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# Genistein protects against UVB-induced senescence-like characteristics in human dermal fibroblast by p66Shc down-regulation

Yi Na Wang [a,1], Wei Wu [b,1], Hong Chao Chen [a], Hong Fang [a,*]

[a] Department of Dermatology, 1st Affiliated Hospital, Zhejiang University School of Medicine, 79# Qing Chun Road, Hangzhou 310003, China
[b] State Key Laboratory for Diagnosis and Treatment of Infectious Disease, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China

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ABSTRACT

*Background:* Genistein, as an active compound of dietary antioxidants, has shown considerable promise as an effective agent against aging process. However, the effect of genistein on skin photoaging and the associated mechanism remain unclear.
*Objective:* To delineate the effect of genistein on UVB-induced senescence in human dermal fibroblasts (HDFs) with emphasis on the mechanism of oxidative pathway regulated by p66Shc involved in the events.
*Methods:* HDFs were induced to premature senescence by repetitive subcytotoxic doses of UVB irradiation. Cellular apoptosis and DNA cell cycle were analyzed using flow cytometry. Intracellular levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were detected by ELISA. Mutation levels of two large deletions of mitochondrial DNA, 4977 bp and 3895 bp deletion, were determined by quantitative PCR. Western blot was applied to detect the expression and activation of p66Shc (the 66-kilodalton isoform of the growth factor adapter Shc) and FKHRL1 (a forkhead protein that is intimately linked with intracellular oxidation).
*Results:* Strong activity of senescence-associated beta-galactosidase (SA-β-gal), high percent of cell apoptosis as well as cell cycle arrest in G0/G1 phase, and increased intracellular oxidative stress were observed in HDFs irradiated by UVB. Genistein exerted dramatically protective effects on HDFs in a dose-dependent manner. Elevated copy numbers of large deletions in mitochondrial DNA were also inhibited by genistein. Down-regulation of total and phosphorylated p66Shc on Ser36, as well as FKHRL1 and its phosphorylation on Thr32, were observed after genistein treatment.
*Conclusion:* The results indicate that genistein protects UVB-induced senescence-like characteristics in HDFs via maintenance of antioxidant enzyme activities and modulation of mitochondrial oxidative stress through down-regulation of a p66Shc-dependent signaling pathway, which may provide potential prevention against skin aging and even photoaging.


## 1. Introduction

It has been noticed that the appearance of facial wrinkling in the Asian population is delayed for about 10 years when compared to the Caucasian population. The pattern and degree of facial wrinkling is different as well. There are many factors contributing to this difference, such as lifestyle, genetic background, and nutrition. The Asian diet is well known for being rich in soy or soy-containing products and the estrogen-like compounds in soy protein, along with their antioxidant activities, are regarded as potential weapons against the aging process.

Isoflavones, a group of polyphenolic compounds found in and isolated from a number of plants, with soybeans and soy products like tofu and textured vegetable protein being the primary food source, have attracted a great deal of interest, especially for possible properties in the prevention and treatment of cancer and chronic disease including cardiovascular diseases and diabetes mellitus [1,2]. Recent studies further suggested that isoflavones might act as a photoprotection and inhibit the initiation and promotion of skin carcinomas [3].

One of the main isoflavones is genistein. Genistein has been reported to modulate molecular functions mainly by acting as a tyrosine kinase inhibitor [4]. Also genistein has anti-oxidation and anti-angiogenesis effects as well as estrogenic activities [5]. Previous evidences have suggested that genistein down-regulates UVB-induced signal transduction cascades in carcinogenesis and confers photo-protective effect in SKH-1 murine skin and in human reconstituted skin [6,7]. Recent studies revealed that genistein prevent UV-induced photoaging and photodamage in human skin [8]. However, although studies have been reported on the

* Corresponding author. Tel.: +86 571 87236340; fax: +86 571 87236385.
*E-mail addresses:* hongfangzy@medmail.com.cn, tango654321@gmail.com (H. Fang).
[1] These authors contributed equally to this work.

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photo-protective effect of genestein on skin tissue, little has been reported about its effects on aging process of skin cells. Here we report the supplementation of genistein, an active isoflavone, in inhibiting UVB-induced cellular senescence-like characteristics of human dermal fibroblasts (HDFs) and the possible mechanisms involved in the activity.

## 2. Materials and methods

### 2.1. Main reagents

Primary human dermal fibroblast (HDFs) was obtained from Biotek Biotechnology Co. (Beijing, China). Genistein was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The annexin V-FITC apoptosis detection kit was from Beckman Coulter Inc. (Beckman Coulter, Fullerton, CA, USA). Primary antibodies and phosphorylated antibodies to shcA, FKHRL1 and secondary antibodies were purchased from Upstate Biotechnology, Inc. (USA). Cytochemical staining kit of SA-β-gal and MTT assay kit was obtained from Beyotime Biotechnology (Haimen, China) and ELISA kit for detection of intracellular SOD and MDA was from Nanjing Jiancheng Biology Science Company (Nanjing, China).

### 2.2. Cell culture, genistein treatment and UVB irradiation

HDFs were cultured in dulbecco's modified eagle's medium (DMEM, Invitrogen, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, UK), penicillin (100 U/ml), and streptomycin (100 mg/l) at 37 °C in a humidified atmosphere containing 5% $CO_2$. Genistein was dissolved in dimethyl sulfoxide (DMSO) and used for the treatment of cells. 50–60% confluent cells were treated with different concentrations of genistein, whereas DMSO treated cells served as control. After 24 h of genistein treatment, cells were subcultured at half confluence ($1 \times 10^4$ cells/$cm^2$) in DMEM + 1% FBS. They were washed once with phosphate-buffered saline (PBS) and exposed to UVB radiation with the lids removed, in a thin layer of PBS using one Philips TL 20 W/01 lamps (Philips, Netherlands) emitting UVB peaking at 311 nm, which were placed 30 cm above the petri dishes. The emitted radiation was checked using a UVR radiometer with a UVB sensor (Bioblock Scientific, Belgium). After irradiation, PBS was replaced by DMEM + 1% FBS. The radiation stress was performed three times a day and the accumulative dose for UVB exposure was 250 mJ/$cm^2$. Control cells were kept in the same culture conditions without UVB exposure.

