Ginsenoside Rk1 protects human melanocytes from H_2O_2-induced oxidative injury via regulation of the PI3K/AKT/Nrf2/HO-1 pathway

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Abstract. Vitiligo is a cutaneous depigmentation disorder caused by melanocyte injury or aberrant functioning. Oxidative stress (OS) is considered to be a major cause of the onset and progression of vitiligo. Ginsenoside Rk1 (RK1), a major compound isolated from ginseng, has antioxidant activity. However, whether RK1 can protect melanocytes against oxidative injury remains unknown. The aim of the present study was to investigate the potential protective effect of RK1 against OS in the human PIG1 melanocyte cell line induced with hydrogen peroxide (H_2O_2), and to explore its underlying mechanism. PIG1 cells were pretreated with RK1 (0, 0.1, 0.2 and 0.4 mM) for 2 h followed by exposure to 1.0 mM H_2O_2 for 24 h. Cell viability and apoptosis were determined with Cell Counting Kit-8 and flow cytometry assays, respectively. The activity levels of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were analyzed using ELISA kits. Protein expression levels, including Bax, caspase-3, Bcl-2, phosphorylated-AKT, AKT, nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), cytosolic Nrf2 and nuclear Nrf2, were analyzed using western blot analysis. In addition, the expression and localization of Nrf2 were detected by immunofluorescence. RK1 treatment significantly improved cell viability, reduced the apoptotic rate and increased the activity levels of SOD, CAT and GSH-Px in the PIG1 cell line exposed to H_2O_2. In addition, RK1 treatment notably induced Nrf2 nuclear translocation, increased the protein expression levels of Nrf2 and HO-1, and the ratio of phosphorylated-AKT to AKT in the PIG1 cells exposed to H_2O_2. Furthermore, LY294002 could reverse the protective effect of RK1 in melanocytes against oxidative injury. These data demonstrated that RK1 protected melanocytes from H_2O_2-induced OS by regulating Nrf2/HO-1 protein expression, which may provide evidence for the application of RK1 for the treatment of vitiligo.

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Introduction

Vitiligo, one of the most common acquired autoimmune skin disorders, is caused primarily through the selective destruction of melanocytes (1). Oxidative stress (OS), defined as an imbalance between radical-generating and radical-scavenging activity, has been associated with a variety of diseases, including hypertension, diabetes, cancer and cardiovascular diseases (2,3). An increasing number of studies have indicated that the pathogenesis of vitiligo is associated with OS-mediated toxicity in melanocytes (4-7). Previous studies have indicated that the state of OS commonly results in increased intracellular reactive oxygen species (ROS) production, which influences various signaling pathways leading to inhibition of melanin synthesis, destruction of cell structures and reduced survival of melanocytes (8-11). Therefore, improving oxidative status may be an effective therapeutic strategy for vitiligo.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that regulates the protein synthesis of phase II antioxidants, and is commonly known to play a contributory role in protecting cells from oxidative damage (12). A previous study confirmed that Nrf2 protects cells from damage by scavenging free radicals in cells (13). Under normal physiological conditions, Nrf2 binds to kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm and remains inactivated. Upon OS, Nrf2 dissociates from Keap1 and Nrf2 is activated (14). The activated Nrf2 translocates into the nucleus, to induce heme oxygenase-1 (HO-1) expression. As a member of the intracellular phase II enzyme family, HO-1 is considered to play an important role in the regulation of redox balance. Furthermore, HO-1 has been demonstrated to exert anti-apoptotic and anti-inflammatory effects (15). Thus, activation of the Nrf2/HO-1 signaling pathway is critical for the protection of human melanocytes. It has previously been revealed that PI3K/AKT is a key pathway for promoting cell

survival and metabolism. Recent results have demonstrated that PI3K/AKT, as an upstream signaling pathway, can control the defense system of cells against inflammation and oxidative damage by mediating the Nrf2/HO-1 signaling pathway (16).

