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Original Article

Hyperoside elevates the melanin content and promotes the migration of human melanocytes

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Abstract: Vitiligo is a dermatological disorder characterized by portions of skin losing their pigment. Cuscutae semen, the dry root of *Cuscuta australis* and *Cuscuta chinensis*, showed favorable effect on vitiligo treatment recorded by Chinese pharmacopoeia, while the material basis remains unclear. In the present study, we found hyperoside, a compound found from Cuscutae semen, significantly increased melanin content and the migration of human melanocytes. After treated with hyperoside (2, 10 and 50 μg/ml) for 48 h, the protein level of tyrosinase (TYR), microphthalmia-associated transcription factor (MITF), tyrosinase related protein 1 (TYRP 1) and tyrosinase related protein 2 (TYRP 2) were notably increased. Moreover, knockdown of MITF by siRNA transfection notably reduced the effects of hyperoside on melanin content. In human melanocytes transfected with MITF-siRNA, the increase range of MITF, TYR, TYRP 1 and TYRP 2 by hyperoside was significantly lower. In conclusion, hyperoside could stimulate melanogenesis by MITF/TYR/TYRP 1/TYRP 2 signaling and it might be a useful therapeutic agent in the treatment of vitiligo.

Keywords: Hyperoside, melanogenesis, MITF/TYR/TYRP 1/TYRP 2

Introduction

Vitiligo is a chronic dermatosis characterized by white patches of skin on different parts of the body [1, 2]. Vitiligo can be caused by autoimmune, genetic, oxidative stress or viral infection [3]. Although the actual cause of vitiligo remains unknown, the main considered factors are the destruction of melanocytes, which are in charge for skin pigmentation, in the skin, mucous membranes and the retina [1, 2]. Therefore, repairing of the injured melanocytes and renewal of melanocytes is the key point in vitiligo treatment. Nowadays, leucoderma treatment is mainly focused on drug therapy, surgical treatment, systemic phototherapy, and so on. Transplantation of cultured autologous pure melanocytes contributes greatly in leucoderma therapy [4].

Melanin, macromolecular derivative of skin and hair, is produced by melanocytes. Melanogenesis involves complicated physiological processes including the migration, division and mature of melanocytes, formation of melanosome, as well as the transportation and excretion of melanin. Melanocyte-specific microphthalmia-associated transcription factor (MITF) is considered to play a crucial role in melanin synthesis [5-8]. MITF also works as molecular switchboard in mediating the expression of key regulating enzymes in melanin synthesis, including tyrosinase (TYR), tyrosinase-related protein 1 (TYRP 1) and TYRP 2 [8, 9]. Stimulating the melanogenesis at the molecular level will contribute significantly in vitiligo therapy.

Traditional Chinese medicine has been applied for various disease therapies in China for thousands of years. Cuscutae semen is the dry root of *Cuscuta australis* and *Cuscuta chinensis*, which has been used for tonifying kidney and strengthening essence in Chinese [10, 11]. In addition, it has also been long used for drinking [12]. According to Chinese pharmacopoeia, Cuscutae semen shows favorable capability on vitiligo treatment and it is involved in Chinese herbal compound prescription called Chi Tu Ting which is extensively used for leucoderma

treatment [13]. Bioactive compounds including alkaloids, anthraquinones, hyperoside, flavonoids, glycosides, sterols, tannic acid and saccharides are secondary metabolites found in Cuscutae semen [14, 15]. However, little investigation was performed on screening the specific compounds closely related to the treatment effect of Cuscutae semen on vitiligo. We previously obtained 6 compounds from Cuscutae semen including quercetin, astragalin, quercetin-3-O-β-D-galactoside-7-O-β-glucoside, β-carotene, lutein and hyperoside [2-(3,4-dihydroxyphenyl)-3-(B-D-gala-ctopyranosyloxy)-5,7-dihydroxy], and hyperoside exhibited significant ability in melanogenesis.