### 2.3. Cell viability assay (MTT dye assay)

Proliferation of cells was determined by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, approximately 10,000 HDFs were plated in each well of 96-well plates. After overnight incubation, the cells were treated with genistein (0–160 μg/ml) for 12 h, 24 h, 36 h and 48 h. At the various times following genistein treatment, the medium was removed and MTT (20 μl of 5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The plates were spun, and the purple colored precipitates of formazan were dissolved in 150 μl of DMSO. Absorbance was measured at 490 nm using an ELISA plate reader. The reduction in viability of in genistein-treated HDFs was expressed as a percentage compared to non-genistein (DMSO) treated cells and control cells were considered to be 100% viable.

### 2.4. Cytochemical staining

Senescence-associated beta-galactosidase (SA-β-gal) activity was determined at 24 h later after UVB irradiation as described by Dimri et al. [9]. Briefly, the cells were washed with PBS and fixed in 0.2% glutaraldehyde solutions for 5 min. After dilution with PBS, the cells were washed again with PBS and stained with X-gal solution for 6–24 h at 37 °C. The population of SA-β-gal-positive cells was determined by counting 400 cells per dish and photographs were taken using a phase-contrast microscope at 100× and 400× magnification (Olympus, Japan). The proportions of cells positive for the SA-β-gal activity are given as percentage of the total number of cells counted in each dish. The results are expressed as mean of triplicates ± SD.

### 2.5. Flow cytometry

A quantitative assessment of apoptosis and DNA cell cycle was made 24 h later after UVB exposure using the annexin V-FITC apoptosis detection kit as described by the manufacturer. Briefly, for apoptosis analysis, $(0.5–1) \times 10^6$ cells were resuspended at a concentration of $1 \times 10^6$ cells/ml in ice-cold PBS for three times and suspended in 100 μl of binding buffer solution (0.1 M Hepes/NaOH pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$). Cells were then treated with 5 μl of annexin V-FITC and 5 μl of propidium iodide (PI) and placed in the dark at room temperature for 15 min. Fluorescence-activated cell sorting (FACS) analysis was done on a cytometer (Beckman Coulter, Fullerton, CA, USA). For DNA cell cycle analysis, about $1 \times 10^6$ cells were fixed with ice-cold 70% ethanol over night at 4 °C. The cells were subsequently resuspended in PBS for three times and added with 2 ml of Coulter DNA-Prep reagent at room temperature for 30 min. Then cells were stained with PI (50 μg/ml in PBS) for 30 min and data was acquired on a Beckman Coulter XL (Beckman Coulter, Fullerton, CA, USA).

### 2.6. ELISA assay

Intracellular activity of SOD and level of MDA were also detected by ELISA kit under the instruction of manufacturer. The intracellular activity of SOD and level of MDA were calculated directly from the rate of absorbance of the sample versus the average rate of the blank control using the provided ratio table, repeated at least three times for each sample.

### 2.7. Quantitative real-time PCR

Quantitative real-time PCR was performed on an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA, USA) after extraction of genomic DNA from HDFs by total DNA extraction kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. Mutation levels of two large deletions of mitochondrial DNA (mtDNA), 4977 bp deletion and 3895 bp deletion, were determined by quantitative PCR by using specific primers: 4977 bp deletion (8469–13,446 nt in mitochondrial DNA), 5′-ACTACGGTCAATGCTCTG-3′ (sense primer) and 5′-GGAGGTTGAAGTGAGAGGTATG-3′ (antisense primer), 315 bp; 3895 bp deletion (548–4443 nt in mitochondrial DNA) 5′-GCTTCTGGCCACAGCACTTA-3′ (sense primer) and 5′-TAGCGCTGTGAT GAGTGTGC-3′ (antisense primer), 323 bp. The presence of mitochondrial DNA was assessed by PCR amplification of a 83 bp conservative region of the mitochondrial DNA as an internal control by using the following primers: 5′-GATTTGGGTACCACCCAAGTATTG-3′ (sense primer) and 5′-AATATTCA TGGTGGCTGGCAGTA-3′ (antisense primer) [10]. A BioEasy SYBR Green I PCR Kit (Bioer Technology, Hangzhou city, China) was used in this study. The PCR amplification cycles consisted of an initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; and a final extension for 10 min at 72 °C. The dissociation curve for each amplification was analyzed to confirm that there were no non-specific PCR products. The

comparative cycle threshold (Ct) method ($2^{\Delta-Ct}$) was established for the relative quantification of large deletions in mtDNA.

### 2.8. Western blot analysis

Cells were harvested at 24 h following UVB treatment as described above, washed and lysed with lysis buffer. Protein concentration in the resulting lysate was determined using the Lowry assay [11]. Appropriate amounts of protein (about 40 μg) were resolved by electrophoresis in 12% Tris–glycine polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked by 5% bovine albumin serum in TBST (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20) and then incubated overnight with the appropriate primary antibody at 1:1000 or 1:200 dilution (total or phosphorylated antibodies). They were next washed and incubated with the corresponding horseradish peroxidase conjugated secondary antibody at 1:1000 dilution in TBST. Bound secondary antibody was detected using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology Inc., Rockford, IL, USA). Membranes were exposed to light-sensitive film. As a control, the corresponding β-actin levels were determined in the same cell lysates using the antibody for β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### 2.9. Statistical analysis

All values are expressed as means ± SD. Comparisons between treatment groups were made by performing *t*-tests on data derived from triplicates. *P* values of $<0.05$ were considered statistically significant. The results are representative of at least three independent experiments with reproducible results.

