1.42–5.69 mmol/L) decreased the expression of iNOS (P<0.05) confirming that PCF can inhibit UVB-induced apoptosis of HaCaT cells partly through inhibition of iNOS.

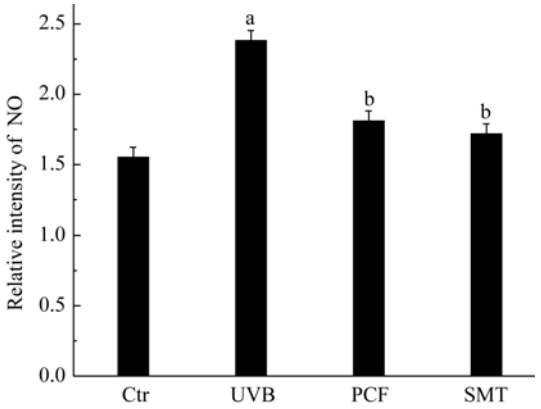

Fig.2 The effect of PCF on the time-dependent release of NO from HaCaT cells exposed to UVB irradiation

Ctr: Control; UVB: Cells were irradiated with 20 mJ/cm UVB; PCF: Cells were pretreated with 2.84 mmol/L PCF for 2 hours prior to UVB irradiation. After irradiation, the cells were incubated for 18 hours, and NO was detected by ESR. Results are expressed as the relatively intensity of NO. P_a<0.01 compared with the control group. P_b<0.01 compared with the UVB group

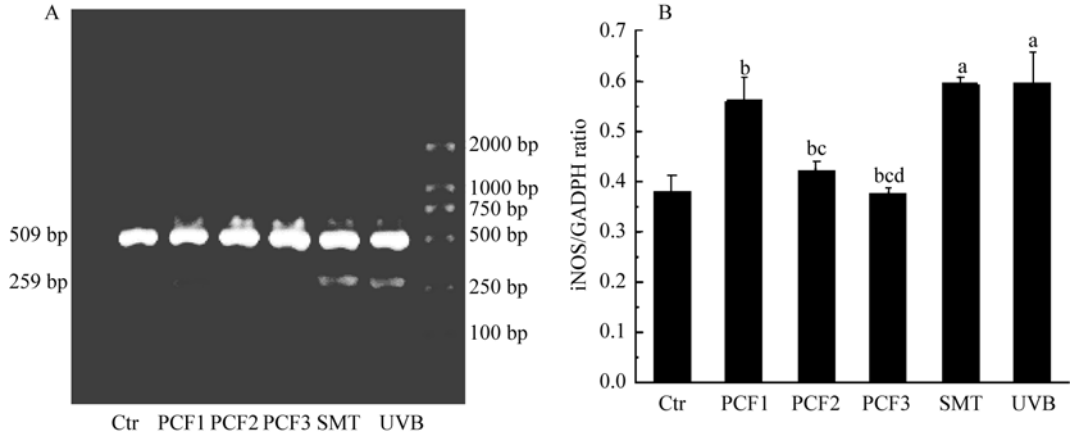

Fig.3 Effects of PCF on the expression of iNOS mRNA induced by UVB irradiation in HaCaT cells

Ctr: Control; UVB: Cells were irradiated with 20 mJ/cm UVB; PCF1–3: Cells were pretreated with 1.42, 2.84 or 5.69 mmol/l PCF, respectively, for 2 hours prior to UVB irradiation. After irradiation, the cells were incubated for 12 hours, and iNOS was detected by RT-PCR. The results shown are representative of three independent experiments. (B) Quantification of the results in (A). Results are expressed as the ratio of the expression level of iNOS over GAPDH. P_a<0.01 compared with the control group. P_b<0.05 compared with the UVB irradiation group. P_c<0.01 compared with the UVB+PCF1 group. P_d<0.05 compared with the UVB+PCF2 group

3.4 Effect of PCF on the expression of HSP90 induced by UVB irradiation in HaCaT cells

Our results show that UVB irradiation increased the expression of HSP90, while the iNOS inhibitor SMT decreased the expression of HSP90. PCF pretreatment dose-dependently enhanced the expression of HSP90, which might not be via iNOS and NO pathway (Fig.4).

