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Original artcle

Berberine protects immortalized line of human melanocytes from H_2O_2-induced oxidative stress via activation of Nrf2 and Mitf signaling pathway

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ABSTRACT

Background: Oxidative stress plays important roles in the pathogenesis of vitiligo. The removal of hydrogen peroxided (H_2O_2) has been established to be beneficial to vitiligo patients. Berberine (BBR), a natural isoquinoline alkaloid, has antioxidant activity, however, whether BBR can defend human melanocytes against oxidative injury remains to be elucidated.
Objective: In the present study, we investigated the potential protective effect of BBR against oxidative stress on an immortalized normal human melanocyte cell line PIG1.
Methods: Generally, PIG1 cells were pretreated with various concentrations of BBR for 1 h followed by exposure to 1.0 mM H_2O_2 for 24 h. Cell apoptosis, intracellular reactive oxygen species (ROS) levels were assessed through flow cytometry. Cell apoptosis, melanogenesis and the activation of Nrf2-ARE and Mitf signaling pathway were assayed.
Results: Our results showed that cell viability rose and intracellular ROS generation, cell apoptosis of melanocytes decreased significantly in response to H_2O_2 through pretreatment with BBR. Furthermore, We found that BBR can dramatically induce Nrf2 nuclear translocation, increase total Nrf2 levels and enhance ARE activity. Besides, Nrf2-siRNA transfection can abrogate the protection of BBR in melanocytes against oxidative injury. At last, we verified that BBR could facilitate melanogenesis function via modulation of Mitf and its target proteins.
Conclusion: The results above suggest that BBR can protect melanocytes against oxidative stress via its anti-oxidative activity. Also, we found H_2O_2-induced activation of NFκB was inhibited by BBR. Therefore, it is worthy of investigation BBR as a potential drug for treatment of vitiligo.

1. Introduction

Vitiligo, affecting about 1% of the people worldwide, is a chronic depigmentation disorder of skin through the selective destruction of melanocytes [1–3]. Widely accepted evidence shows that oxidative injury mediates as an initial pathogenic trigger in melanocytes degeneration in patients with vitiligo [4–6]. Furthermore, it can inhibit melanin synthesis, initiate target injury of melanocytes by the immune system and compromise their survival [7]. Overproduction of ROS can also activate NFκB which could further induce melanocytes to secrete IL-6 and IL-8. Secretion IL-6 and IL-8 by melanocytes can attract of T cell infiltration which could magnify the inflammatory reaction and play a vital role in melanocyte death [8,9]. All of these changes may inhibit melanogenesis and lead to depigmentation in vitiligo lesions.

The Nrf2 is a master transcription factor that plays a vital role in protecting cells from oxidative injury [10]. Our previous studies have verified that Nrf2-ARE pathway is critical for the protection of human melanocytes against H_2O_2-induced oxidative stress lesions, and its primary effector is HO-1 [10]. Thus, it is a pivotal approach for vitiligo treatment by targeting on the

Abbreviations: ARE, anti-oxidant response element; CAT, catalase; Bax, B cell lymphoma 2-associated X protein; BBR, berberine; Bcl-2, B cell lymphoma-2; DCT, L-dopachrome Delta-isomerase; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; Mitf, microphthalmia-associated transcription factor; NFκB, nuclear factor κ B; NQO1, NADH quinone oxidoreductase 1; Nrf2, nuclear erythroid 2-related factor; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; TRP1, tyrosinase-related protein 1; TYR, tyrosinase; PBS, phosphate buffer solution; SOD, superoxide dismutase.

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Nrf2-ARE pathway. The melanin synthesis capacity of the epidermis melanocytes was considered to be weakened in patients with vitiligo [11]. Particularly, suppression of Mitf could lead to further inhibition of numerous genes about pigmentation and melanocyte differentiation (e.g., TYR, TRP1 and DCT), as well as essential genes for keeping cellular environmental homeostasis, as well as maintaining genes encoding proteins such as the Bcl2 family that regulate apoptosis [12]. NFκB, a master regulator of proinflammatory responses, was recognized as a redox-regulated transcription factor which can be activated by H_2O_2 [8]. By catalyzing the phosphorylation and degradation of IκBs at specific amino acid residues, NFκB can translocate from cytoplasm to nucleus and further activate immune mediators such as IL-6 and IL-8 [13].

Given the above, the purpose of our study was to investigate whether BBR could exert protective effects on human melanocytes from H_2O_2-induced oxidative stress and apoptosis and to elucidate the underlying molecular mechanism involved. Here, we show that BBR could protect human melanocytes against H_2O_2-induced oxidative stress through activation of Nrf2-ARE, potentiating function of melanin synthesis of melanocytes and ameliorating activation of NFκB.

2. Materials and methods

2.1. Cell culture and treatment

The PIG1 immortalized human melanocyte cell line (given as a present by Dr. Caroline Le Poole, Loyola University, Chicago, USA) were cultured in Medium 254 from Invitrogen (Portland, OR, USA) supplemented with Human Melanocyte Growth Supplement from Invitrogen, 5% fetal bovine serum from Invitrogen in a humid atmosphere of 5% CO_2 and maintain the temperature at 37 °C. Oxidative injury in PIG1 cells was inducted by treatment of 1.0 mM H_2O_2 from the Sigma- Aldrich Chemical Company (St. Louis, MO, USA) for 24 h. BBR (Sigma–Aldrich) was added at indicated concentrations and time (Supplementary material, Fig. S1B).

