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Hyperoside protects human primary melanocytes against H_2O_2-induced oxidative damage

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Abstract. Cuscutae semen has been shown to have beneficial effects in the treatment of vitiligo, recorded in the Chinese Pharmacopoeia, whereas the effects of its constituent compounds remains to be elucidated. Using a tetrazolium bromide assay, the present study found that hyperoside (0.5-200 μg/ml) significantly increased the viability of human melanocytes in a time- and dose-dependent manner. The present study used a cell model of hydrogen peroxide (H_2O_2)-induced oxidative damage to examine the effect of hyperoside on human primary melanocytes. The results demonstrated that hyperoside pretreatment for 2 h decreased cell apoptosis from 54.03±9.11 to 17.46±3.10% in the H_2O_2-injured melanocytes. The levels of oxidative stress in the mitochondrial membrane potential of the melanocytes increased following hyperoside pretreatment. The mRNA and protein levels of B-cell lymphoma-2/Bcl-2-associated X protein and caspase 3 were regulated by hyperoside, and phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase signaling were also mediated by hyperoside. In conclusion, the results of the present study demonstrated that hyperoside protected the human primary melanocytes against oxidative damage.

Introduction

Vitiligo is a common type of dermatosis, characterized by depigmentation of the skin and mucosae due to the loss of melanocytes, most likely as a result of autoimmune effects (1). It is predominantly caused by the destruction of melanocytes in the skin, mucous membranes and the retina, which results in white patches of skin on different parts of the body (1,2). The hair growing in these vitiligo-affected areas usually turns white. At present, leucoderma treatment is predominantly focused on drug therapy, surgical treatment and physical therapy (3). The transplantation of cultured autologous pure melanocytes contributes significantly in leucoderma therapy. Melanocytes are considered to be more vulnerable to the damaging effects of oxidative stress, compared with keratinocytes and fibroblasts (4-6). Oxidative stress is also one of the inducing factors causing vitiligo (7). Therefore, the repair of injured melanocytes and the renewal of melanocytes are key for the treatment of vitiligo.

Traditional Chinese medicine has been used for various disease therapies in China for thousands of years (8). Cuscutae semen is the dry root of *Cuscuta australis* and *Cuscuta chinensis*, which has been used for treating various kidney conditions in China (9,10). It has also long been used for drinking (11). According to the Chinese Pharmacopoeia (2005, 2010) (12,13), Cuscutae semen has favorable effects on vitiligo treatment, and it is Component of the Chinese herbal compound prescription, Chi Tu Ting, which is used extensively in the treatment of leucoderma (14). Bioactive compounds, including alkaloids, anthraquinones, hyperoside, flavonoids, glycosides, sterols, tannic acid and saccharides are secondary metabolites found in Cuscutae semen (15,16). However, there has been little screening of the specific compounds closely associated with the effect of Cuscutae semen on vitiligo. In our previous study, six compounds obtained from Cuscutae semen, including quercetin, astragalin, quercetin-3-O-β-D-galactoside-7-O-β-glucoside, β-carotene, lutein and hyperoside [2-(3,4-dihydro xyphenyl)-3-(B-D-gala-ctopyranosyloxy)-5,7-dihydroxy] and hyperoside (Fig. 1) exhibited significant effects in melanogenesis.

In the present study, the protective ability of hyperoside against hydrogen peroxide (H_2O_2)-induced damage in melanocytes was investigated, and the possible mechanisms involved was examined. The results of these investigations aimed to provide novel direction and understanding for the treatment of vitiligo.

Materials and methods

Melanocyte culture. Human primary epidermal melanoyctes (cat. no. PCS-200-012) were purchased from

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American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in dermal cell basal medium supplemented with a melanocyte growth kit and antimicrobials/antimycotics (0.5 ml gentamicin-amphotericin B and 0.5 ml penicillin-streptomycin-amphotericin B) (ATCC). All cultures were incubated in a humidified incubator with 5% CO_2 at 37˚C.

Hyperoside. Hyperoside, with a purity of 98.78%, was obtained as a canary yellow needle-shaped crystal (Nanjing Zelang Medical Technological Co., Ltd., Nanjing, China). The hyperoside was dissolved in an appropriate volume of dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and diluted to the desired concentrations prior to use, with the final concentration of DMSO maintained <0.5%.