## 3. Results

### 3.1. Inhibitory effect of genistein and UVB irradiation on HDFs cell viability

The cytotoxic effect of genistein on HDFs cells was determined with varying concentrations of genistein and times (12–48 h) by MTT assay. As shown in Fig. 1A, no inhibitory effect of genisterin on HDFs cell viability was observed when concentration of genistein was lower than 80 μg/ml. The lowest concentration of genistein that exhibited an inhibitory effect on cell viability was 120 μg/ml for 36 h. Based on these observations, we selected a maximum dose of 80 μg/ml and a time period of 24 h treatment for further mechanistic studies. We also examined the effect of UVB exposure on HDFs cell viability. There was a decrease in the viability of HDFs with increasing doses of UVB radiation (50–350 mJ/cm$^2$) (Fig. 1B), and UVB at doses of 300 and 350 mJ/cm$^2$ significantly inhibited the cell viability. Therefore the subcytotoxic dose of UVB used throughout this study was 250 mJ/cm$^2$.

### 3.2. UVB-induced SA-β-gal activity is suppressed by genistein

At present, the most commonly used method to detect senescent cells is a modified β-gal assay [9]. Detectable β-gal at pH 6 was found to increase during replicative senescence of fibroblast cultures in vitro and in vivo and was absent in immortal cell cultures. This was termed senescence-associated beta-galactosidase or SA-β-gal. Our study revealed strong activity of SA-β-gal in UVB-irradiated HDFs, which, as one of the biomarkers of senescence, indicated that cells were induced to a senescence-like state by UVB exposure. The effect of genistein on SA-β-gal activity was also observed and genistein was found to effectively suppress the expression of SA-β-gal in a dose-dependent manner (Fig. 2).

### 3.3. UVB-induced apoptosis and cell cycle arrest is inhibited by genistein

We next determined the effect of genistein on UVB-induced apoptosis by PI staining and the annexin V method using flow cytometry. As shown in Fig. 3A, UVB irradiation resulted in induction of apoptosis on HDFs, while genistein had an anti-apoptotic effect in a dose-dependant manner. It was observed that treatment of HDFs with 40 and 80 μg/ml of genistein for 24 h decreased the number of early apoptotic cells (LR) significantly when compared to 18.1% in vehicle control (0 μg/ml) group. The number of late apoptotic cells (UR) decreased from 15.9% in 0 μg/ml control group to 3.8% (80 μg/ml of genistein-treated cells). The total percent of apoptotic cells (UR + LR) decreased from 34% in 0 μg/ml control HDFs to 8.5% with 80 μg/ml of genistein treatment for 24 h. Slow proliferation serves as a biochemical event during aging process, and it has been demonstrated that cells can be arrested in some certain phase of cell cycle [12]. For these reasons, we next quantified the extent of cell cycle arrest by flow-cytometric analysis of genistein-treated cells labeled with PI and annexin V. As shown by PI staining and the annexin V method, we

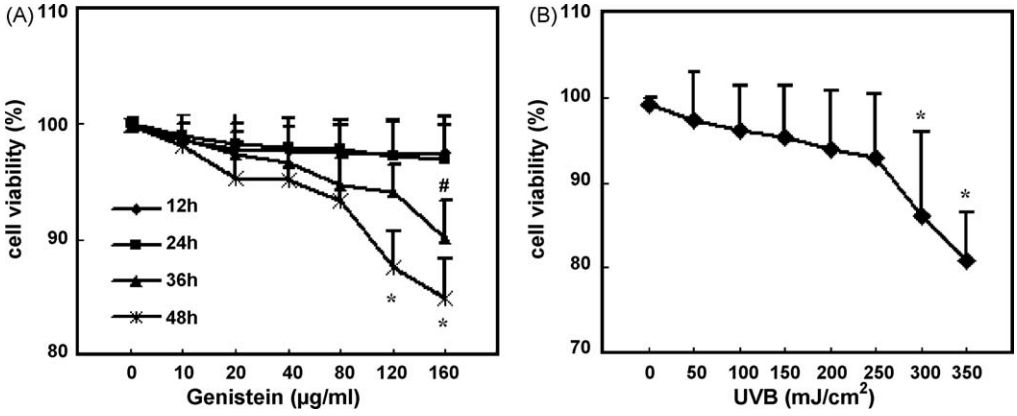


**Fig. 1.** Genistein shows little inhibitory effect on HDFs cell viability, while UVB irradiation induces decrease of HDFs cell viability in a dose-dependent manner. (A) Cell viability was not affected by genistein when concentration was lower than 80 μg/ml. Reduced cell viability of HDFs was observed with genistein treatment (120–160 μg/ml) at 36 and 48 h (compared with 0 μg/ml control group, $^{\#}p < 0.05$ at 36 h, $^{*}p < 0.05$ at 48 h). Viability of cells was determined by the MTT assay as described in materials and methods. (B) Dose-dependent effect of UVB irradiation on HDFs cell viability. Reduced cell viability was observed with UVB irradiation (50–350 mJ/cm$^2$), and UVB at doses of 300 and 350 mJ/cm$^2$ significantly inhibited the cell viability (compared with 0 mJ/cm$^2$ group, $^{*}p < 0.05$). The data are presented as means ± SD ($n = 8$) and all experiments were done in triplicate.