2.2. Determination of cell viability by CCK-8 assay

Cell viability was assessed by using Cell Counting Kit-8 (CCK-8) purchased from Beyotime Institute of Biotechnology (Haimen, China) to count living PIG1 cells as depicted previously [14]. PIG1 cells were plated initially into 96-well plates at a density of 2×10^4 cells per wel. After incubation with the drugs mentioned above, 10 μl of CCK-8 was added into 100 μl cell Medium 254 and cultured for 90 min. The results of the color change, reflecting melanocytes viability, was assessed by spectrophotometer reader from Bio-Rad (Hercules, CA, USA) at an absorbance of 450 nm.

2.3. Determination of apoptosis assay

Melanocytes were plated into 6-well plates at the density of about 5×10^5 cells per well. By using the kits from MaiBio (ShangHai, China), Cell apoptosis rates (FITC and PI fluorescence were measured and analyzed by flow cytometer from Beckman Coulter (Miami, FL, USA) [15].

2.4. Measurement of intracellular ROS

PIG1 cells were seeded into 6-well plates at the density of 5×10^5 cells per well. Following drug treatment mentioned above, the intracellular ROS level was determined by the General Oxidative Stress Indicator (CM-H2DCFDA, Invitrogen, Carlsbad, CA, USA), and details are mentioned detailed previously [14].

2.5. Detection the activity of SOD and CAT

To detect the activity of SOD and CAT, the cells after treatment were lysed to obtain the total protein and was further detected the activity of SOD and CAT by using the total SOD analysis kit and total CAT analysis kit (Beyotime Biotechnology, China) [16]. The SOD and CAT activity in the total cell proteins was assessed by spectrophotometer reader from Bio-Rad (Hercules, CA, USA) at an absorbance of 550 nm.

2.6. Western blot

The concentration of proteins was quantified by using the Thermo Scientific Pierce BCA Protein Assay Kit (Rockford, IL, USA). Same amounts of protein (20 μg per lane) were separated by 10% SDS-PAGE (Bio-Rad) and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking in a solution of 5% non-fat milk diluted in TBST for 1 h, the PVDF membranes were incubated with primary antibodies for 24 h on the 4 °C constant temperature shaker (Supplementary material, Table S1). Then, the membranes were incubated with secondary antibodies for 1 h at room temperature. Then, by using ECL kit, the bounding antibodies were detected with western blotting detection system (Bio-Rad).

2.7. Immunofluorescence

As previously reported, PIG1 cells were cultured with different treatment mentioned above in the single layer glass slides [14]. After the incubation with antibodies and the nuclear dye (DAPI), the fluorescent images were obtained by using the Olympus confocal microscope (FV-1000/ES, Tokyo, Japan).

2.8. Transfection of short interfering RNAs (siRNAs)

Three short interfering RNAs (siRNAs) specific to the Nrf2 gene and two short interfering RNAs (siRNAs) specific to the Mitf gene (Supplementary Material, Table S1) were designed and manufactured by Genechem (Shanghai, China). Transfection of them into PIG1 cells was carried out by using INTERFERinTM (Polyplus-transfection, France) in the antibiotic-free medium according to the user's guidance. The details are described previously [14].

2.9. Detection of IL-6 and IL-8 level by melanocytes

Melanocytes were treated with the method mentioned above. After 24 h, cell culture supernatant was collected and centrifuged for store at −80 °C until analysis. The amount of IL-6 or IL-8 protein was determined using the Quantikine R & D System ELISA kits (Minneapolis, MN, USA) according to the instructions. The sensitivity of the kits was 0.7 pg/ml. All tests were performed in three times.

2.10. Measurement of tyrosinase activity and melanin content

As described previously by Qiong et al., tyrosinase activity was measured with the same methods [17]. The cells after incubation with test substances and then lysed with 1%Triton-X/PBS at −80 °C for 30 min. Then, thawing the lysate were mixed with 10 μl of 1% L-DOPA at 37 °C for 2 h.The absorbance was acquired by using spectrophotometer reader from Bio-Rad (Hercules, CA, USA) at an absorbance of 492 nm. As described by Qiong et al., the melanin content was measured [17]. In Brief, PIG1 cells were treated with test substances and dissolved in 0.5 ml of 1 M NaOH at 100 °C for 30 min and centrifuged for 20 min at 16 000×*g*. Then, the melanin contents were acquired by using spectrophotometer reader from Bio-Rad (Hercules, CA, USA) at an absorbance of 410 nm.

2.11. Statistical analyses

Comparison of data was performed with GraphPad Prism version 5.0 software (San Diego, CA, USA) by using the Student's unpaired *t*-test or one-way analysis of variance (ANOVA) with Dunnett's post-tests ($n \geq 3$). P values below 0.05 were considered to have statistic difference. The results require at least three independent experiments.