Cell viability. A standard tetrazolium bromide (MTT) assay was used to assess cell viability. Briefly, the melanocytes ($5x10^3$ cells/well) were seeded into 96-well plates. The cells were treated with hyperoside (0, 2, 10 and 50 μg/ml) for 2 h at 37˚C, following which the melanocytes were exposed to H_2O_2 (200 μM) for 24 h at 37˚C. Subsequently, 50 ml MTT (Sigma-Aldrich, St. Louis, MO, USA) solution (2 mg/ml) in phosphate-buffered saline (PBS) was added to each well and incubated for an additional 4 h at 37˚C. The medium was then removed, and the cells were incubated with 200 μl DMSO in the dark for 30 min to dissolve the violet crystals. The absorbance was read at 570 nm on an automatic microplate reader (550; Bio-Rad Laboratories, Inc., Hercules, CA, USA), with DMSO as a blank control. All assays were performed in multiples of give and repeated at least three times.

Cell apoptosis assessment. Following treatment with hyperoside (0, 2, 10 and 50 μg/ml) for 2 h, the H_2O_2 (200 μM)-treated melanocytes were stained with Annexin V-propium iodide (Beyotime Institute of Biotechnology, Shanghai, China), and apoptosis rates were analyzed using a flow cytometer (FACS-Calibur; BD Biosciences, Hercules, CA, USA).

Mitochondria membrane potential (MMP). Rhodamine-123 (Rho-123) dye (Sigma-Aldrich) was used to detect the changes in MMP in the cells. The cells ($5x10^4$ cells/well) were cultured in a 24-well plate. Following hyperoside (0, 2, 10 and 50 μg/ml) pretreatment for 2 h, and H_2O_2 exposure for 24 h, the cells were washed with PBS, incubated with Rho-123 (10 mg/ml) at 37˚C for 20 min and subsequently subjected to flow cytometry.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using TRIzol reagent (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reverse transcription reactions were performed using M-MuLV reverse transcriptase (Promega Corporation, Madison, WI, USA), following the same protocol as Baek *et al* (17). Samples were pretreated with DNase (Promega Corporation) for 1 h at 37˚C in order to avoid contamination. The qPCR reaction was performed on an ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) thermal cycler using 5 μl cDNA template, 1 μl primer pairs (10 μM) and 10 μl of a standard SYBR Green PCR kit (Thermo Fisher Scientific, Inc.) to a final reaction volume of 20 μl. The following cycling parameters were used:

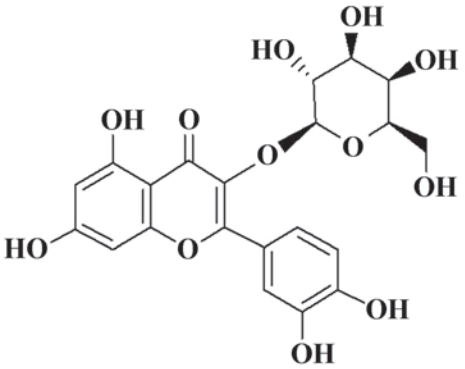

Figure 1. Chemical structure of hyperoside.

95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 45 sec and a final extension step at 72˚C for 5 min. The relative mRNA expression levels of target genes were compared with GAPDH, which were calculated using the $2^{-\Delta\Delta Cq}$ method (18). The primers used for each gene (synthesized by Generay Biotech Co., Ltd., Shanghai, China) were as follows: B cell lymphoma-2 (Bcl-2), forward 5'-AGACCGAAGTCCGCA GAACC-3' and reverse 5'-GAGACCACACTGCCCTGTTG-3' (product 113 bp); Bcl-2-associated X protein (Bax), forward 5'-GCGACTGATGTCCCTGTCTC-3' and reverse 5'-GGCCTC AGCCCATCTTCTTC-3' (product 132 bp); caspase 3, forward 5'-AACTGGACTGTGGCATTGAG-3' and reverse 5'-ACA AAGCGACTGGATGAACC-3' (product 161 bp); and GAPDH, forward 5'-ATCACTGCCACCCAGAAG-3' and reverse 5'-TCC ACGACGGACACATTG-3' (product 191 bp). The experiment was repeated three times.