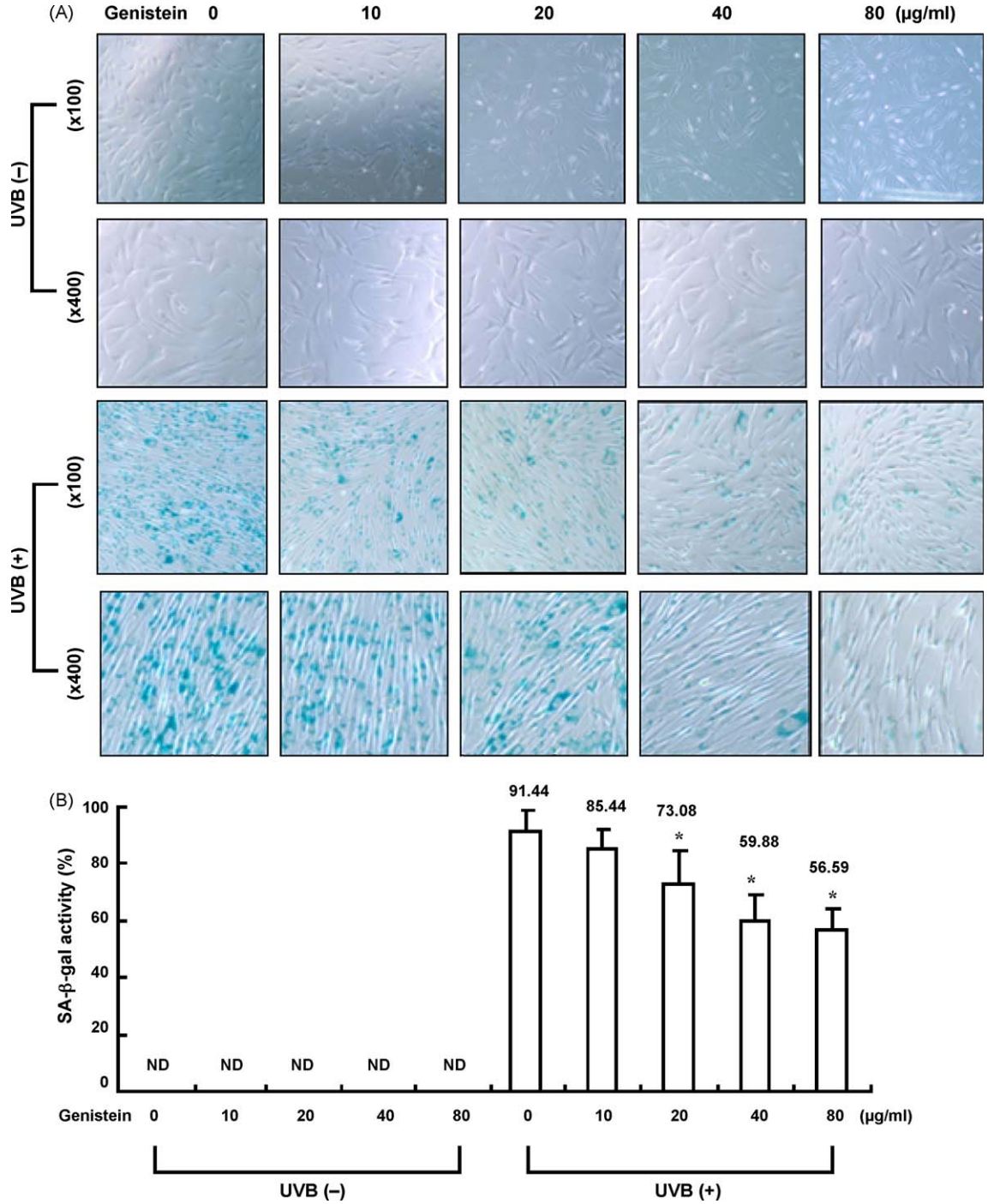


**Fig. 2.** UVB irradiation induces SA-β-gal expression in HDFs and protective effect of genistein on it. The expression of SA-β-gal was detected using cytochemical staining method as described in materials and methods, and cell in blue was considered as positive for SA-β-gal staining. Genistein treatment for 24 h inhibits the activity of SA-β-gal in a dose-dependent manner in HDFs, and a significant inhibitory effect on SA-β-gal activity was observed at doses of 20, 40 and 80 μg/ml (compared with 0 μg/ml control group, $^{*}p < 0.05$). The data are presented as means ± SD ($n = 8$) and all experiments were done in triplicate. ND, not detectable. A representative staining image is shown from three independent experiments with identical results.

found UVB irradiation caused cell cycle arrest, with most HDFs being arrested on G0/G1 phase, and it might play a key role in aging process by initiating cell aging (Fig. 3B). Fig. 3B also shows that HDFs in G0/G1 phase was diminished in quantity upon different doses of genistein (20 μg/ml, 40 μg/ml, and 80 μg/ml), indicating a dose-dependent inhibition in cell cycle arrest by genistein. Taken together, flow cytometry analysis indicated that treatment of HDFs cells with genistein resulted in a dose-dependent inhibitory effect of UVB-induced apoptosis and cell cycle arrest.

### *3.4. Genistein has regulatory effect on decreased activity of intracellular SOD and increased level of MDA induced by UVB*

SOD, as a primary defense, could be a crucial enzyme in eliminating oxygen free radicals and reducing the oxidative stress, while MDA, a pro-oxidative product, was regarded as a marker for free radicals-induced lipid peroxidation [13,14]. Therefore, decreased activity of SOD and increased level of MDA were often used as an indication of oxidative damage. In this experiment, we also