3. Results

3.1. Berberine ameliorates H_2O_2-induced oxidative damage in human melanocytes

In our study, we first assessed the effect of BBR on PIG1 cell proliferation and viability. As shown in the Fig. 1A and B, melanocytes pretreated with 0.1 μM–5.0 μM BBR induced cell proliferation in a time-dependent mode. Nevertheless, melanocytes pretreated with 10.0 μM–40.0 μM BBR signally suppressed cell growth compared to untreated groups. Besides, there is no apparent morphologic change in melanocytes pretreated with 5.0 μM BBR (Fig. 1C). PIG1 cells are treated with 1.0 mM H_2O_2 with or without BBR (0.1, 1.0 and 5.0 μM). After treatment with 1.0 mM H_2O_2 for 24 h, cells shrinkage (the dendrites of melanocytes shortened or disappeared) and obvious membrane blebbing are noted. While, pretreatment with BBR (0.1, 1.0 and 5.0 μM) for 1 h can surprisingly attenuate H_2O_2-induced oxidative injury in a dose-dependent manner, as represented by the decreased number of cells shrinkage and membrane blebbing (Fig. 1C) and an increase of cell viability is also noted (Fig. 1D). As shown in Fig. 1E, treatment with H_2O_2 markedly induced a robust increase in intracellular ROS (9.08-fold compared with the comparison group). Pretreatment with 0.1–5.0 μM BBR for 1 h sharply reduced the H_2O_2-induced ROS accumulation in PIG1 cells in a dose-dependent mode (Fig. 1F). Defects of antioxidant enzymes are critical for the generation of intracellular ROS. Essential antioxidant enzymes, such as CAT and SOD, can downregulate ROS level via eliminating free radicals in cells. The enzymatic activity of CAT and SOD can be prevented by BBR in a dose-dependent mode (Fig. 1G and H). Taken together, BBR was capable of improving H_2O_2-induced oxidative injury of human melanocytes.

3.2. Berberine protects human melanocytes from H_2O_2-induced apoptosis

To investigate whether BBR protects against H_2O_2-induced apoptosis, PIG1 cells were pretreated with various concentrations of BBR (0.1, 1.0, 5.0 μM) for 1 h before 1.0 mM H_2O_2 was added. By using flow cytometry analysis, the percentage of apoptosis PIG1 cells increased to 43.5% from a baseline of 4.1% after H_2O_2 treatment, whereas pretreatment with BBR(0.1, 1.0, 5.0 μM) markedly reduced apoptotic rate as the concentration gradually rose (Fig. 2A and B). To further investigate whether BBR can reduce apoptotic rate via modulating the Bcl-2 family after treatment with H_2O_2 in PIG1 cells, we also determined anti-apoptotic protein Bcl-2 and Bax expression levels with Western Blot. Previous studies suggest that downregulation of pro-apoptotic proteins such as Bax and PARP or upregulation of anti-apoptotic protein Bcl-2 play an important role in preventing oxidative induced apoptosis of melanocytes [18]. As shown in Fig. 2C and E, the level of Bcl-2 was significantly reduced, and the level of Bax increased after exposure to H_2O_2 for 24 h. While, pretreatment with 5.0 μM BBR did invert the change in Bcl-2 and Bax, leading to a decrease in the Bax/Bcl-2 ratio. Besides, suppression PARP cleavage with 5.0 μM BBR could further prevent H_2O_2-induced melanocytes apoptosis (Fig. 2D and F). These results indicate that BBR could protect human melanocytes from H_2O_2-induced apoptosis.