Western blot analysis. The treated and untreated melanocytes were harvested and washed twice with PBS, followed by lysis in ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) with freshly added 0.01% protease inhibitor cocktail (Sigma-Aldrich) and incubation on ice for 30 min. The cell lysate was centrifuged at 12,000 x g for 10 min at 4˚C. The supernatant (20-30 μg protein) was run on a 10% SDS-PAGE gel and transferred electrophoretically onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were then blocked with 5% skim milk, followed by incubation with primary antibodies at 4˚C. Antibodies against rabbit polyclonal Bcl-2 (cat. no. Sc-492; 1:400), and rabbit polyclonal Bax (cat. no. Sc-493; 1:400) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), rabbit polyclonal caspase 3 (cat. no. ab44976; 1:500) was purchased from Abcam (Cambridge, UK). Antibodies against rabbit monoclonal phosphorylated (p)-AKT (cat. no. 4060; 1:1,000), rabbit polyclonal AKT (cat. no. 9272; 1:1,000), rabbit monoclonal p-p38 (cat. no. 4511; 1:1,000), rabbit monoclonal p38 (cat. no. 8690; 1:1,000) and rabbit monoclonal GAPDH (cat. no. 5471; 1:1,500) were purchased from Cell Signaling Technology (Danvers, MA, USA). The blots were then incubated for 1 h at room temperature with goat anti-mouse secondary antibody (cat no. A0216; 1:1,000; Beyotime Institute of Biotechnology) or polyclonal goat anti-rabbit secondary antibody (cat no. A0208; 1:1,000; Beyotime Institute of Biotechnology) and visualized using enhanced chemiluminescence (EMD Millipore).

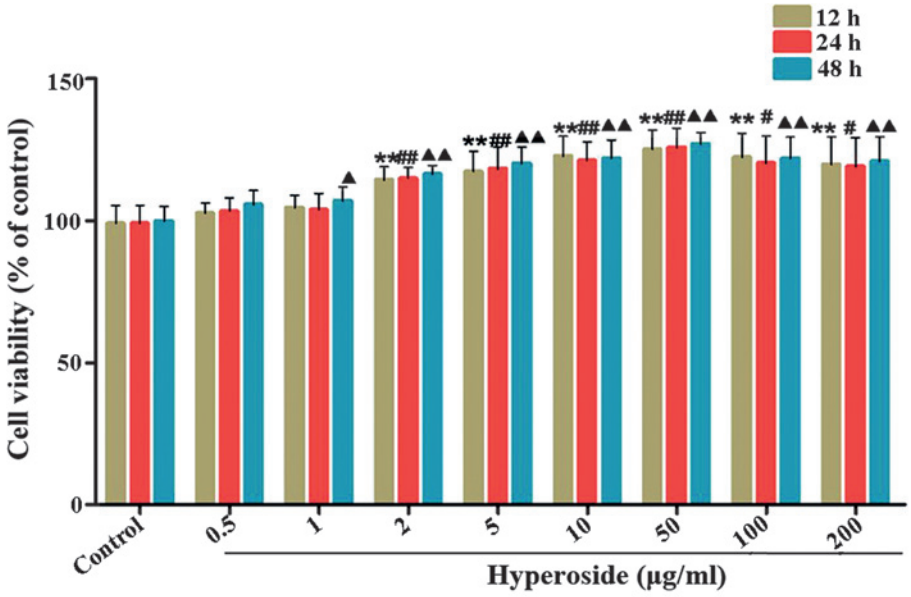

Figure 2. Effects of hyperoside on the proliferation of melanocytes. (A) Following exposure of melanocytes to various concentrations of hyperoside (0, 0.5, 1, 2, 5, 10, 50, 100 and 200 μg/ml) for 12, 24 and 48 h, cell viability was determined using a tetrazolium bromide assay. Data are expressed as the mean ± standard deviation (n=6), $^{\#,\blacktriangle}$P<0.05 and $^{**,\#\#,\blacktriangle\blacktriangle}$P<0.01, vs. control.