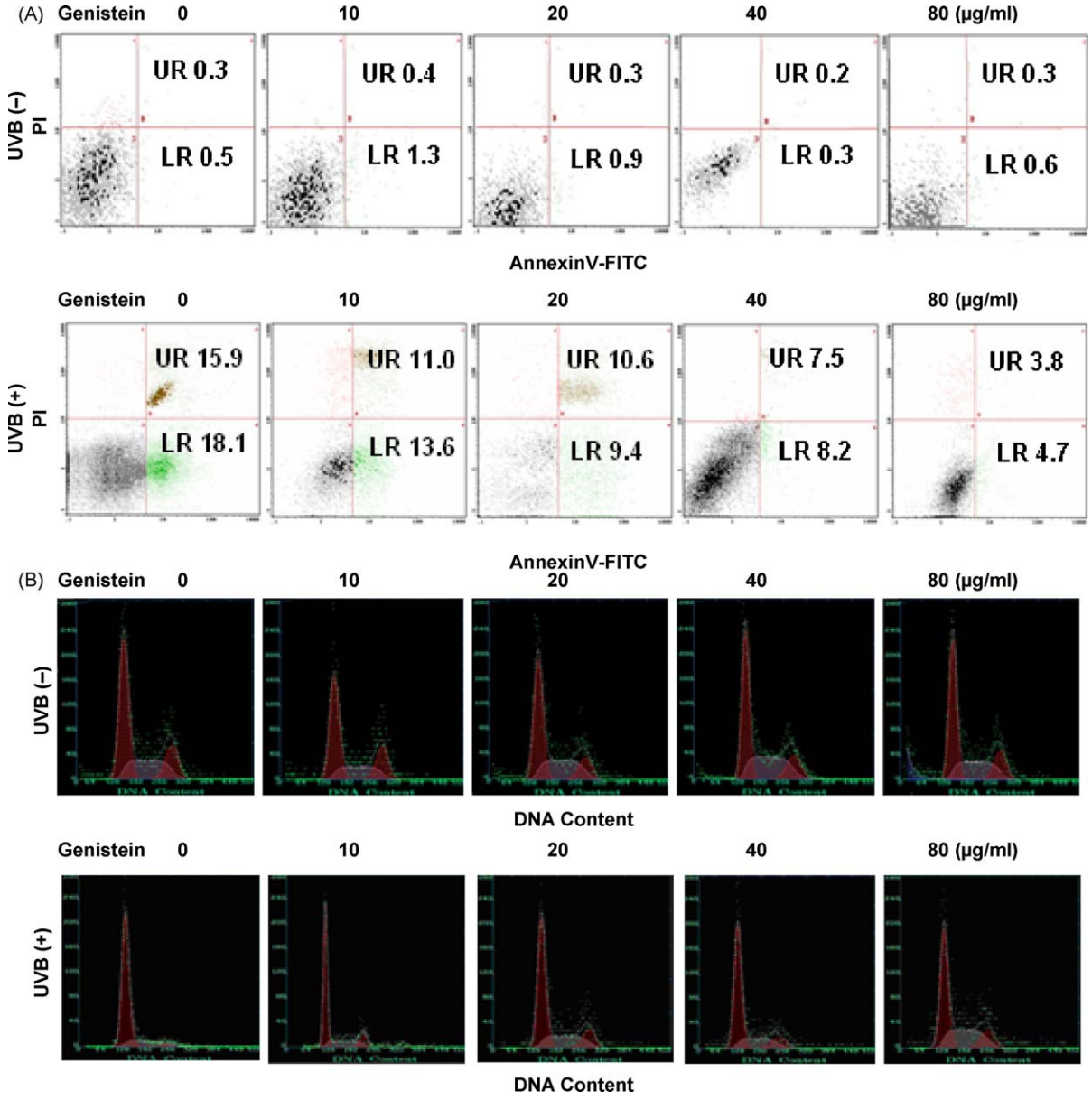


**Fig. 3.** Genistein treatment dose-dependently inhibits UVB-induced apoptosis and cell cycle arrest in HDFs. (A) Flow cytometry of genistein-treated HDFs using a double-staining method with FITC-conjugated annexin V and PI. The LR quadrant indicates the percentage of early apoptotic cells (annexin V-stained cells) and the UR quadrant the percentage of late apoptotic cells (annexin V + PI-stained cells). (B) Genistein treatment positively regulates cell cycle activity in HDFs. Cell cycle was analyzed by flow cytomety using DNA content analysis kit. A representative figure is shown from three independent experiments with identical results.

examined the effect of genistein treatment on the intracellular activity of SOD and level of MDA in HDFs irradiated by UVB. As shown in Fig. 4, even in the absence of UVB irradiation, genistein treatment induced the activity of SOD and inhibited the intracellular level of MDA in HDFs. Repeated exposure to subcytotoxic doses of UVB resulted in a significant decrease in intracellular activity of SOD and a concomitant increase in intracellular level of MDA, strongly suggesting an increased oxidative stress response of HDFs to UVB. However, genistein treatment of HDFs was found to have regulatory effects on the expression of intracellular SOD and MDA. Our data clearly show that genistein treatment caused an increase in intracellular SOD activity, and a significant decrease in MDA level at the highest dose (80 μg/ml). Thus there was an overall shift in the ratio of anti-oxidative and pro-oxidative products following genistein treatment.

### 3.5. UVB-induced mtDNA mutations are down-regulated by genistein

Mitochondrial processes – especially those involving free radical production, damage and propagation – are deeply implicated in the advance of aging [15]. Previous work has provided support for the significance of mtDNA mutation during aging process. There is evidence to suggest that deletions in mtDNA accumulate with age, and the most common and also the most often-assayed mtDNA deletion mutation is 4,977 bp deletion (also known as common deletion) [16,17]. Furthermore, recent investigation revealed a possible link between another kind of large deletion, 3895 bp mtDNA deletion and UVR exposure [10]. Therefore, we next determined the relative copy number of 4977 bp deletion and 3895 bp deletion in mtDNA using real-time quantitative PCR. Products from real-time PCR were confirmed by DNA gel electrophoresis (Fig. 5A). As shown in Fig. 5B, compara-