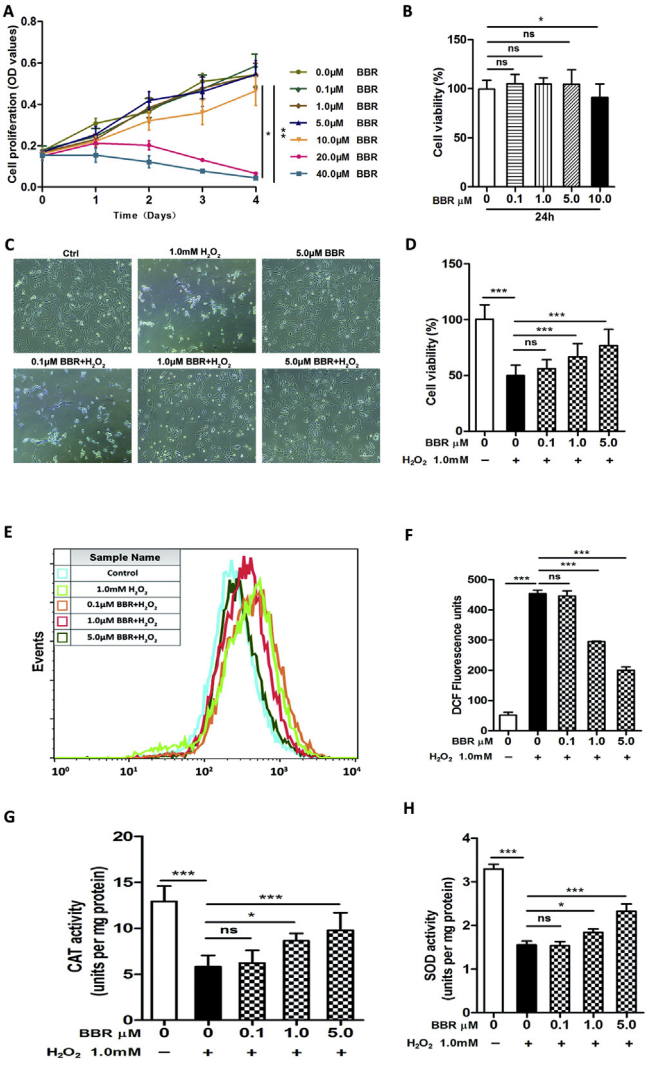

Fig. 1. Berberine ameliorates H_2O_2-induced oxidative damage in human melanocytes.
(A and B) Melanocytes were treated with different concentrations of berberine for indicated times. Cell proliferation (A) and viability (B) were determined by CCK8 assay. (C and D) Melanocytes were pretreated with different concentrations of berberine for 1 h and then exposed to 1.0 mM H_2O_2 for 24 h. The morphological features of melanocytes were detected by the microscope. Each field shown is a representative image of at least nine similar fields from three independent experiments, Scale bar = 200 μm (C). Cell viability (D) determined by CCK-8 assay, respectively. Melanocytes were exposed to various concentrations of BBR for 1 h and were further treated with 1.0 mM H_2O_2 for another 24 h. (E and F) Intracellular ROS production determined by flow cytometry assay. The fluorescence intensity of ROS level is shown right. (G and H) The activity of antioxidant enzymes CAT and SOD. All data are presented as the mean ± SD across three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ns means no significance.

3.3. Berberine protects human melanocytes from H_2O_2-induced injury through activation of Nrf2-ARE

To test whether BBR could downregulate intracellular ROS and protect melanocytes from H_2O_2-induced injury through activating the Nrf2-ARE pathway. By using immunofluorescence staining and immunoblotting, we found that BBR can dramatically promote the Nrf2 expression in melanocytes in a concentration-dependent manner. As it was shown in Fig. 3A and B, we showed that BBR rose the expression of nuclear Nrf2 while declined that of cytosolic Nrf2 respectively. We found that BBR could significantly increase the protein expression of HO-1 and NQO-1(Fig. 3C). Besides, BBR can also increase the level of SOD (Fig. 3C). These results illustrated

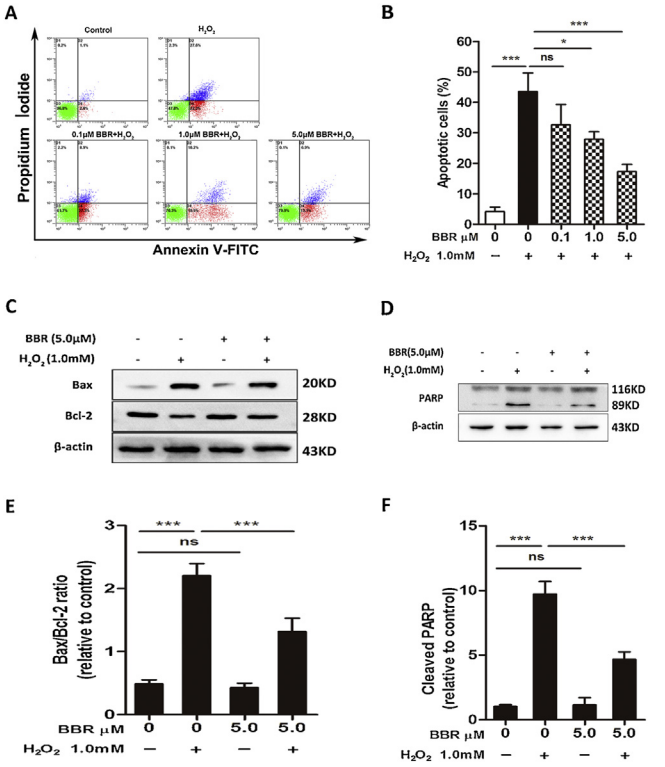

Fig. 2. Berberine protects human melanocytes from H_2O_2-induced apoptosis.
Melanocytes were exposed to 5.0 μM BBR for 1 h and were further treated with 1.0 mM H_2O_2 for another 24 h. (A and B) The percentage of apoptosis cells were determined by flow cytometry assay. (C and E) Effects of BBR on H_2O_2-induced Bax and Bcl-2 expression. (D and F) Effects of BBR on H_2O_2-induced PARP cleavage, All data are presented as the mean ± SD across three independent experiments. *P < 0.05, ***P < 0.001, ns means no significance.