4 DISCUSSION

The mechanism mediating cell apoptosis by NO is one of the most interesting fields. UVB radiation is a

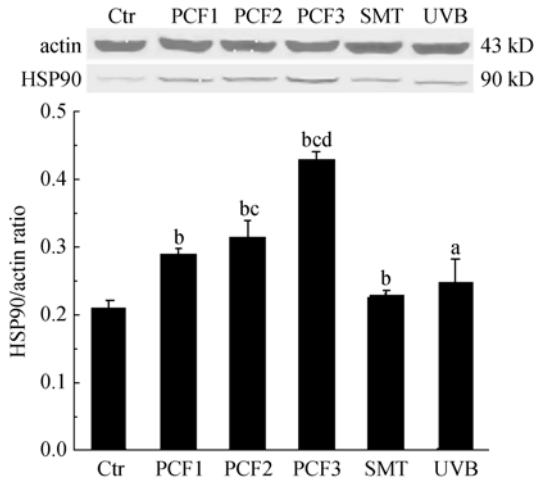

Fig.4 Effects of PCF on the expression of HSP90 induced by UVB irradiation in HaCaT cells

Ctr: Control; UVB: Cells were irradiated with 20 mJ/cm UVB; PCF1–3: Cells were pretreated with 1.42, 2.84 or 5.69 mmol/L PCF, respectively, for 2 hours prior to UVB irradiation. After irradiation, the cells were incubated for 24 hours, and HSP90 was detected by Western blotting. The results shown are representative of three independent experiments. The results are expressed as the ratio of the expression level of iNOS over GAPDH. P_a<0.05 compared with the control group. P_b<0.01 compared with the UVB irradiation group. P_c<0.05 compared with the UVB+PCF1 group. P_d<0.01 compared with the UVB+PCF2 group

potent inducer of NO, which has been implicated in various skin disorders. Our results showed that 20 mJ/cm^2 UVB could significantly induce apoptosis in HaCaT cells at 18 hours after irradiation, which indicates that UV irradiation from daylight can induce apoptosis in HaCaT cells. At the same time, we also determined using ESR that the release of NO was increased at 18 hours after UVB irradiation. We also found that this phenomenon could be reduced by pretreatment with the iNOS inhibitor SMT. Therefore, we can presume that NO plays an important role in UVB-induced apoptosis in HaCaT cells, which might be mediated by iNOS.

The iNOS gene is primarily under transcriptional control and is induced in a variety of conditions such as oxidative stress, and produces NO in 1000-fold larger quantities than the constitutive enzymes eNOS and nNOS (Beck et al., 1999). Prior studies have revealed that the expression of iNOS was induced by UVB irradiation in HaCaT cell and was significant from 24 h, and longer, after UVB irradiation (Chang et al., 2002; Song et al., 2006); however, we observed marked DNA fragmentation in the HaCaT cells and release of NO at 18 hours after UVB irradiation, which could be inhibited by the iNOS inhibitor SMT. To confirm the source of NO production, we assessed iNOS activation in HaCaT cells cultivated 12 hours after UVB irradiation by RT-PCR. We found that the expression of iNOS was increased at 12 hours after UVB irradiation. Thus, iNOS and its product may participate in UVB-induced apoptosis in HaCaT cells.

HSPs, also known as chaperones, play crucial roles in the folding/unfolding of proteins, assembly of multiprotein complexes, transport/sorting of proteins into correct subcellular compartments, cell-cycle control and signaling, and protection of cells against stress/apoptosis. It was reported that low or physiological concentrations of NO could protect cells from apoptosis and the antiapoptotic mechanism could involve the expression of protective genes such as HSPs (Chung et al., 2001). Our results revealed that the expression of HSP90 was increased in HaCaT cells after UVB irradiation. However, this effect was not significant when the cells were pretreated with the iNOS inhibitor SMT. Therefore, it appears that HSP90 is activated by high concentrations of NO induced by iNOS after 20 mJ/cm^2 of UVB irradiation to promote its anti-apoptotic effect in HaCaT cells.