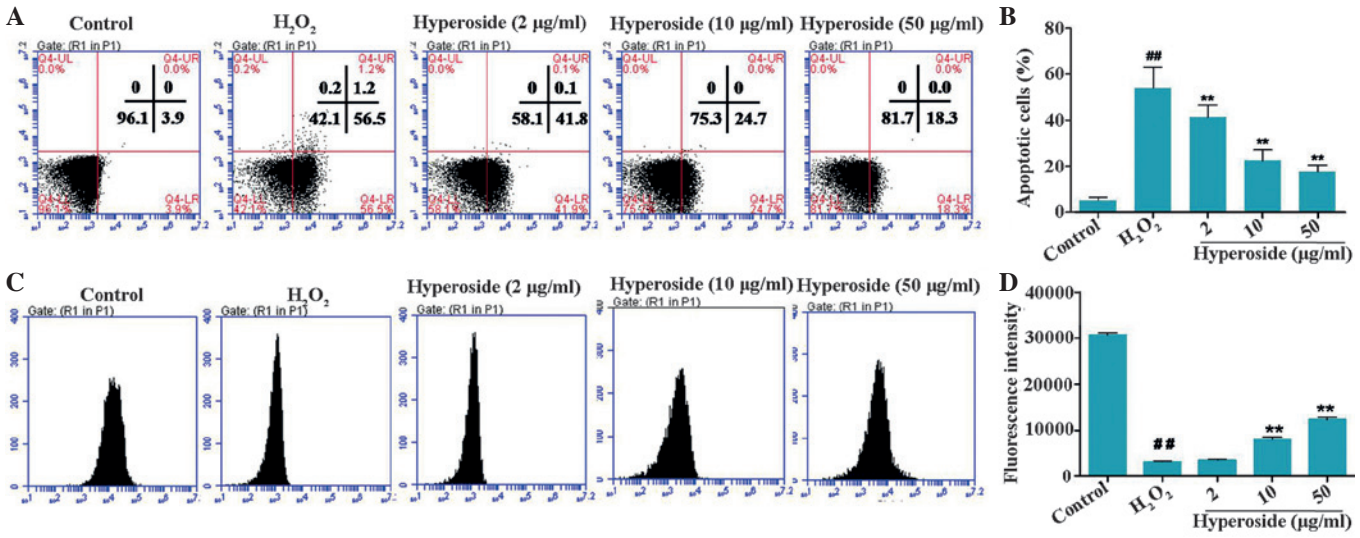

Figure 3. Effects of hyperoside on H_2O_2-induced apoptosis and MMP of human primary melanocytes. (A and B) Melanocytes were treated with different concentrations of hyperoside (0, 2, 10 and 50 μg/ml) for 2 h, and then exposed to H_2O_2 (200 μM) for 24 h. An Annexin V assay was used for apoptosis detection. (C and D) Melanocytes were treated with different concentrations of hyperoside (0, 2, 10 and 50 μg/ml) for 2 h, anf then exposed to H_2O_2 (200 μM) for 6 h. A Rhodamine-123 assay was used for MMP detection. Data are presented as the mean ± standard deviation (n=3). $^{\#\#}$P<0.01, vs. control; **P<0.01, vs. H_2O_2-treated melanocytes. H_2O_2, hydrogen peroxide; MMP, mitochondrial membrane potential.

Statistical analysis. The GraphPad Prism 5.0 software system (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. Data are expressed as the mean ± standard deviation. Student's t-test was used to compared the differences between two groups, and one-way analysis of variance was used for comparing more than two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Hyperoside stimulates melanocyte proliferation. For the purpose of evaluating the proliferation promoting ability of hyperoside on melanocytes, cell viability was detected following treatment with different concentrations of hyperoside. As shown in Fig. 2, hyperoside significantly increased the proliferation of the melanocytes in a time- and dose-dependent manner, compared with the control group. However, treatment with high concentrations of hyperoside (100 and 200 μg/ml) had no significant effects on cell viability, compared with the 50 μg/ml hyperoside treatment group. As a result, doses of 5, 10 and 50 μg/ml were selected for treatment in the subsequent investigations.