In the present study, we have evaluated the effects of hyperoside on the melanogenesis of human primary melanocytes and the mechanisms involved in. Our study may provide new thoughts for the vitiligo treatment.

Materials and methods

Melanocytes culture

Human primary epidermal melanoyctes (ATCC, PCS-200-012) were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in dermal cell basal medium supplemented with growth kit and antimicrobials/antimycotics. 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). It was dissolved in an appropriate amount of dimethylsulfoxide (DMSO) and diluted to the desired concentrations before utilization, with the final concentration of DMSO kept below 0.5%.

Measurement of cellular melanin contents

Human melanocytes were treated with hyperoside (0, 2, 10 and 50 μg/ml) for 48 h. Melanocytes were collected, washed with phosphate buffered saline (PBS) and solubilized in 1 mol/L NaOH at 95°C for 1 h as previously described [16]. The optical densities were measured at 490 nm using a microplate reader (Epoch, BioTek, Luzern, Switzerland).

Transfection of cells with MITF-siRNA

MITF-siRNA sequence (siRNA) and a non-specific scramble siRNA sequence (siNC) was designed and synthesized by JRDUN (Shanghai, China). Melanocytes were transfected with MITF-siRNA or negative control siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Melanocytes were exposed to siRNA in DMEM for 6 h, after which the medium was replaced and the melanocytes were incubated for another 48 h.

Cell migration assay

The migration assay was performed by using Transwell Chambers (BD Bioscience, San Jose, CA, USA). Melanocytes were added to the upper chamber and lower chambers were filled with (0, 2, 10 and 50 μg/ml) hyperoside. After 48 h of incubation, the melanocytes on the upper surface of the filter were completely removed. The migrated cells were washed with PBS, fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Cells were photographed and counted in five random fields under microscopy.

Reverse transcription and western blot

Treated and untreated melanocytes were harvested, washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, St. Louis, MO, USA). After incubated on ice for 30 min, cell lysis was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Bredford, USA). The blots were blocked with 5% skim milk, followed by incubation with primary antibodies. Antibodies against MMP-2, MMP-9, MITF, TYR, TYRP 1 and TYRP 2 were purchased from Abcam (Cambridge, MA, USA). GAPDH were purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA). Blots were then incubated with corresponding secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Millipore).

Statistical analysis

The GraphPad Prism 5.0 software was employed for statistical analysis. Data are expressed

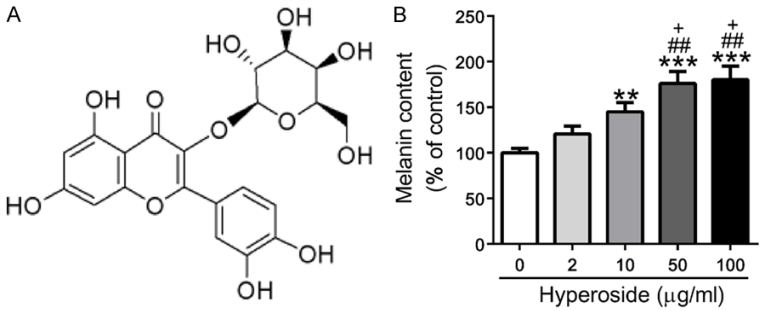

Figure 1. Effects of hyperoside on melanin content of human primary melanocytes. A. Chemical structure of hyperoside. B. After melanocytes were exposed to various concentrations of hyperoside (0, 2, 10, 50 and 100 μg/ml) for 48 h, melanin content was determined as described in Materials and Methods. Data are expressed as mean ± SD, n=6, ***P*<0.01, ****P*<0.001 *versus* control; ##*P*<0.01 *versus* 2 μg/ml group; +*P*<0.05 *versus* 10 μg/ml group.

as the mean ± standard error. Student's *t* test was used to compare the differences between two groups, while one-way analysis of variance was used when more than two groups were compared. *P*<0.05 was taken as statistical significance.