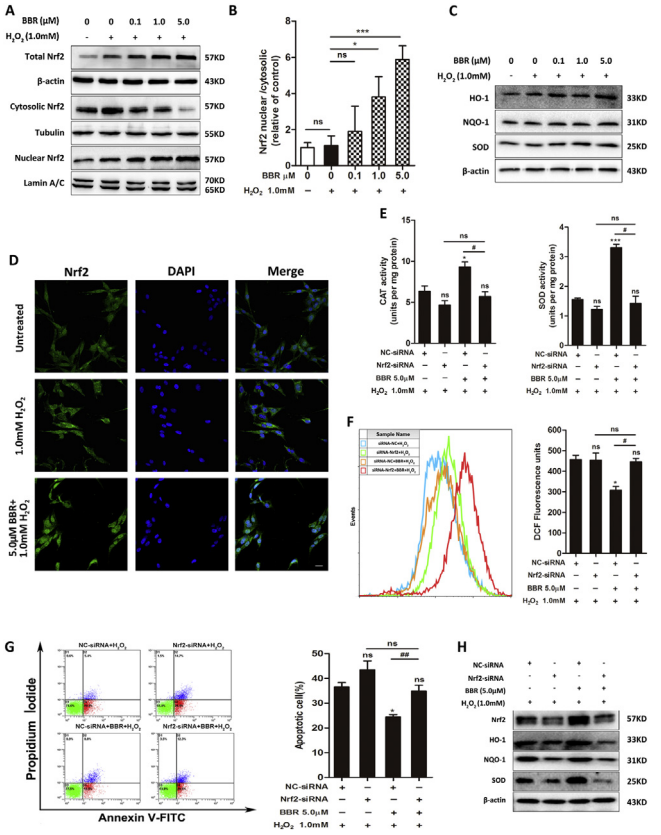

Fig. 3. Berberine promotes the activation of Nrf2 and its downstream genes in H_2O_2-treated melanocytes.
Melanocytes were exposed to different concentrations of BBR for 1 h and were further treated with 1.0 mM H_2O_2 for another 24 h. (A and B) Western blots of total, nuclear and cytoplasmic fractions of Nrf2. The intensity of each band was quantified by densitometry analysis. (C) Western blots of HO-1, NQO1, and SOD. (D) The ratio of nuclear/cytosolic of Nrf2 is shown right. Nrf2 localization in melanocytes was observed by laser confocal scanning microscopy. (E–H) Melanocytes transfected with siRNA against Nrf2 for 24 h, the antioxidative enzyme activity intracellular ROS levels, percentage of apoptosis cells and the protein levels were measured after treatment with 5.0 μM BBR for 1 h and were further treated with 1.0 mM H_2O_2 for another 24 h. All data are presented as the mean ± SD across three independent experiments. *P < 0.05, ***P < 0.001, ##P < 0.01, ### P < 0.001, ns means no significance.

that BBR exerted its antioxidant effect through activating Nrf2-ARE pathway and prevented melanocytes from H_2O_2-induced injury. As shown in Fig. 3D, We further found that obvious nuclear translocation of Nrf2. Furthermore, to testify if Nrf2 is required for the protection effects of BBR against oxidative damage or not, PIG1 cells were transfected with specific Nrf2-siRNA for 48 h (Fig. S1B). We have selected Nrf2-siRNA#2 to silence Nrf2 for further experiments as it has the highest transfection efficiency among three Nrf2-siRNAs. It is noteworthy that the upregulation of SOD and CAT activity induced by BBR was significantly restrained by Nrf2-siRNA (Fig. 3E). Moreover, the level of intracellular ROS was remarkably raised by BBR with Nrf2 deficiency in PIG1 cells compared with 5.0 μM BBR with 1.0 mM H_2O_2 group (Fig. 3F). Furthermore, Nrf2-siRNA abolished the protection of BBR against H_2O_2-induced apoptosis (Fig. 3G). Consistently, Nrf2 deficiency markedly restrained the up-regulation of the protein expression of HO-1, SOD and NQO-1 in BBR group under oxidative injury (Fig. 3H). Consequently, these results suggested that Nrf2 activation was indispensable for the anti-oxidative ability of BBR in human melanocytes."

3.4. Berberine improves H_2O_2-induced downregulation of Mitf in melanocytes

It was reported that dysfunction of melanocytes was observed under oxidative injury [19]. Thus, we were intended to find out whether BBR can modulate the function of melanocytes after treatment with H_2O_2. In our study, we found that in PIG1 cells, 1.0 mM H_2O_2 treatment for 24 h could inhibit the activity of tyrosinase (TYR) which is the rate-limiting enzyme in melanogenesis and significantly decreased the content of melanin, while, BBR could reverse the phenomenon obviously (Fig. 4A and B). By using Western blotting analysis, we observed the down-regulation of Mitf and its downstream targets such as TYR, TRP1, DCT in oxidative injury group which can be improved by BBR pretreatment (Fig. 4C). To testify whether Mitf is required for the effects of BBR modulate a function of melanocytes after treatment with H_2O_2, PIG1 cells were transfected with specific Mitf-siRNA (Fig. S1C) for 48 h. Subsequently, the PIG1 cells were treated with 5.0 μM BBR for 24 h and 1.0 mM H_2O_2 respectively. We have selected Mitf-siRNA#2 to silence Mitf for further experiments. It is noteworthy that, Mitf deficiency abolished the up-regulation of the activity of TYR, the content of melanin, and the expression of TYR, TRP1 and DCT in BBR group (Fig. 4D, E and F). These results suggested that Mitf activation was necessary for the improvement of the function of melanocytes by BBR.

3.5. Berberine ameliorates H_2O_2-induced NFκB activation

It was reported that oxidative stress triggers inflammatory response through the upregulation of levels of IL-6 and IL-8 in the medium which plays essential roles in vitiligo [9]. As shown in