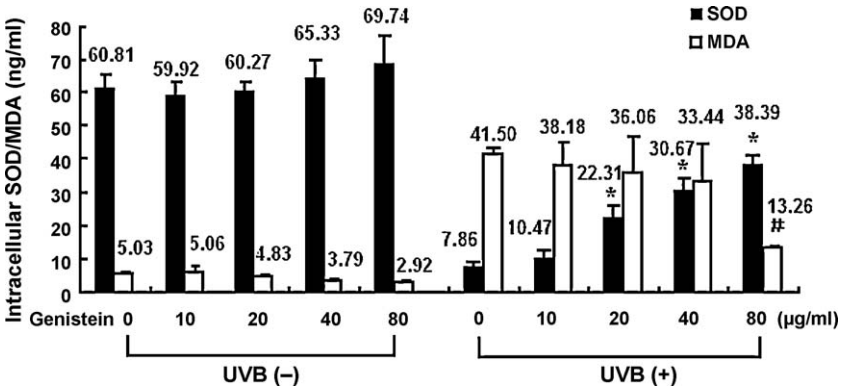


**Fig. 4.** Genistein treatment increases intracellular SOD activity and decreases MDA intracellular level in HDFs. Cells were treated with different concentrations of genistein for 24 h as indicated. Intracellular levels of SOD as well as MDA in HDFs were analyzed by ELISA method as detailed in Section 2. Reduced SOD activity and increased MDA level were observed in HDFs with UVB exposure, while genistein treatment at concentrations of 20, 40 and 80 μg/ml significantly up-regulates the intracellular activity of SOD (compared with 0 μg/ml control group, $^{*}p < 0.05$). Genistein treatment at concentration of 80 μg/ml was observed to down-regulate intracellular level of MDA (compared with 0 μg/ml control group, $^{\#}p < 0.05$). The data are presented as means ± SD ($n = 8$) and all experiments were done in triplicate.

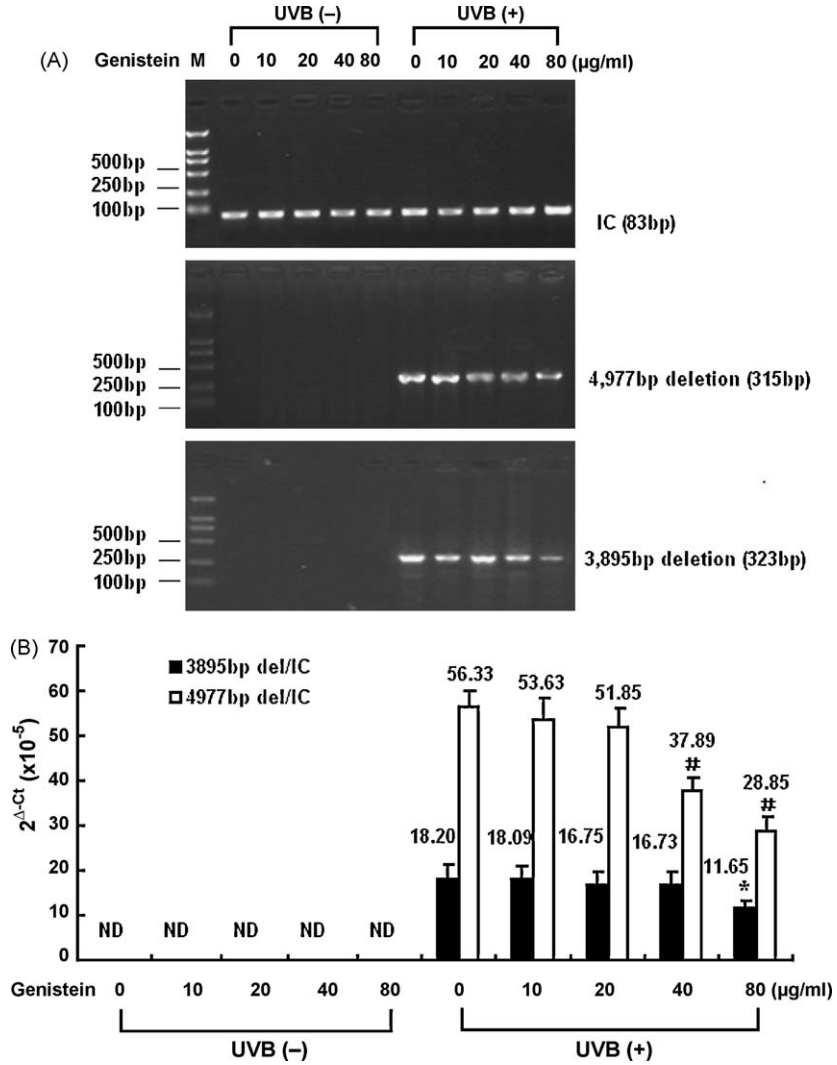


**Fig. 5.** Quantitative polymerase chain reaction analysis revealed that genistein treatment decreases the relative copy number of common deletion (4977 bp deletion) and 3895 bp deletion of mtDNA in UVB-exposed HDFs. (A) Two large deletions of mtDNA were detected in HDFs after UVB irradiation using real-time PCR method and the products were electrophoresised in 2% agarose gel. (B) Genistein treatment in concentrations of 40 and 80 μg/ml significantly down-regulates the relative copy number of common deletion (compared with 0 μg/ml control group, $^{\#}p < 0.05$). Also genestein was found to down-regulate the relative copy number of 3895 bp deletion of mtDNA at 80 μg/ml (compared with 0 μg/ml control group, $^{*}p < 0.05$). The data are presented as means ± SD ($n = 8$) and all experiments were done in triplicate. ND, not detectable.