Hyperoside protects melanocytes against H_2O_2-induced apoptosis. Oxidative damage to human melanocytes is one

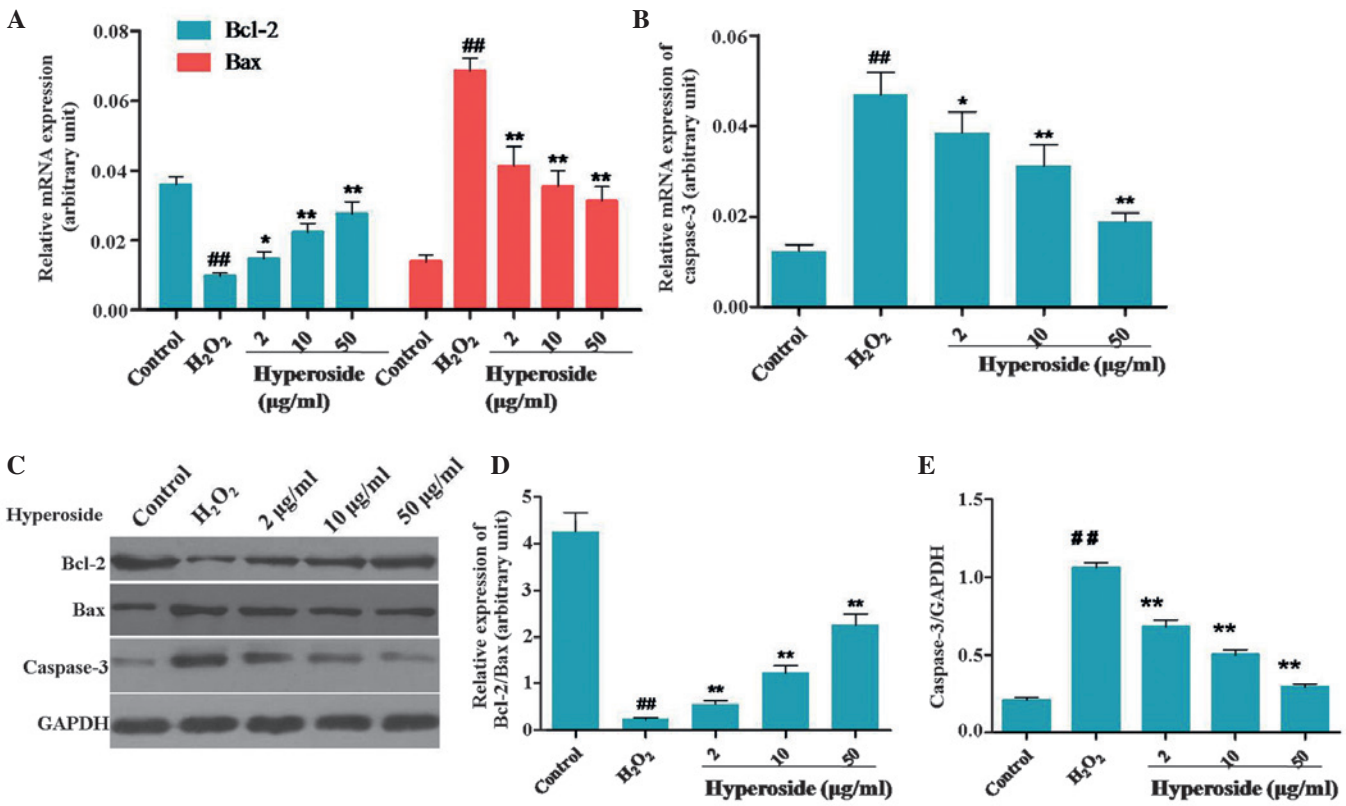

Figure 4. Effect of hyperoside on the expression levels of Bcl-2, Bax and caspase 3 in H_2O_2-treated melanocytes. (A and B) Melanocytes were treated with different concentrations of hyperoside (0, 2, 10 and 50 μg/ml) for 2 h, and then exposed to H_2O_2 (200 μM) for 3 h. Reverse transcription-quantitative polymerase chain reaction analysis was used to determine the mRNA expression levels of Bcl-2, Bax and caspase 3. (C-E) Following the treatment with H_2O_2 for 6 h, the protein levels of Bcl-2, Bax and caspase 3 were detected using Western blotting. Data are presented as the mean ± standard deviation (n=6), $^{##}$P<0.01, vs. control; *P<0.05 and **P<0.01 vs. H_2O_2-treated melanocytes. Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein; H_2O_2, hydrogen peroxide.