Results

Effects of hyperoside on melanogenesis

To evaluate the effects of hyperoside on melanogenesis, melanin content in human melanocytes was detected after treated with different concentrations of hyperoside (**Figure 1B**). Hyperoside treatment (2, 10, 50 and 100 μg/ml) increased mean melanin content to 120.8%, 145.0%, 175.9% and 180.1% of the control cells (100%), respectively (**Figure 1B**). Treatment with 100 μg/ml hyperoside did not significantly increase the accumulation of melanin compared with treatment with 50 μg/ml hyperoside. As a result, the doses of 2, 10 and 50 μg/ml were chosen for further investigations.

Hyperoside stimulated the migration of melanocytes

Melanocyte migration plays an important role in re-pigmentation of vitiligo skin. To explore the effects of hyperoside on cell migration, we performed cell migration assays by using Transwell (**Figure 2**). As shown in **Figure 2A**, the addition of hyperoside led to a significant increase in the number of migrated melanocyte (*P*<0.001). These results demonstrated that hyperoside can enhance melanocyte migration. Moreover, the protein levels of important factors to regulate cell migration were then estimated by western blot. The protein levels of MMP-2 and MMP-9 were significantly increased by hyperoside treatment in a dose-dependent manner. Thus, hyperoside had direct stimulatory effects on cell migration and melanogenesis, which made it a possible treatment for vitiligo.

Hyperoside stimulated the MITF/TYR/TYRP signaling

Hyperoside exhibited a favorable effect on melanogenesis, thus we further investigated the effects of hyperoside on MITF/TYR/TYRP signaling, which is pivotal in melanin synthesis [17]. Human primary melanocytes were exposed to different concentrations of hyperoside for 48 h, and western blot was then performed to measure the protein expression of MITF, TYR, TYRP 1 and TYRP 2. Hyperoside (2, 10 and 50 μg/ml) dramatically gave rise to the protein levels of MITF and down-stream target genes, TYR, TYRP 1 and TYRP 2 in a dose-dependent manner compared with that of control group (**Figure 3**).

Hyperoside enhanced melanogenesis via regulating MITF/TYR/TYRP signaling

MITF, TYR, TYRP 1 and TYRP 2 are involved in the procession of melanin formation [18, 19]. To further identify the effects of hyperoside on melanogenesis, siRNA targeting MITF was synthesized and transfected into melanocytes. The expression of MITF in MITF-siRNA-transfected melanocytes was dramatically descended compared with WT and scrambled-siRNA group (**Figure 4A**). As shown in **Figure 4B**, MITF-siRNA transfection notably reduced melanin content. The increase range of melanin content by hyperoside treatment (10 μg/ml) was suppressed by MITF-siRNA transfection. After transfected with MITF-siRNA, the increase range of MITF, TYR, TYRP 1 and TYRP 2 by hyperoside (10 μg/ml) was significantly lower (**Figure 4C**). These data suggested that hyperoside enhanced melanogenesis via regulating through regulating MITF/TYR/TYRP signaling.