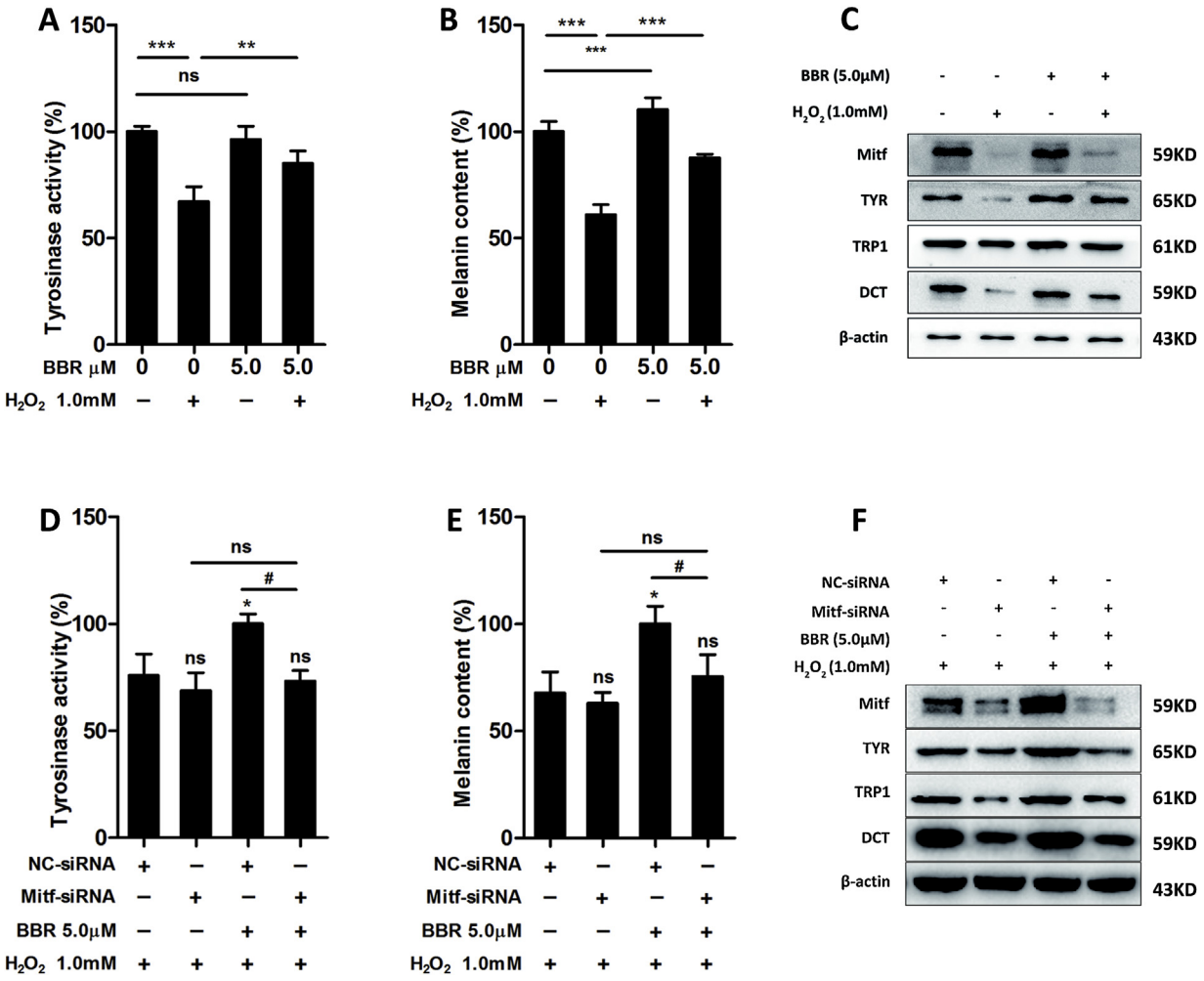

Fig. 4. Berberine improves H_2O_2-induced dysfunction of melanocytes.
Melanocytes were exposed to 5.0 μM of BBR for 1 h and were further treated with 1.0 mM H_2O_2 for another 24 h. (A) Melanin content and (B) tyrosinase activity were measured by using an ELISA reader. (C) The level of Mitf, TYR, TYRP1 and DCT in melanocytes was measured by western blotting. Melanocytes were transfected with siRNA against Mitf for 24 h exposed to 5.0 μM of BBR for 1 h and were further treated with 1.0 mM H_2O_2 for another 24 h. (D) Melanin content and (E) tyrosinase activity were measured by using an ELISA reader. (F) The level of Mitf, TYR, TYRP1 and DCT in melanocytes was measured by western blotting. Data are representative of three independently performed experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ns means no significance.

Fig. 5A and B, we found that the levels of IL-6 and IL-8 were significantly downregulated by BBR after treatment with H_2O_2. Next, we were intended to clarify whether BBR could inhibit secretion of IL-6 and IL-8 induced by H_2O_2 through the upstream gene-NFκB. We found that H_2O_2-induced activation of p65 and nuclear translocation was markedly reversed by BBR (Fig. 5C). By using Western blot analysis, the p-p65/p65 ratio increased significantly after exposure to H_2O_2, while, pretreatment with BBR substantially reversed the change of p-p65/p65 ratio (Fig. 5D). These results indicated that BBR might play a role in ameliorating NFκB activation and downstream pro-inflammatory cytokine genes (Fig. 6).

3.6. The schematic mechanism of berberine in ameliorating H_2O_2-induced oxidative injury of in melanocytes

We found that BBR can dramatically induce Nrf2 nuclear translocation, increase total Nrf2 levels and enhance ARE activity. Besides, the reduction Mitf and its downstream targets such as TYR, TRP1, DCT in oxidative injury group which can be improved by BBR pretreatment were also noted. BBR could protect H_2O_2-induced oxidative injury through activation of Nrf2-ARE and Mitf. H_2O_2-induced activation of p-p65 and nuclear translocation was markedly reversed by BBR. The phenomenon indicated that BBR might play a role in ameliorating NFκB activation and downstream pro-inflammatory cytokine.