Panax ginseng (PG) is one of the most ancient traditional herbs, which has been used as an herbal remedy in Asia for its tonic and restorative actions for years (17). Studies have revealed that PG has various pharmacological activities, including antioxidant, anti-inflammatory, anti-viral, neuroprotective and anticancer effects (18-21). Ginsenosides are the major biologically active ingredients of ginseng (the root of PG), which are divided into protopanaxadiol [ginsenosides Rk1 (RK1), Rg5 and Rg3] and protopanaxatriol (ginsenoside Rg1) on the basis of their steroidal structure and sugar moieties (22). A previous study reported that RK1 may protect the liver from OS and apoptosis caused by paracetamol in rats (23). However, to the best of our knowledge, the protective effect of RK1 against hydrogen peroxide (H_2O_2)-induced oxidative damage of melanocytes has not yet been reported, either *in vitro* or *in vivo*. Thus, the aim of the present study was to investigate whether RK1 could protect against H_2O_2-induced oxidative damage of human melanocytes via activation of the PI3K/AKT/Nrf2/HO-1 signaling pathway.

Materials and methods

Reagents. RK1 (cat. no. 42754) and LY294002 (cat. no. L9908) were purchased from Sigma-Aldrich (Merck KGaA) and the purity was ≥95 and ≥98%, respectively. Additional reagents used in the present study were commercially available and of analytical purity.

Cell culture. The PIG1 immortalized human melanocyte cell line was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences, and cultured in Medium 254 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C in a humidified atmosphere with 5% CO_2.

Cell viability assay. Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Dojindo, Molecular Technologies, Inc.) assay. The PIG1 cell line was seeded into 96-well plates at a density of $7x10^3$/well. When 60-70% confluence was reached, the cells were pretreated with different concentrations of RK1 (0, 0.05, 0.1, 0.2, 0.4, 0.8 or 1.6 mM) for 2 h, and then treated with 1.0 mM H_2O_2 for 24 h at 37˚C with 5% CO_2. Subsequently, 10 μl CCK-8 solution was added to each well and cultured for 90 min. The absorbance was measured using a microplate reader (Molecular Devices, LLC) at 450 nm. Cell viability was calculated according to the following formula: Viability rate (%) = (absorbance of test sample-absorbance of blank)/(absorbance of control-absorbance of blank) x100. In addition, following treatment with RK1 (0.1,0.2 and 0.4 mM) for 2 h, and treatment with 1.0 mM H_2O_2 for 24 h at 37˚C with 5% CO_2, cell morphology was observed using an inverted light microscope (Olympus Corporation).

Determination of apoptosis. Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)-PI apoptosis detection kit (BD Biosciences). Briefly, PIG1 cells were seeded into 6-well plates at a density of $3x10^5$/well and cultured until they had reached 60-70% confluence. The cells were exposed to 1 mM H_2O_2 for 24 h after pretreatment with different concentrations of RK1 (0, 0.1, 0.2 or 0.4 mM) or LY294002 (10 μM) for 2 h at 37˚C with 5% CO_2. Then, the collected PIG1 cells were resuspended in 100 μl binding buffer ($1x10^5$ cells), stained with 5 μl Annexin V-FITC and 5 μl PI, and incubated at room temperature (20-25˚C) for 15 min in the dark. The cell apoptosis rate (early + late apoptosis) was detected using a FACSCalibur flow cytometer and CellQuest software (version 3.3; BD Biosciences) within 1 h.

Detection of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activity levels. The cells from each group were disrupted using an ultrasonic cell disruptor (3 times; each for 3 sec with an interval of 1 sec on ice) and the lysate was centrifuged at 4˚C at 16,000 x g for 5 min to obtain the total protein. The activity levels of SOD, CAT and GSH-Px were determined using a SOD (cat. no. A001-3-2), CAT (cat. no. A007-1-1), GSH-Px (cat. no. A005-1-2) analysis kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Western blot analysis. Total protein from PIG1 cells in each group was extracted using RIPA lysis buffer. Nuclear and plasma proteins were extracted using the Nuclear Protein Extraction Kit (cat. no. R0050; Beijing Solarbio Science & Technology Co., Ltd.). Protein concentrations were determined using a BCA kit (cat. no. AR0146; Wuhan Boster Biological Technology, Ltd). Protein samples (20 μg) were separated via SDS-PAGE on 10% gels, and then transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk in TBS-Tween-20 (0.05%) for 1 h at room temperature, followed by incubation at 4˚C overnight with primary antibodies against Bax (cat. no. ab53154; 1:500), caspase-3 (cat. no. ab4051; 1:500), Bcl-2 (cat. no. ab196495; 1:500), phosphorylated (p)-AKT (cat. no. ab38449; 1:500), AKT (cat. no. ab18785; 1:500), Nrf2 (cat. no. ab137550; 1:500), HO-1 (cat. no. ab13243; 1:2,000), lamin A/C (cat. no. ab227176; 1:1,000), tubulin (cat. no. ab6130; 1:2,000) and β-actin (cat. no. ab8227; 1:1,000) (all from Abcam). Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. BA1054; Wuhan Boster Biological Technology, Ltd.) at 1:5,000 dilution for 1 h at room temperature. The chemiluminescent signals were developed using an enhanced chemiluminescence kit (MilliporeSigma). The images of the proteins were collected and the intensity was analyzed using ImageJ software (version 2.0; National Institutes of Health). β-actin was used as a whole cell internal reference, lamin A/C was used as a nuclear internal reference and tubulin was used as a cytosolic internal reference.