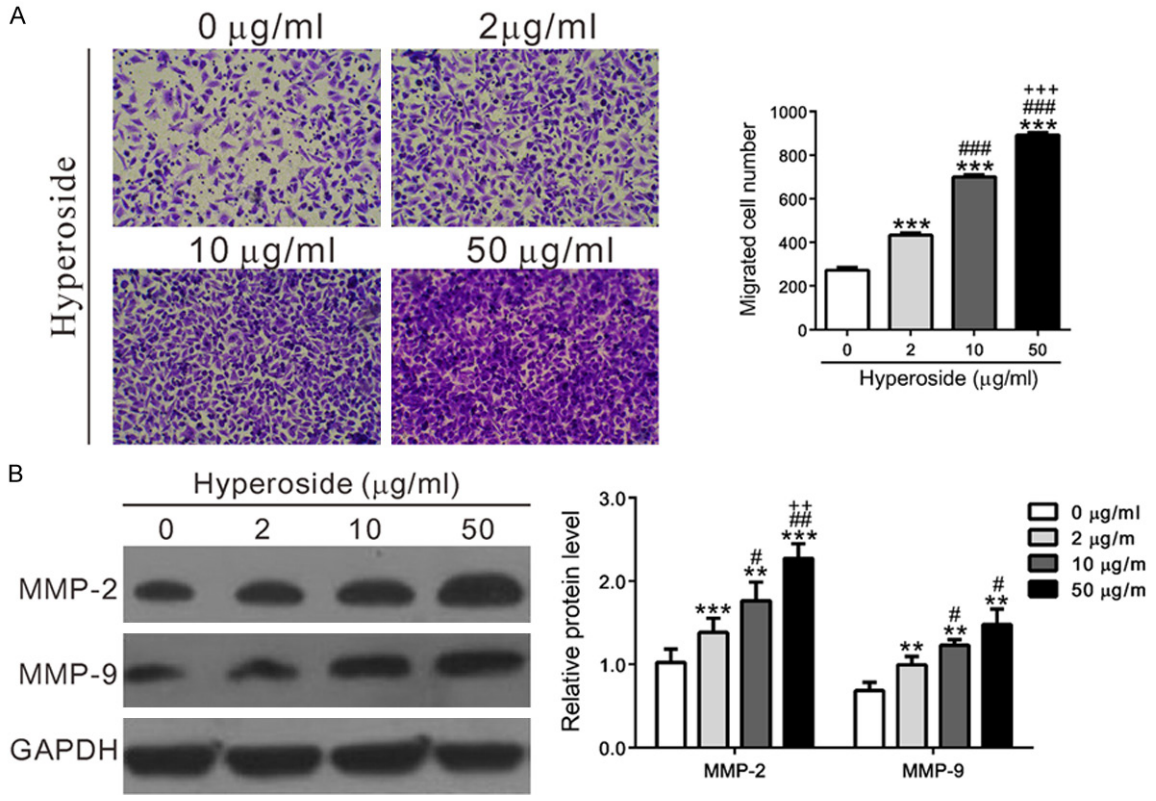

Figure 2. Hyperoside stimulated the migration of human primary melanocytes. A. Migration was measured by Transwell invasion assay with hyperoside (0, 2, 10 and 50 μg/ml) in the lower chambers. The migrated cells were photographed and counted. B. Melanocytes were exposed to hyperoside (2, 10 and 50 μg/ml) for 48 h, protein levels of MMP-2 and MMP-9 were measured by western blot. Data were presented as mean ± SD, n=6. ***P*<0.01, ****P*<0.001 *versus* control; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 *versus* 2 μg/ml group; ++*P*<0.01, +++*P*<0.001 *versus* 10 μg/ml group.