4. Discussion

In our study, we studied the effect of BBR on meliorating H_2O_2-induced oxidative injury in human melanocytes and clarified some of the mechanisms involved. We found that BBR exerts a cytoprotective influence on PIG1 cells by improving anti-oxidant enzymes, downregulating intracellular ROS, reducing Bax/Bcl-2 ratio and suppressing PARP cleavage. Furthermore, we found that BBR could protect melanocytes against oxidative stress by activating Nrf2-ARE, such as HO-1, NQO-1, and SOD. Besides, we verified that BBR could ameliorate dysfunction of melanocytes induced by oxidative stress by potentiating expression on Mitf. At last, activation of NFκb and its downstream proteins such as IL-6 and IL-8 by H_2O_2 was suppressed by BBR. Our study demonstrates that BBR might be a potent antioxidant and anti-inflammatory agent for treatment of vitiligo.

Vitiligo has complex pathogenesis with the multifactorial polygenic disorder, in which accumulating evidence has indicated that oxidative stress plays an important role in the onset and

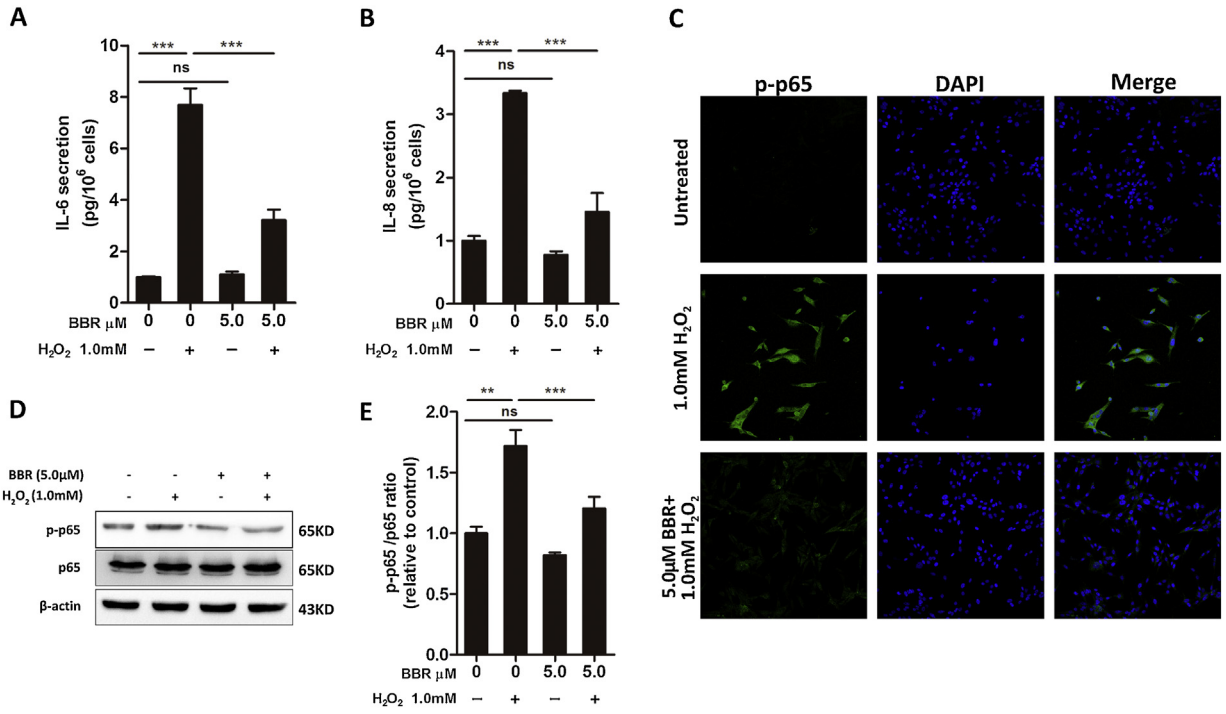

Fig. 5. Berberine ameliorates H_2O_2-induced NFκB activation.
Melanocytes were exposed to 5.0 μM of BBR for 1 h and were further treated with 1.0 mM H_2O_2 for another 24 h. (A and B) The Culture medium was collected, and the IL-6 levels and IL-8 levels were measured with ELISA kit and expressed as the percentage of the negative control. (C) p-p65 localization in melanocytes was observed by laser confocal scanning microscopy. (D) Western blots of p-p65 and p65. The intensity of each band was quantified by densitometry analysis. The ratio of p-p65 and p65 is shown. All data are presented as the mean ± SD across three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, ns means no significance.

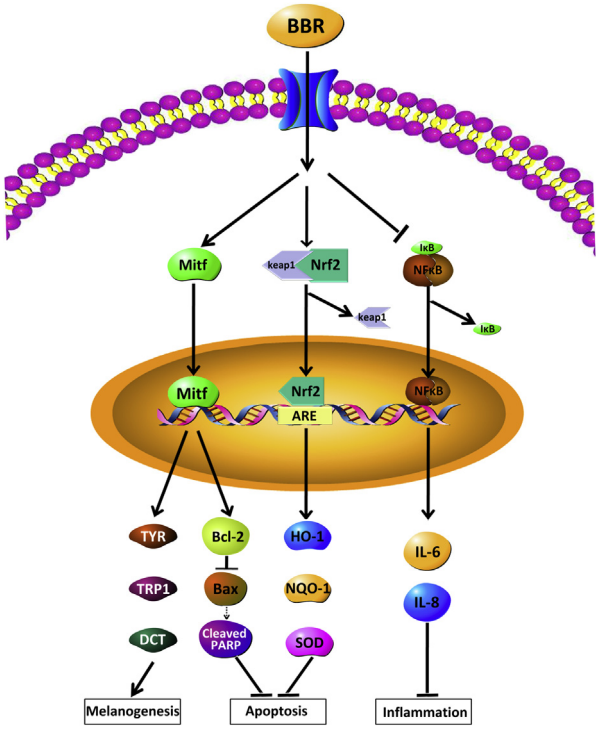

Fig. 6. Protection effect of BBR on H_2O_2-induced oxidative stress and Mitf activation in human melanocytes.
BBR could exert a protective effect on human melanocytes from H_2O_2-induced oxidative stress and apoptosis. BBR could attenuate H_2O_2-induced oxidative injury by activating Nrf2 and ameliorates activation of NFκB. Besides, it could facilitate melanogenesis function via upregulation of Mitf.