of the inducing factors causing vitiligo (19). The results of the Annexin V/propidium iodide staining in the present study showed that treatment of the melanocytes with H_2O_2 (200 μM) resulted in a significant increase in apoptotic rates, compared with the control group (Fig. 3). Pretreatment with hyperoside (2, 10 and 50 μg/ml) for 2 h led to a notable decrease in the apoptotic rates of the melanocytes, compared with the H_2O_2-treated group. These results indicated the protective effects of hyperoside against H_2O_2-induced apoptosis in melanocytes.

The breakdown in MMP is an early stage of the apoptotic process. In the present study, the effects of hyperoside on the MMP were evaluated using a Rho-123 assay. As shown in Fig. 3B, a notable reduction in the MMP was observed in the H_2O_2-treated human primary melanocytes. Compared with the H_2O_2-treated control cells, 1.91-fold and 3.45-fold increases in MMP were detected in the 10 and 50 μg/ml hyperoside pretreatment groups, respectively.

Hyperoside mediates the expression levels of Bcl-2, Bax and caspase 3 in H_2O_2-induced melanocytes. The relative expression levels of Bcl-2/Bax and caspase 3 are crucial in the process of cell apoptosis (20,21). In the present study, the mRNA and protein expression levels of Bcl-2/Bax and caspase 3 were measured using RT-qPCR and Western blot analyses, respectively. As shown in Fig. 4A, the expression of Bcl-2 was downregulated, and the expression of Bax was upregulated, in the melanocytes exposed to H_2O_2. Hyperoside effectively increased the relative mRNA expression levels of Bcl-2 and decreased those of Bax. As shown in Fig. 4B, the mRNA expression levels of caspase 3 were enhanced in the melanocytes exposed to H_2O_2, whereas hyperoside (2, 10 and 50 μg/ml) led to a dose-dependent reduction in the mRNA expression of caspase 3, by 17.23, 64.78 and 79.23%, respectively. As shown in Fig. 4C and D, the relative protein expression levels of Bcl-2/Bax were decreased by H_2O_2 treatment, whereas hyperoside treatment effectively upregulated the levels of Bcl-2/Bax, in a dose-dependent manner. On examination of the protein levels of caspase 3 by Western blotting, the protein expression levels increased significantly following exposure to H_2O_2, and decreased significantly following hyperoside treatment (Fig. 4B).

Hyperoside regulates PI3K/AKT and MAPK signaling H_2O_2-treated melanocytes. PI3K/AKT and MAPK signaling are crucial in cell apoptosis, proliferation, differentiation and various cellular functions (22,23). The phosphorylation of AKT exerts a protective effect in cell apoptosis, whereas the phosphorylation of p38 MAPK stimulates the process of apoptosis (24,25). In the present study, Western blot analysis was performed to evaluate the phosphorylation of AKT and p38. As shown in Fig. 5A and B, the levels of p-AKT/AKT in the melanocytes treated with H_2O_2 were markedly lower, compared with those in the control, and the expression