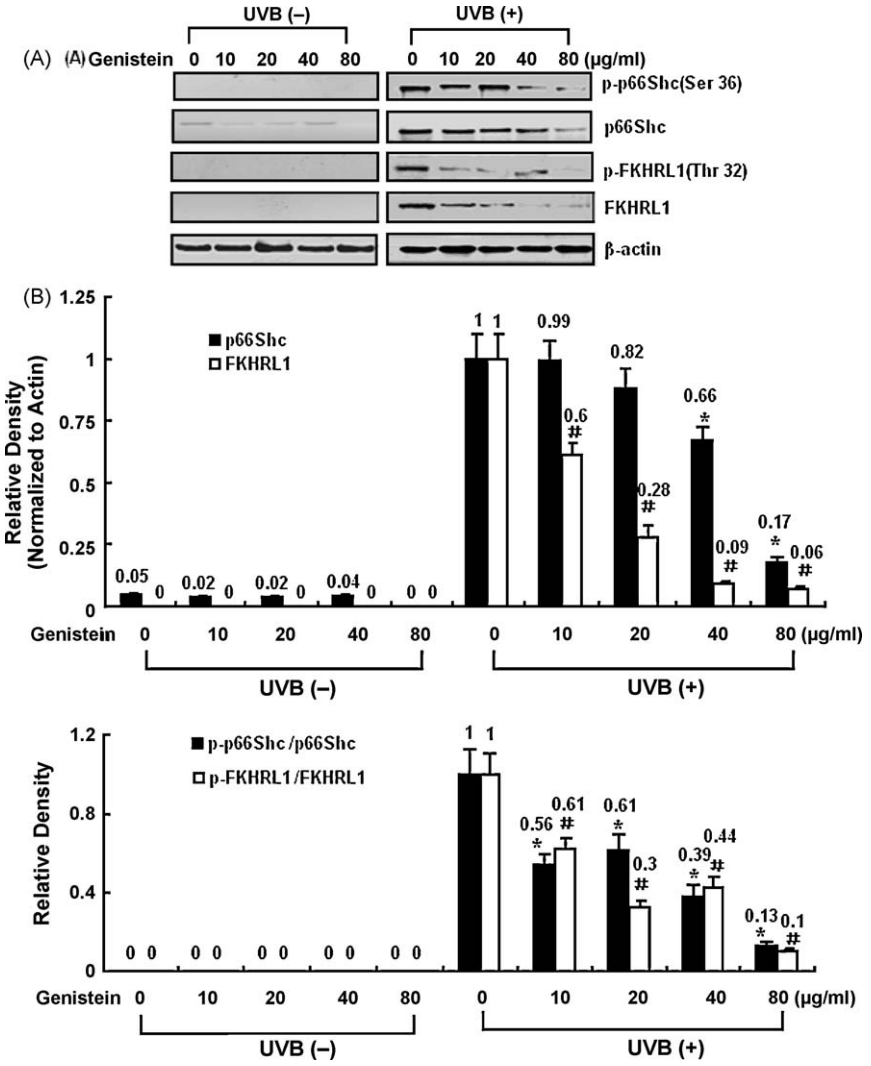


**Fig. 6.** Genistein treatment reduces the expression of p66Shc and FKHRL1 in UVB-exposed HDFs. Cells were treated with different concentrations of genistein for 24 h as indicated. The expression of proteins in treated cells was analyzed by Western blotting as detailed in Section 2. (A) Representative blots are shown from three independent experiments with identical results. (B) Protein expression levels of p66Shc and FKHRL1 were normalized to that of β-actin and are presented as the fold change compared to 0 μg/ml control group. UVB irradiation induced increased expression of p66Shc and FKHRL1, while genistein treatment down-regulates the relative expressions of p66Shc and FKHRL1 dose-dependently (compared with 0 μg/ml control group, $^{\#}p < 0.05$, $^{*}p < 0.05$). (C) Relative expression levels of phospho-Ser36-p66Shc/p66Shc and phospho-Thr32-FKHRL1/FKHRL1 are presented as the fold change compared to 0 μg/ml control group. Increased expression of phosphorylated p66Shc and FKHRL1 were detected in HDFs after UVB irradiation. Genistein treatment at concentrations of 10, 20, 40 and 80 μg/ml significantly down-regulates the expressions of phosphorylated p66Shc (compared with 0 μg/ml control group, $^{*}p < 0.05$) and phosphorylated FKHRL1 (compared with 0 μg/ml control group, $^{\#}p < 0.05$).

tively higher levels of 4977 bp deletion and 3895 bp deletion were found in UVB-induced HDFs, while 24 h-treatment of genistein at dose of 80 μg/ml led to remarkable down-regulations in the relative copy number of these two large deletions of mtDNA.

### 3.6. p66Shc and FKHRL1 is involved in anti-aging effect of genistein

The 66-kilodalton isoform of the growth factor adapter Shc (p66Shc) has been shown to intimately associate with cellular stress, mitochondrial proapoptotic activity and cellular senescence, and it appears to regulate the transcription of antioxidant proteins that scavenge superoxide and hydrogen peroxide. Studies have also linked p66Shc in a signaling pathway that includes FKHRL1, another mammalian forkhead protein that tightly regulates the transcription of antioxidant genes such as MnSOD and catalase [18,19]. Therefore, to further examine whether p66Shc and FKHRL1 was involved in UVB-induced senescence of HDFs and mechanism underlying anti-aging effect of genistein, we analyzed the expression and activation pattern of p66Shc as well as FKHRL1 protein by western blot. As shown in Fig. 6, p66Shc and FKHRL1 were significantly up-regulated by UVB irradiation, and genistein treatment of HDFs resulted in a dose-dependent decrease in their expression. The phosphorylation of p66Shc and FKHRL1 were also down-regulated by different concentrations of genistein, which indicated genistein, as one of antioxidants, counteracts the UVB-induced oxidative stress at least in part by regulating the expression and activation of p66Shc and FKHRL1 protein.