progression of vitiligo [20]. It has found that vitiligo patients have compromised antioxidant responses such as reduced enzyme activity of CAT and SOD. Previous reports have suggested that BBR could protect macrophages, endothelial cells, and neuron cells by decreasing ROS production. In our study, we for the first time found that in melanocytes BBR was able to downregulate intracellular ROS by increasing CAT and SOD enzymatic activity.

The Bcl-2 family, in which Bcl-2 and Bax is antiapoptotic protein and proapoptotic protein respectively, play the critical role in the regulation of apoptosis through releasing of cytochrome c from mitochondrial into the cytosol and further activates executor protein of apoptosis such as the degradation of PARP. Given the functional antagonism between the pro- and anti-apoptotic Bcl-2 family members, it was found that the ratio of their activity levels is a critical determinant of the cells' susceptibility to apoptosis. In this study, we found that pretreatment with BBR could inverse the ratio of Bax/Bcl-2 and further downregulated cleavage of PARP. These data demonstrate that the anti-apoptotic of BBR may partially result from the deactivation of cleavage of PARP through modulation of the ratio between Bax and Bcl-2 expression in oxidative injury melanocytes.

Our previous studies have revealed that the defective Nrf2 activation in melanocytes plays important role in the pathophysiology [14]. Translocation of Nrf2 from the cytoplasm to nuclear can promotes the expression of a variety of antioxidant enzymes, including HO-1, CAT, GPx and SOD [21]. Slominski et.al found that melatonin and its metabolites protect human melanocytes through mediation of Nrf2 mediated pathways against UVB-induced damage [22,23]. Our studies have proved that Nrf2-ARE activation is necessary for the protection of melanocytes from oxidative stress. Recent studies have revealed that Nrf2 is considered as a crucial mediator of BBR in reducing oxidative damage in macrophages and nerve cells [21,24,25]. In our studies, we found that BBR could not only enhance translocation of Nrf2 from the

cytoplasm to nuclear but also increase the expression of its downstream anti-oxidant genes including HO-1, NQO-1, and SOD. Our results indicate that BBR could protect melanocytes from H_2O_2-induced oxidative injury through activating the Nrf2-ARE pathway.

The NFκB is a key transcription factor that regulates the activities of inflammation, oncogenesis, and apoptosis [26]. H_2O_2 was found to stimulate NFκB activation through enabling proteasomal degradation by translocation from cytoplasm to the nucleus through post-translational modifications (e.g., ubiquitination and phosphorylation) of IκB. Activation of NFκB may, therefore, induce the expression of the downstream genes, including various kinds of cytokines genes such as IL6 and IL8. It indicates that NFκB serves as a pivotal link between oxidative injury and production of pro-inflammatory cytokines [27]. NFκB signal pathways are the main signal pathways involving in an H_2O_2-induced expression of IL-6 by various types of cell [28,29]. Increasing level of IL-6 and IL-8 could also immediately suppress the growth of melanocytes and attracts cytotoxic T lymphocytes to infiltrate into vitiligo lesions, which will play a vital role in the melanocytes death [30].It was found that BBR could significantly reduce the level of hs-CRP and IL-6 in the peripheral blood sample from those patients with coronary heart disease [31]. Consistently, we found that BBR could inhibit the activation of NFκB and its downstream genes IL6 and IL8. In brief, BBR could protect melanocytes from H_2O_2-induced oxidative stress through anti-inflammatory behavior.

Mitf is a very important transcription factor for melanocytes to produce melanin and regulates cellular responses to ROS [32]. Various downstream genes of Mitf have been explored, including melanogenesis proteins such as TYR, TRP1 and DCT [32]. The most critical enzyme for the whole process is tyrosinase [33]. It was found that there are defective of Mitf regulation of melanocytes in patients with vitiligo. Those defective pathways may bring death to melanocytes [12]. Besides, it has been proved that Mitf plays the critical role for melanocyte survival via upregulation of Bcl-2. In our study, we found that the reduction of enzymatic activity of tyrosinase and the melanin content in melanocytes could be reversed successfully by BBR after oxidative injury induced by H_2O_2. In summary, restoration the expression of Mitf after pretreatment with BBR followed by oxidative injury could potentiate melanogenesis function of human melanocytes.

In conclusion, BBR could ameliorate H_2O_2-induced oxidative damage through activation of Nrf2-ARE and Mitf. Also, we note that BBR could exert anti-inflammatory activity through inhibition of IL-6 and IL-8 by inhibiting nuclear translocation of NFκB. These results suggest that BBR's antioxidative and anti-inflammatory activity plays a significant role in protecting the injury induced by H_2O_2. More studies ought to be performed to confirm its precise mechanisms in vitro and in vivo.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.03.007.

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