Hyperoside protects human primary melanocytes against $H_2O_2$-induced oxidative damage

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Abstract. Cuscuta 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 ($0.5$–$200$ $\mu$g/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 $54.03\pm9.11$ to $17.46\pm3.10\%$ 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 ($8$). Cuscuta 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 ($2005, 2010$) ($12,13$), Cuscuta 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 Cuscuta semen ($15,16$). However, there has been little screening of the specific compounds closely associated with the effect of Cuscuta semen on vitiligo. In our previous study, six compounds obtained from Cuscuta semen, including quercetin, astragalin, quercetin-3-O-β-D-galactoside-7-O-β-glucoside, β-carotene, lutein and hyperoside [2-(3,4-dihydroxyxyphenyl)-3-(B-D-gala-cotypranosyloxy)-5,7-di hydroxy] 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 melanocytes (cat. no. PCS-200-012) were purchased from...
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% CO₂ 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 (5x10⁴ 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 H₂O₂ (200 μM) for 24 h at 37°C. Subsequently, 50 μl 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 five and repeated at least three times.

**Cell apoptosis assessment.** Following treatment with hyperoside (0, 2, 10 and 50 μg/ml) for 2 h, the H₂O₂ (200 μM)-treated melanocytes were stained with Annexin V-propium iodide (Beyotime Institute of Biotechnology, Shanghai, China), following the same protocol as Baek et al (17). The experiment was repeated three times.

**Mitochondria membrane potential (MMP).** Rhodamine-123 (Rho-123) dye (Sigma-Aldrich) was used to detect the changes in MMP in the cells. The cells (5x10⁴ cells/well) were cultured in a 24-well plate. Following hyperoside (0, 2, 10 and 50 μg/ml) pretreatment for 2 h, and H₂O₂ 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: 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 2ΔΔCt method (18). The primers used for each gene (synthesized by Genery Biotech Co., Ltd., Shanghai, China) were as follows: B cell lymphoma-2 (Bcl-2), forward 5'-AGACCGAAGTCCGCA GAACC-3' and reverse 5'-GAGACCATGACCTGTTGTG-3' (product 113 bp); Bcl-2-associated X protein (Bax), forward 5'-GCGACTGATGTCCCTGTCTC-3' and reverse 5'-GGGCTC AGCCCATCTTCTTC-3' (product 132 bp); caspase 3, forward 5'-AACCAGCTGTGGCATTAG-3' and reverse 5'-ACA AAGCGACTGGATGAAC-3' (product 161 bp); and GAPDH, forward 5'-ATCACTGGCACCCCAGAG-3' and reverse 5'-TCC AGCGGACACATGG-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 1. Chemical structure of hyperoside.](image-url)
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 compare 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$_2$O$_2$-induced apoptosis. Oxidative damage to human melanocytes is one...
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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$_2$O$_2$ (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$_2$O$_2$-treated group. These results indicated the protective effects of hyperoside against H$_2$O$_2$-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$_2$O$_2$-treated human primary melanocytes. Compared with the H$_2$O$_2$-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$_2$O$_2$-induced melanocytes. The relative expression levels of Bcl-2/Bax and caspase 3 were 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$_2$O$_2$. 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$_2$O$_2$, 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$_2$O$_2$ 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$_2$O$_2$, and decreased significantly following hyperoside treatment (Fig. 4B).

Hyperoside regulates PI3K/AKT and MAPK signaling H$_2$O$_2$-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$_2$O$_2$ were markedly lower, compared with those in the control, and the expression
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 H₂O₂-treated group. By contrast, the expression of p-p38/p38 was increased on exposure to H₂O₂, 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 pharmacodynamic 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 H2O2. 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). H2O2-treatment significantly decreased the phosphorylation of AKT, but increased the phosphorylation of p38. Pretreatment 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 H2O2-induced apoptosis via the regulation of PI3K/AKT and p38 signaling.

Mitochondrial dysfunction caused by oxidative stress can result in a decrease in MMP levels (30). In the present study, the MMP levels of the H2O2-treated melanocytes pretreated with hyperoside were notably increased, comparison with those of the H2O2-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, Apat-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 caspase 3 in the H2O2-treated melanocytes with hyperoside pretreatment were significantly decreased, compared with those in the H2O2-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.