## 4. Discussion

In contrast to other tissues, the skin is subject to both chronological aging as well as environmental insult in the form of UVR, which triggers increased intracellular levels of reactive oxygen species (ROS) and other markers of oxidative stress (e.g., 8-oxo-guanosine, isoprostane, nitrotyrosine), and finally contributes to the aging process of the skin (photoaging). Although UVB is mostly absorbed in the epidermis and predominantly affects epidermal cells, about 10–30% of UVB can penetrate the epidermis,

reach the upper dermis, and do harm to fibroblasts and extracellular matrix as well [20]. Chronic exposure to high intensity UVB causes cumulative damage over time, and it is regarded as one of the most important environmental hazards affecting human skin. Recent studies have observed that UVB has far more potent carcinogenic potential and can also generate ROS, and the molecular mechanisms which are responsible for UVB-induced gene expression differ from those involved in UVA-induced gene regulation [21,22]. However, the exact mechanisms of UVB on skin aging and related signaling pathways are still a matter of debate. In 2005, stress-induced premature senescence (SIPS) of human diploid fibroblasts (HDFs) was induced by a series of subcytotoxic exposures to UVB [23], which helps to explore the mechanisms of UVB-induced senescence, and it has been proved to be an unique and attractive in vitro model for photoaging study [24,25].

In our study, based on this in vitro model system, we evaluated the protective effect of genistein against photoaging in HDFs and its mechanism of action. Consistent with previous studies [23–25], we found that repetitive subcytotoxic doses of UVB irradiation successfully induces the expression of SA-β-gal, causes cell apoptosis and cell cycle arrest in HDFs, while genistein protects cells against senescence-like state in a dose-dependant manner. The result clearly demonstrated the anti-aging effect of genistein in premature HDFs induced by UVB exposure. Genistein is well known for its diverse biological actions including protective effects against cellular aging [26]. Accumulating evidence from cell culture and laboratory animal experiments also indicates that genistein has the potential to prevent or delay the photoaging process [27]. However, we are interested in not only the anti-aging effect in HDFs of genistein but also the underlying molecular mechanism. Previous studies have been observed that genistein might protect against the cellular aging process by modulating oxidative stress and subsequent events [28,29]. It has been well established that cellular aging is accompanied by increased oxidative stress, DNA damage (including mtDNA damage) and altered expression of aging-related genes. Oxidative stress, as the major event in the pathogenesis of the aging process, in turn, increases the occurrence of cell damages, especially by apoptosis, which contributes to cellular aging [30]. Our data showed that genistein treatment resulted in significant inhibition of elevated levels of MDA as well as copy number of large deletion mutations (including 4977 bp deletion and 3895 bp deletion) in mtDNA, and increased the intracellular activity of SOD in UVB-irradiated HDFs. The 4977 bp "common" deletion and 3895 bp deletion in mtDNA have been proposed to be valuable as biomarkers of photodamage or photoaging in skin, and to reflect the dysfunctions of mitochondria [31–34], which are both generators and targets of radical damage in aging. Our results supported the hypothesis that genistein protects skin fibroblasts against senescence by inducing antioxidant enzymes and preventing intracellular oxidative stress that originates in the mitochondria.

Still, it is unclear what events are involved in genistein's effects of protecting both intracellular oxidative stress and mitochondrial damages. In this respect, it is of interest that p66Shc adaptor protein has been involved in mitochondrial signal transduction pathways that regulate cellular responses to oxidative stress. It has been demonstrated that p66Shc plays a critical role in linking mitochondrial functions, oxidative stress and cellular aging. p66Shc translates oxidative damage into cell death by acting as reactive oxygen species producer within mitochondria [35–37], and causes alterations of mitochondrial responses and three-dimensional structure, thus inducing apoptosis [38,39]. Mice lacking expression of p66Shc are less susceptible to oxidative stress and have an extended life span [40]. Phosphorylation of p66Shc at serine 36 in the N-terminal CH2 domain is critical for the cell aging response elicited by oxidative damage, such as UV and $H_2O_2$ treatment [19,41,42]. These findings prompted us to investigate whether p66Shc was involved in the anti-aging effect of genistein in HDFs. We found that expression and phosphorylation of p66Shc on Ser 36 in cultured HDFs increased significantly upon UVB irradiation, while a decrease in total and phosphorylated p66Shc was observed in HDFs after treatment with genistein. These data support the concept that p66Shc is correlated with oxidative stress and plays a pivotal role in the cell aging process, and it participates in genistein's protective effect against senescence-like characteristics at least as an important signaling molecule. Therefore, genistein might block the oxidative and aging pathway by a mechanism regulated by p66Shc.

Additionally, p66Shc protein seems to interfere with oxidative stress-induced aging by regulating forkhead transcription factors [43]. Forkhead family members are well known for their role in regulating stress responses [44–46]. They can be activated by a wide range of stress stimuli, in response to which they can regulate a broad variety of cellular mechanisms (e.g., DNA repair, scavenging of ROS, cell cycle progression, apoptosis, or metabolism) [45–48]. Recent studies revealed that deletion of p66shc largely blocks phosphorylation of FKHRL1, a homologue of the forkhead family members, indicating the functional interaction with FKHRL1 of p66shc [49]. To determine whether FKHRL1 expression is regulated by p66Shc in human HDFs during genistein's anti-aging process, total and phosphorylated FKHRL1 on T1 (Thr 32) were assessed by western blot in this study. Our data show UVB irradiation of HDFs induced up-regulation of not only p66Shc but also FKHRL1 as well as its phosphorylation. Similar with the expression of p66Shc protein, a down-regulation of total and phosphorylated FKHRL1 expression was also observed with the treatment of genistein. These data suggested the possibility that redox-dependent forkhead activation was regulated by intracellular oxidative stress in a p66Shc-dependent pattern, and this interplay may modulate the anti-aging effect of genistein on HDFs.

Overall, this study presents evidence to explore the protective effect of genistein against senescence-like characteristics on HDFs and the mechanism underlying it. The above data indicate that genistein reverses the senescence process in HDFs by anti-oxidative action, which is mediated through down-regulation of p66Shc protein that involves forkhead protein suppression. Therefore, targeting of this pathway may benefit skin-aging (including photoaging and photodamage) treatment. Given that oxidative stress has been implicated in a lot of human aging processes besides skin aging, this role for p66Shc as signaling agents may have important therapeutic implications.

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