Immunofluorescence. The PIG1 cell line was seeded into 12-well plates containing single layer glass slides and cultured until it reached 60-70% confluence. The cells were then pretreated with 0.4 mM RK1 or 10 μM LY294002 for 2 h, and with 1 mM H_2O_2 for 24 h at 37˚C with 5% CO_2. Following treatment, the PIG1 cell line was fixed with 4% formaldehyde

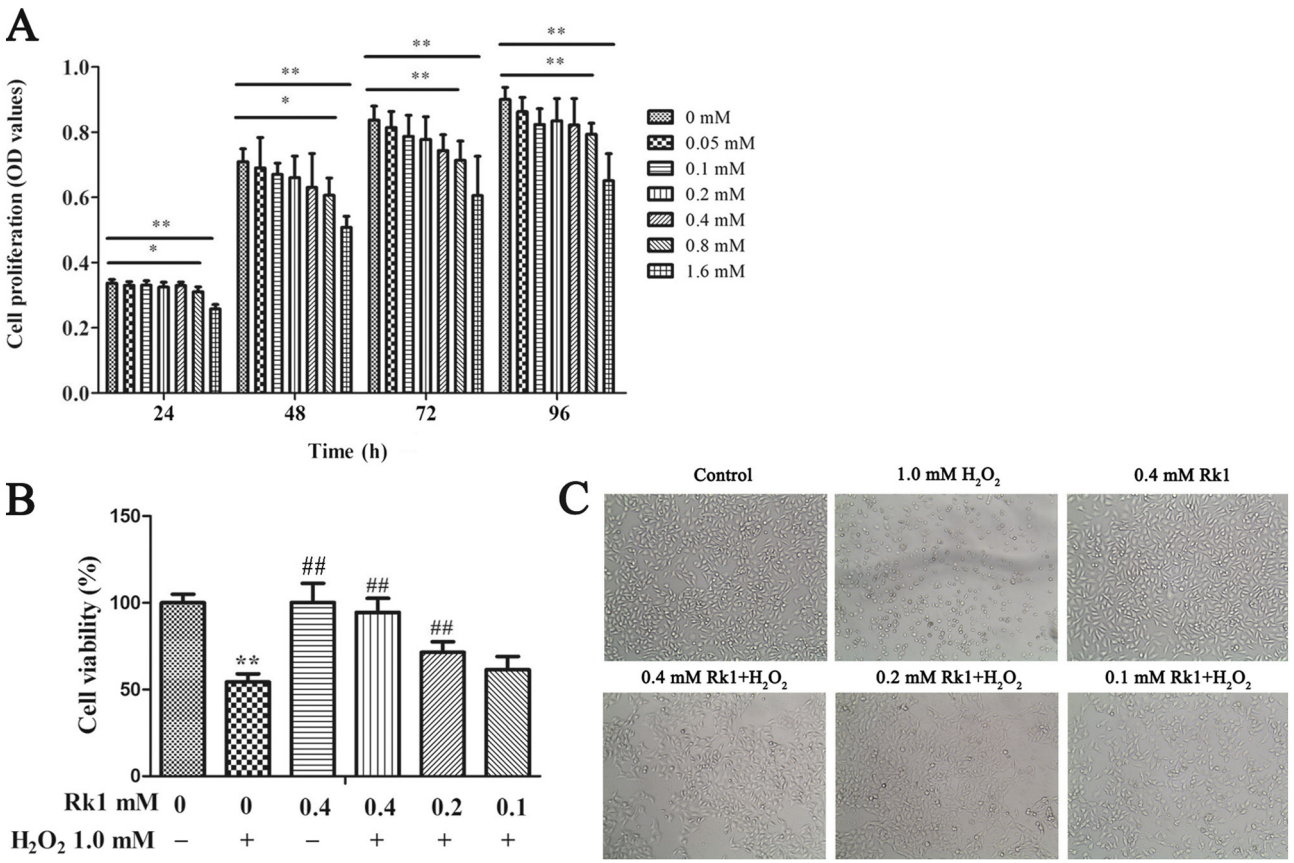

Figure 1. Protective effects of RK1 against H_2O_2-induced cytotoxicity in human melanocytes. (A) PIG1 cells were treated with RK1, at the indicated concentrations, for 24-96 h, then cell viability was determined using a CCK-8 assay. (B) PIG1 cells were pretreated with different concentrations of RK1 for 2 h, exposed to 1.0 mM H_2O_2 for 24 h, then cell viability was determined using a CCK-8 assay. (C) Effect of RK1 on H_2O_2-induced morphological features in human melanocytes (magnification, x200). All the data are presented as the mean ± SD. *P<0.05 and **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. H_2O_2 group. RK1, ginsenoside Rk1; H_2O_2, hydrogen peroxide; CCK-8, Cell Counting Kit-8.

solution for 20 min at room temperature, permeabilized with 0.1% Triton X-100/PBS for 5 min at room temperature, blocked with 10% newborn calf serum (cat. no. 22012-8612; Zhejiang Tianhang Biotechnology Co., Ltd.) for 30 min at room temperature and incubated with an anti-Nrf2 primary antibody (cat. no. ab137550; 1:500; Abcam) overnight at 4˚C. The slides were washed twice with PBS/0.1% Tween-20 and incubated with Alexa Fluor® 488 goat anti-rabbit IgG (green color; cat. no. ab150077; 1:1,000; Abcam) for 1 h at room temperature. DAPI was used to stain the cell nuclei (blue) for 15 min at room temperature. The protein expression of Nrf2 was observed using an Olympus confocal microscope (Olympus FV300; Olympus Corporation).