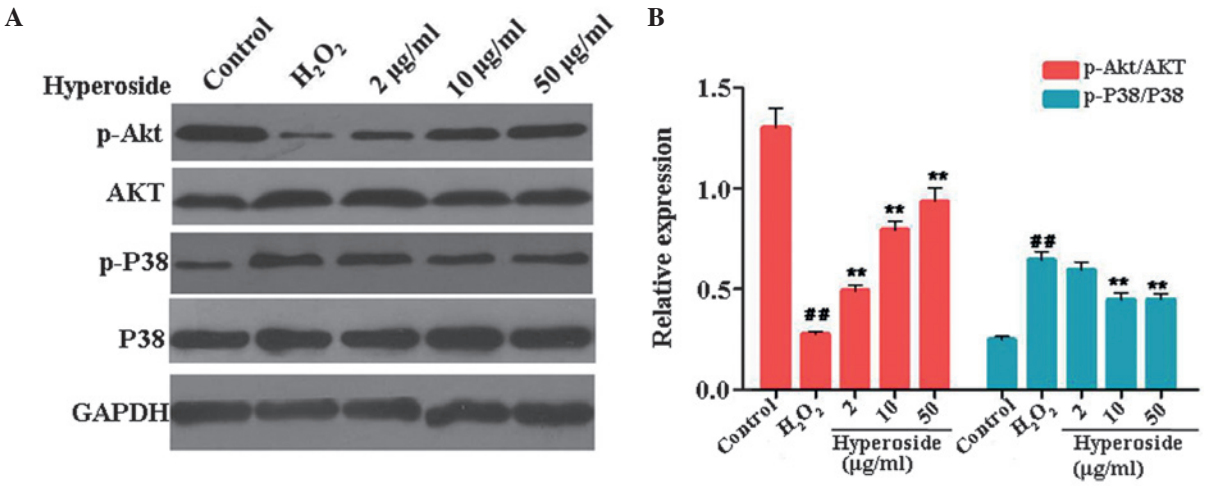

Figure 5. Effect of hyperoside on the expression levels of p-AKT and p-p38 in H_2O_2-treated melanocytes. (A and B) Melanocytes were treated with different concentrations of hyperoside (0, 2, 10 and 50 μg/ml) for 2 h, and then exposed to H_2O_2 (200 μM) for 6 h. Western blot analysis was performed to identify the protein levels of p-AKT, AKT, p-p38 and p38 in the menlanocytes, and GAPDH was detected as a sample loading control. Data are presented as the mean ± standard deviation (n=3). $^{\#\#}$P<0.01, vs. control; **P<0.01, vs. H_2O_2-treated melanocytes. p-, phosphorlyated; H_2O_2, hydrogen peroxide.

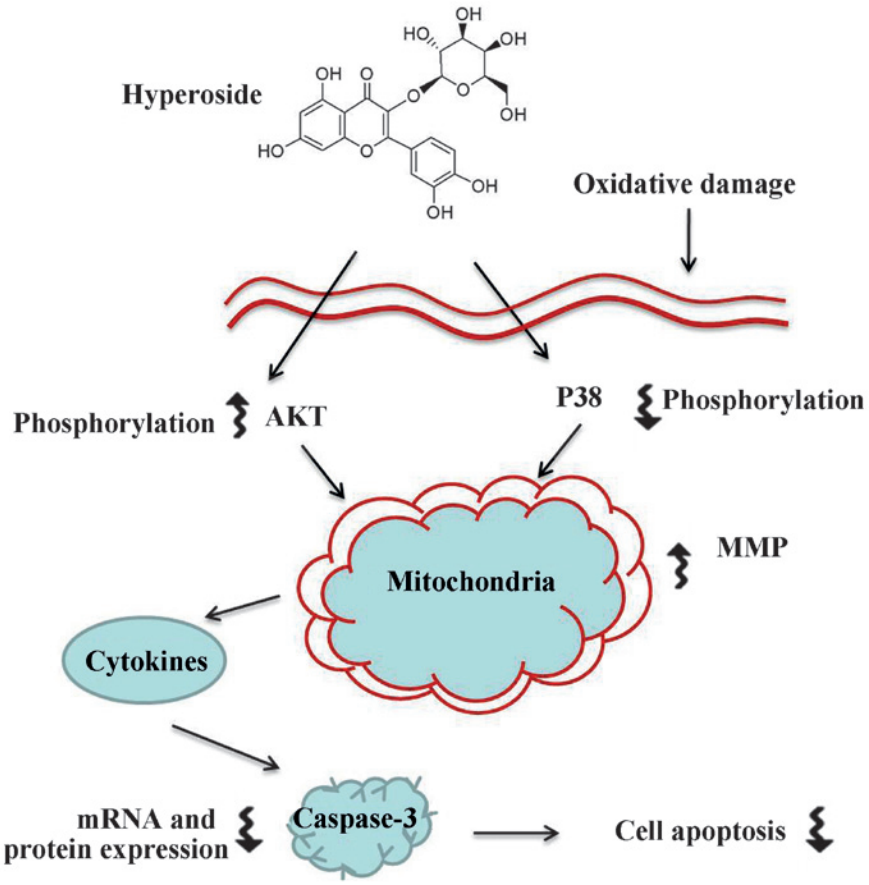

Figure 6. Mechanisms of hyperoside-induced enhancement of melanogenesis, and the protection of human primary melanocytes against oxidative stress. MMP, mitochondrial membrane potential.