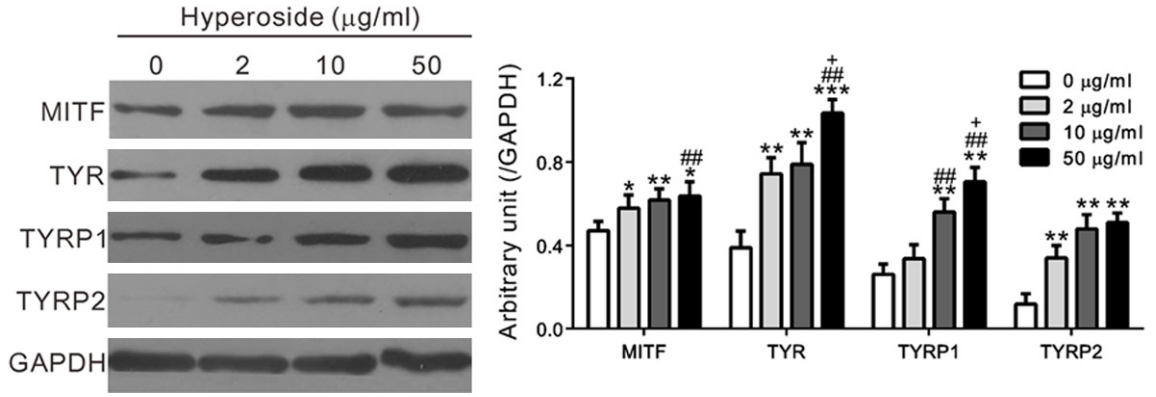

Figure 3. Effects of hyperoside on MITF/TYR/TYRP 1/TYRP 2 signaling. Melanocytes were exposed to hyperoside (2, 10 and 50 μg/ml) for 48 h, protein levels of MITF, TYR, TYRP 1 and TYRP 2 were measured by western blot. Data were presented as mean ± SD, n=6. **P*<0.05, ***P*<0.01 *versus* control; #*P*<0.05, ##*P*<0.01 *versus* 2 μg/ml group; +*P*<0.05 *versus* 10 μg/ml group.

Discussion

Stimulation of melanogenesis of melanocytes is the most important driving forces for the vitiligo treatment. As Chinese pharmacopoeia recorded, Cuscutae semen shows favorable capability on the vitiligo treatment, which is also involved in Chinese herbal compound pre-