Statistical analysis. SPSS v20.0 software (IBM Corp.) was used for statistical analysis. All data are presented as the mean ± standard deviation from three separate experiments. Differences among multiple groups were compared by one-way analysis of variance with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

RK1 protects against H_2O_2-induced human melanocyte death. To identify the cytotoxic concentration of RK1, the effects of RK1 on PIG1 cell viability were assessed using a CCK-8 assay. As shown in Fig. 1A, compared with that in the control group, there were no significant changes in cell viability at doses of RK1 between 0.05 and 0.4 mM. Nevertheless, RK1 at a concentration of 0.8 and 1.6 mM significantly suppressed cell viability. Therefore, 0.4 mM RK1 was used as the highest concentration in the subsequent experiments. To investigate the protective effect of RK1 on the PIG1 cell line, the cells were treated with RK1 (0, 0.1, 0.2 and 0.4 mM) for 2 h followed by exposure to 1.0 mM H_2O_2 for 24 h. It was previously found that 1 mM H_2O_2 was the optimal dose for inducing a sufficient cytotoxic effect on melanocytes (24,25). As shown in Fig. 1B and C, treatment with 1.0 mM H_2O_2 for 24 h significantly inhibited cell viability, and the dendrites of the melanocytes were also shortened compared with that in the control cells. However, pretreatment with RK1 (0.2 and 0.4 mM) for 2 h significantly increased cell viability and decreased the amount of cell shrinkage compared with that in cells treated with H_2O_2. Taken together, these data suggested that RK1 could effectively reduce H_2O_2-induced human melanocyte death.