levels of p-AKT/AKT in the groups pretreated with different doses of hyperoside (2, 10 and 50 μg/ml) were increased by 83.3, 103.1 and 236.5%, respectively, compared with those of the H_2O_2-treated group. By contrast, the expression of p-p38/p38 was increased on exposure to H_2O_2, and reduced following hyperoside treatment (Fig. 5A and B).

Discussion

The repair of injured melanocytes is one of the most important driving forces in the treatment of vitiligo. As recorded in the Chinese Pharmacopoeia (2005, 2010), Cuscutae semen shows beneficial effect in vitiligo treatment, which is also contained

within the prescribed Chinese herbal compound, Chi Tu Ding, which is used extensively for the treatment of leucoderma (14). Wang *et al* reported that an ethanol fraction from Cuscutae semen significantly affected melanogenesis by regulating the enzymatic activity of tyrosinase in zebrafish (26). Ma *et al* (27) demonstrated that the ethanol extract of Cuscutae semen was effective in inducing the adhesion and migration of melanocytes, and offered potential in the treatment of vitiligo treatment. However, the pharmacodyamic material basis of Cuscutae semen in the vitiligo treatment remains to be elucidated. In the present study, six compounds from *Cuscuta australis* were obtained, and hyperoside was found to exhibit marked effects on the induction of melanogenesis in human primary melanocytes.

Melanocytes are considered to be more vulnerable to the damaging effects of oxidative stress, compared with keratinocytes and fibroblasts (4-6), and oxidative stress is one of inducing factors causing vitiligo. In the present study, it was shown that hyperoside significantly reduced the apoptosis of cultured human melanocytes treated with H_2O_2. PI3K/AKT and MAPK signaling are reported to be important regulators of cell apoptosis. The phosphorylation of AKT exerts protective effects in cell apoptosis, whereas the phosphorylation of p38 MAPK stimulates the process of apoptosis (28,29). H_2O_2-treatment significantly decreased the phosphorylation of AKT, but increased the phosphorylation of p38. Pretreament with hyperoside partially reversed these effects of on the phosphorylation of AKT and p38. Taken together, these data demonstrated that hyperoside protected the human primary melanocytes against H_2O_2-induced apoptosis via the regulation of PI3K/AKT and p38 signaling.

Mitochondrial dysfunction caused by oxidative stress can result in a decease in MMP levels (30). In the present study, the MMP levels of the H_2O_2-treated melanocytes pretreated with hyperoside were notably increased, comparison with those of the H_2O_2-only treated melanocytes. The loss of MMP causes an increase in the permeability of the MMP, followed by the release of pro-apoptotic molecules, including cytochrome *c*. The release of cytochrome *c* from the mitochondria interacts with ATP, Apaf-1 and caspase 9, and subsequently activates caspase 3, which consequently elicits caspase-dependent apoptotic cell death (31). In the present study, the mRNA and protein expression levels of casepase 3 in the H_2O_2-treated melanocytes with hyperoside pretreatment were significantly decreased, compared with those in the H_2O_2-treated melanocytes without pretreatment. These results indicated that hyperoside showed protective effects towards the human primary melanocytes from oxidative damage by inhibiting the mitochondrial apoptotic pathway.

Taken together, the results of the present study lead to the hypothesis that hyperoside protects melanocytes against oxidative damage by activating AKT, inhibiting p38 phosphorylation and suppressing mitochondrial apoptosis signaling,. These findings may provide further insight into vitiligo therapy (Fig. 6), and hyperoside may be a useful therapeutic agent in the treatment of vitiligo.

Acknowledgements

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