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References

1. Ruiz-Arleguies A, Brito GJ, Reyes-Izquierdo P, Pérez-Romano B and Sánchez-Sosa A: Apoptosis of melanocytes in vitiligo results from antibody penetration. J Autoimmun 29: 281-286, 2007.
2. Silverberg NB: Recent advances in childhood vitiligo. Clin Dermatol 32: 524-530, 2014.
3. Guerrera L, Deldamabe E, Brescia S and Raskovic D: Vitiligo: Patho-genetic hypotheses and targets for current therapies. Curr Drug Metab 11: 451-467, 2010.
4. Liu CF, Hu CL, Chiang SS, Tseng KC, Yu RC and Pan TM: Beneficial preventive effects of gastric mucosal lesion for soy-skim milk fermented by lactic acid bacteria. J Agric Food Chem 57: 4433-4438, 2009.
5. Hoogduijn MJ, Cemeli E, Robb K, Anderson D, Thody AJ and Wood JM: Melanin protects melanocytes and keratinocytes against H2O2-induced DNA strand breaks through its ability to bind Ca2+. Exp Cell Res 290: 60-67, 2004.
6. Valverde P, Manning P, Todd C, McNeil CJ and Thody AJ: Tyrosinase may protect human melanocytes from the cytotoxic effects of the superoxide anion. Exp Dermatol 5: 247-253, 1996.
7. Laddha NC, Dwivedi M, Munsri MS, Gani AR, Ansarullah M, Ramachand AV, Dalai S and Begum R: Vitiligo: Interplay between oxidative stress and immune system. Exp Dermatol 22: 245-250, 2013.
8. Li X, Yang W, Ye M, Wang Q and Guo D: Differentiation of Cuscuta chinensis and Cuscuta australis by HPLC-DAD-MS analysis and HPLC-UV quantitation. Planta Med 77: 1950-1957, 2011.
9. Tang JL, Liu BY and Ma KW: Traditional chinese medicine. The text 372: 1938-1940, 2001.
10. Yang HM, Shin HK, Kang YH and Kim JK: Cuscuta chinensis extract promotes osteoblast differentiation and mineralization in human osteoblast-like MG-63 cells. J Med Food 12: 85-92, 2009.
11. Yang HM, Shin HK, Kang YH and Kim JK: Cuscuta chinensis extract promotes osteoblast differentiation and mineralization in human osteoblast-like MG-63 cells. J Med Food 12: 85-92, 2009.
12. China Pharmacopoeia Committee. Chinese pharmacopoeia. Chinese Medicine Science and Technology Press, Beijing, China, pp740, 2005.
13. State Pharmacopoeia Committee. Chinese pharmacopoeia. Chinese Medicine Science and Technology Press, Beijing, pp70-71, 2010.
14. Wang et al. (27): In vivo and in vitro evidence for hydrogen peroxide (H2O2) accumulation in the epidermis of patients with vitiligo and its successful removal by a UVB-activated pseudocatalase. The journal of investigative dermatology. Symposium proceedings/the Society for Investigative Dermatology, Inc. and European Society for Dermatological Research 4: 91-96, 1999.
15. Li CP, Li JH, He SY, Li P and Zhong XL: Roles of Fas/Fasl, Bcl-2/Bax, and Caspase-8 in rat nonalcoholic fatty liver disease pathogenesis. Genet Mol Res 13: 3991-3999, 2014.
16. Shadrinali S, Barar J, Hejazi MS and Samadi N: Roles of the Bcl-2/Bax ratio, caspase-8 and 9 in resistance of breast cancer cells to mitoxantrone. Asian Pac J Cancer Prev 15: 8617-8622, 2014.
17. CV SB, Babar SM, Song EI, Oh E and Yoo YS: Kinetic analysis of the MAPK and PI3K/Akt signaling pathways. Mol Cells 25: 397-406, 2008.
18. Lee ER, Kim JY, Kang YJ, Ahn JY, Kim JH, Kim BW, Choi HY, Jeong MY and Cho SG: Interplay between PI3K/Akt and MAPK signaling pathways in DNA-damaging drug-induced apoptosis. Biochim Biophys Acta 1763: 958-968, 2006.
24. Song G, Ouyang G and Bao S: The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 9: 59‑71, 2005.
25. Wada T and Penninger JM: Mitogen‑activated protein kinases in apoptosis regulation. Oncogene 23: 2838‑2849, 2004.
26. Wang TJ, An J, Chen XH, Deng QD and Yang L: Assessment of *Cuscuta chinensis* seeds effect on melanogenesis: Comparison of water and ethanol fractions in vitro and in vivo. J Ethnopharmacol 154: 240‑248, 2014.
27. Ma H, Zhang X, Mu K, Feng J, Niu X, Liu C and Dang Q: Effects of herb extracts on melanocyte adhesion and migration. Zhongguo pifu xingbingxue zazhi 18: 526‑527, 2004. (In Chinese)
28. Sabine VS, Crozier C, Brookes CL, Drake C, Piper T, van de Velde CJ, Hasenburg A, Kieback DG, Markopoulos C, Dirix L, et al: Mutational analysis of PI3K/AKT signaling pathway in tamoxifen exemestane adjuvant multinational pathology study. J Clin Oncol 32: 2951‑2958, 2014.
29. Toda M, Kuo CH, Borman SK, Richardson RM, Inagaki M, Collins A, Schneider K and Ono SJ: Evidence that formation of vimentin mitogen‑activated protein kinase (MAPK) complex mediates mast cell activation following FceRI/CC chemokine receptor 1 cross‑talk. J Biol Chem 287: 24516‑24524, 2012.
30. Cavalcanti BC, da Costa PM, Carvalho AA, Rodrigues FA, Amorim RC, Silva EC, Pohlit AM, Costa‑Lotufo LV, Moraes MO and Pessoa C: Involvement of intrinsic mitochondrial pathway in neosergeolide‑induced apoptosis of human HL‑60 leukemia cells: The role of mitochondrial permeability transition pore and DNA damage. Pharm Biol 50: 980‑993, 2012.
31. Huang WJ, Bi LY, Li ZZ, Zhang X and Ye Y: Formononetin induces the mitochondrial apoptosis pathway in prostate cancer cells via downregulation of the IGF‑1/IGF‑1R signaling pathway. Pharm Biol, 2013.