RK1 protects human melanocytes from H_2O_2-induced apoptosis. To determine the protective effect of RK1 against H_2O_2-induced apoptosis, the PIG1 cell line was pretreated with various concentrations of RK1 (0.1, 0.2 and 0.4 mM) for 2 h, then treated with or without 1.0 mM H_2O_2 for 24 h. As shown in Fig. 2A and B, the number of apoptotic cells was significantly

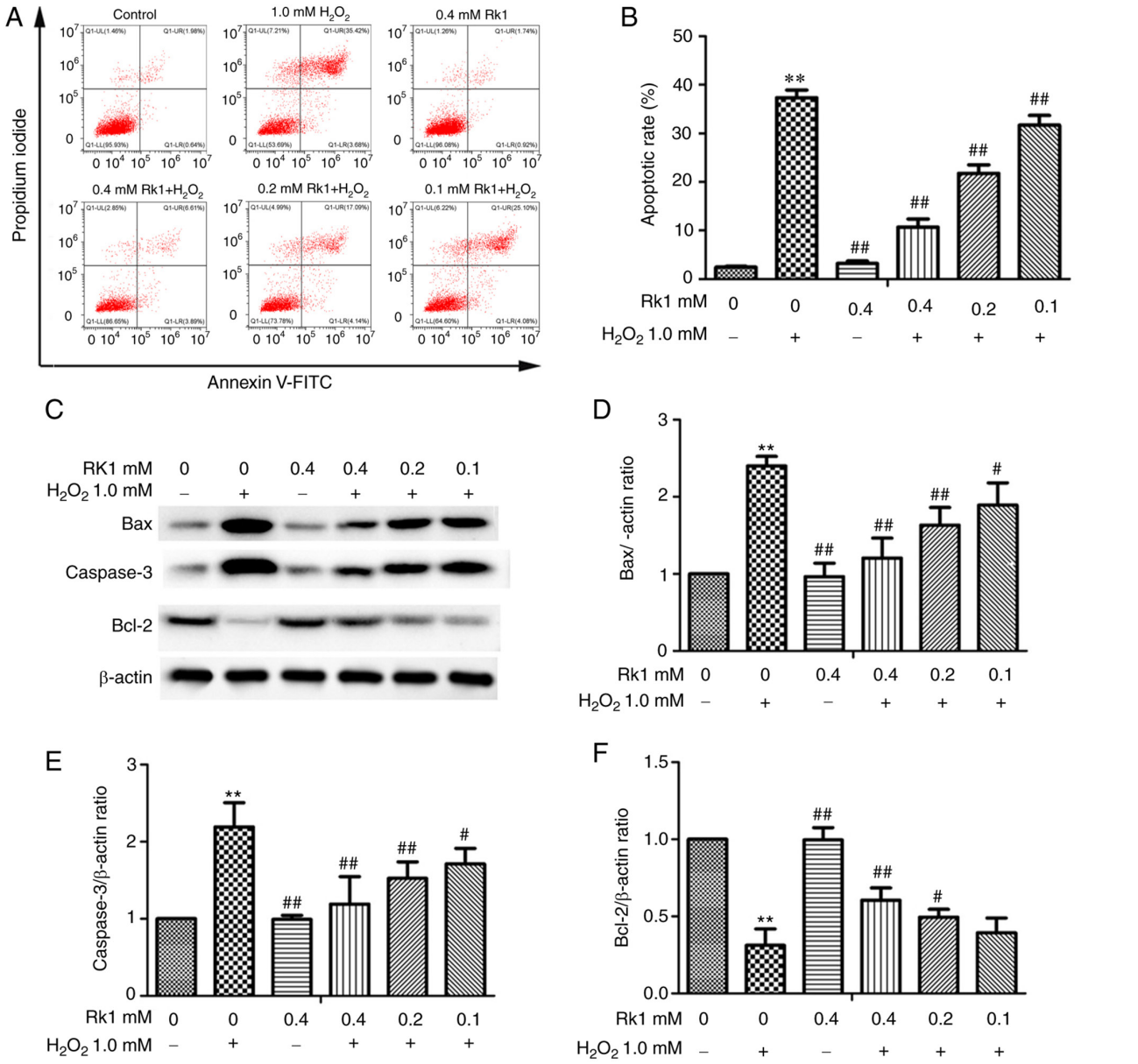

Figure 2. Protective effects of RK1 against H_2O_2-induced apoptosis in human melanocytes. The PIG1 cell line was pretreated with different concentrations of RK1 for 2 h, then exposed to 1.0 mM H_2O_2 for 24 h. Apoptotic cells were stained with Annexin V/FITC and PI, then analyzed using (A) flow cytometry, and the results were subsequently (B) quantified. (C) Protein expression levels of the apoptosis-related proteins were detected using western blotting, and the relative intensity of (D) Bax, (E) caspase-3 and (F) Bcl-2 protein was subsequently semi-quantified. All the data are presented as the mean ± SD. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H_2O_2 group. RK1, ginsenoside Rk1; H_2O_2, hydrogen peroxide; FITC, fluorescein isothiocyanate.

increased in the H_2O_2 group compared with that in the control group; however, RK1 significantly reversed these effects in a dose-dependent manner. In addition, the western blot analysis results revealed that H_2O_2 treatment resulted in a significant increase in the anti-apoptotic protein expression levels of caspase-3 and Bax, and a decrease in the expression of Bcl-2 protein compared with that in the control group. However, treatment with RK1 (0.2 and 0.4 mM) significantly decreased caspase-3 and Bax protein expression levels, and increased Bcl-2 expression levels compared with that in H_2O_2-treated cells (Fig. 2C-F). These data indicated that RK1 could protect human melanocytes from H_2O_2-induced apoptosis.