PCF has been shown to protect HaCaT cells from UV damage via several cell signaling pathways by studies performed in our laboratory. In this study, we pretreated HaCaT cells with PCF to investigate the relationship between the protective effect of PCF and the UVB-induced activation of iNOS and its subsequent functions. Our results show that PCF could protect HaCaT cells from UVB-induced apoptosis. On one hand, PCF inhibited the expression of iNOS and decreased NO production in a dose-dependent manner; on the other hand, PCF upregulated the expression of HSP90 in a dose-dependent manner. As a result, we suggest that inhibition of the iNOS gene and NO release is involved in the mechanism by which PCF protects HaCaT cells from apoptosis induced by UVB. Although HSPs cannot be activated by NO in HaCaT cells pretreated with PCF, HSP90 can be upregulated by PCF to elicit its anti-apoptotic effects.

Taken together, our observations suggest that iNOS, NO and HSP90 are involved in UVB-induced apoptosis in HaCaT cells. Furthermore, PCF may inhibit the expression of iNOS and the release of NO, and upregulate HSP90 gene to protect HaCaT cells from UVB-induced apoptosis.

References

Afaq F, Mukhtar H. 2002. Photochemoprevention by botanical antioxidants. *Skin. Pharmacol. Appl. Skin. Physiol*., **15**: 297-306.

Beck K F, Eberhardt W, Frank S, Huwiler A, Messmer U K, Mühl H, Pfeilschifter J. 1999. Inducible NO synthase: role in cellular signaling. *J. Exp. Biol.*, **202**(Pt 6): 645-53.

Cals-Grierson M M, Ormerod A D. 2004. Nitric oxide function in the skin. *Nitric Oxide. Jun*., **10**(4): 179-193.

Chang D S, Seo S J, Hong C K. 2002. The effect of amniotic membrane extract on the expression of iNOS mRNA and generation of NO in HaCaT cell by ultraviolet B irradiation. *Photodermatol Photoimmunol Photomed.*, **18**(6): 280-286.

Chung H T, Pae H O, Choi B M, Billiar T R, Kim Y M. 2001. Nitric oxide as a bioregulator of apoptosis. *Biochem. Biophys. Res. Commun.*, **282**(5): 1 075-1 079.

Gao M Q, Guo S B, Chen X H, Du W, Wang C B. 2007. Molecular mechanisms of polypeptide from Chlamys farreri protecting HaCaT cells from apoptosis induced by UVA plus UVB. *Acta Pharmacol. Sin.*, **28**(7): 1 007-1 014.

Heck D E, Gerecke D R, Vetrano A M, Laskin J D. 2004. Solar ultraviolet radiation as a trigger of cell signal transduction. *Toxicol. Appl. Pharmacol.*, **195**: 288-297.

Jańczyk A, Garcia-Lopez M A, Fernandez-Peñas P, Alonso-Lebrero J L, Benedicto I, López-Cabrera M, Gonzalez S. 2007. A Polypodium leucotomos extract inhibits solar-simulated radiation-induced TNF-alpha and iNOS expression, transcriptional activation and apoptosis. *Exp. Dermatol.*, **16**(10): 823-829.

Li J L, Liu N, Chen X H, Sun M, Wang C B. 2007. Inhibition of UVA-induced apoptotic signaling pathway by polypeptide from Chlamys farreri in human HaCaT keratinocytes. *Radiat. Environ. Biophys.*, **46**(3): 263-268.

Li B H, Wang H B, Quan X H, Wang Y J, Wang C B. 2008. Polypeptide from Chlamys farreri inhibits UVB-induced HaCaT cells apoptosis via Fas pathway. *Chinese Pharmaceutical Journal*, **43**(15): 1 130-1 136. (in Chinese)

Song X Z, Bi Z G, Xu A E. 2006. Green tea polyphenol epigallocatechin-3-gallate inhibits the expression of nitric oxide synthase and generation of nitric oxide induced by ultraviolet B in HaCaT cells. *Chin. Med. J. (Engl.)*, **119**(4): 282-287.

Weller R. 2003. Nitric oxide: a key mediator in cutaneous physiology. *Clin. Exp. Dermatol*., **28**(5): 511-514.

Xing Y X, Li P, Miao Y X, Du W, Wang C B. 2008. Involvement of ROS/ASMase/JNK signaling pathway in inhibiting UVA-induced apoptosis of HaCaT cells by polypeptide from Chlamys farreri. *Free Radic. Res.*, **42**(1): 12-19.