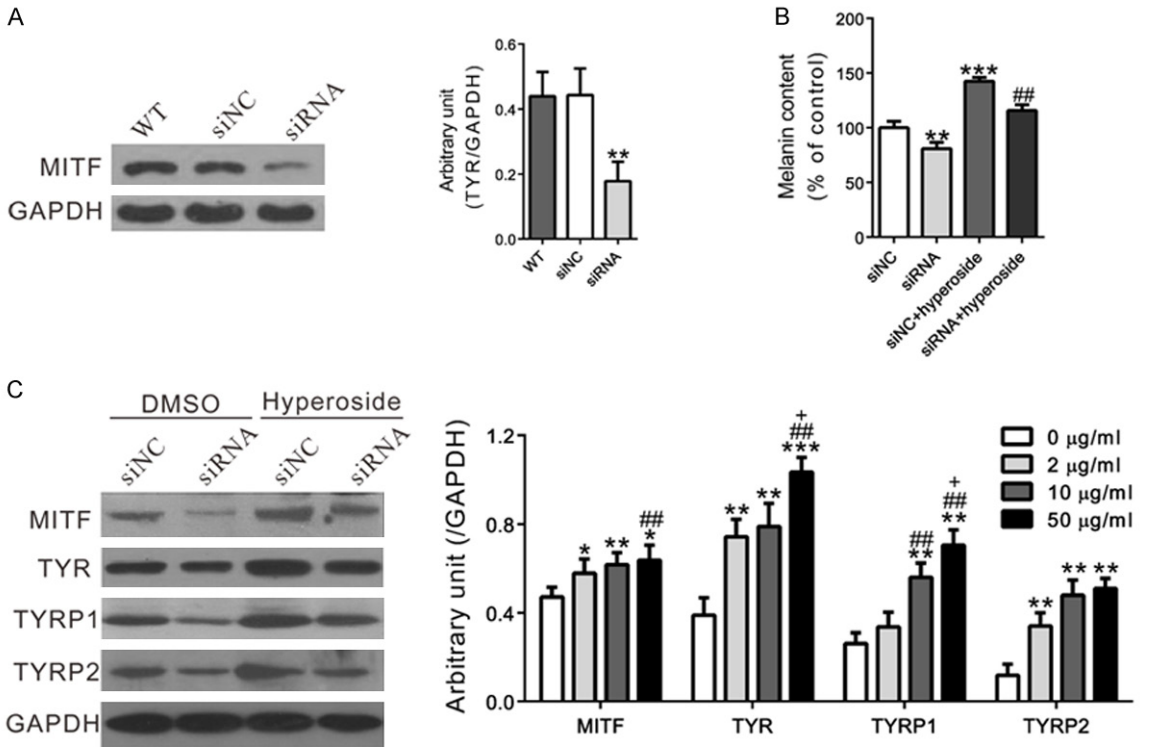

Figure 4. Hyperoside enhanced melanogenesis via regulating MITF/TYR/TYRP signaling. A. Human melanocytes transfected with MITF-siRNA and scrambled-siRNA were incubated for 48 h, relative protein level of MITF was measured by western blot. B. MITF-siRNA group and scrambled-siRNA group treated with DMSO or hyperoside (10 μg/ml) for 48 h, melanin content was then determined. C. MITF-siRNA group and scrambled-siRNA group treated with hyperoside (10 μg/ml) for 48 h, protein levels of MITF, TYR, TYRP 1 and TYRP 2 were assessed by western blot. Data were presented as mean ± SD, n=6. **P*<0.05, ***P*<0.01 *versus* control, ##*P*<0.05, ##*P*<0.01 *versus* scrambled-siRNA group.

scription called Chi Tu Ding extensively used for leucoderma treatment [13]. Wang *et al.* reported that ethanol fraction from Cuscutae semen significantly influenced melanogenesis by regulating enzymatic activity of tyrosinase in zebrafish [20]. However, the pharmacodyamic material basis of Cuscutae semen in the vitiligo treatment remains unknown. In the present study, hyperoside, a compound from Cuscutae semen exhibited outstanding effects on increasing melanin content and the migration of human primary melanocytes. The underlying molecular mechanisms of how hyperoside played a role on the melanogenesis of human primary melanocytes were also investigated.

A previous study showed that the ethanol extract of Cuscutae semen was effective in inducing adhesion and migration of melanocytes [21]. Here, we demonstrated that hyperoside could induce melanocyte migration in a dose-dependent manner (**Figure 2A**). The matrix-degrading metalloproteinases (MMPs) are a family of enzymes involved in the degradation of extracellular matrix components. During tissue remodeling and cell migration, MMPs expression is obviously enhanced [22]. In the present study, we revealed that hyperoside treatment could induce the expression of MMP-2 and MMP-9 (**Figure 2B**), and stimulated melanocyte migration, which may promote repigmentation of vitiliginous skin.

Melanogenesis plays an important role in the treatment of vitiligo. Our result indicated that hyperoside effectively enhanced the melanin content (**Figure 1**) and increased the expressions of MITF, TYR, TYRP 1 and TYRP 2 (**Figure 3**). MITF is regarded as not only a master gene for the development and survival of melanocytes, but also a crucial transcription factor regulating the expression of major melanogenic proteins such as TYR, TYRP 1 and TYRP 2 [17, 23]. To further study the hyperoside effects on

the melanogenesis, MITF expression was suppressed in the melanocytes by transfecting with MITF-siRNA. Our data showed that the increase range of melanin content (**Figure 4B**), as well as protein levels of TYR, TYRP 1 and TYRP 2 (**Figure 4C**) by hyperoside (10 μg/ml) treatment was notably decreased by MITF-siRNA transfection. These results indicated that hyperoside could enhance cellular melanin synthesis by targeting the MITF/TYR/TYRP 1/TYRP 2 signaling, although further exploration is needed.

Taken together, we demonstrated that hyperoside stimulated melanogenesis and melanocyte migration. Moreover, hyperoside enhanced melanin content by inducing the expression of MITF and its downstream target gene including TYR, TYRP 1 and TYRP 2 in human primary melanocytes. Hyperoside might be a useful therapeutic agent in the treatment of vitiligo.

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Disclosure of conflict of interest

None.

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