RK1 strengthens the anti-oxidant ability in human melanocytes. To investigate whether RK1 protects melanocytes against H_2O_2-induced OS, the PIG1 cell line was pretreated with different concentrations of RK1 for 2 h, followed by incubation with 1.0 mM H_2O_2 for 24 h, then the activity levels of SOD, CAT and GSH-Px were determined. As shown in Fig. 3A-C, the activity levels of SOD, CAT and GSH-Px were significantly decreased in the H_2O_2 group compared with that in the control group, whereas RK1 significantly reversed this effect. These results indicated that RK1 could protect melanocytes against H_2O_2-induced oxidative damage.

RK1 activates the AKT/Nrf2/HO-1 signaling pathway and promotes Nrf2 nuclear translocation in human melanocytes. The effect of RK1 on the AKT/Nrf2/HO-1 signaling pathway was further evaluated in the PIG1 cell line. The results showed that H_2O_2 treatment significantly decreased

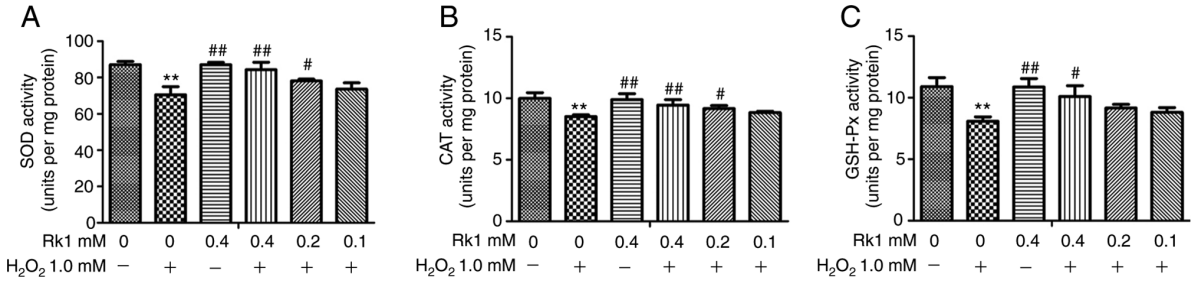

Figure 3. Protective effects of RK1 against H_2O_2-induced oxidative injury in human melanocytes. The PIG1 cell line was pretreated with different concentrations of RK1 for 2 h, then exposed to 1.0 mM H_2O_2 for 24 h. (A) SOD, (B) CAT and (C) GSH-Px activity levels in each group. All the data are presented as the mean ± SD. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H_2O_2 group. RK1, ginsenoside Rk1; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, chloramphenicol acetyltransferase; GSH-Px, glutathione peroxidase.

the protein expression levels of HO-1, the ratio of p-AKT to AKT, and Nrf2 nuclear to cytosolic translocation in the PIG1 cell line (Fig. 4A-D). In addition, compared with that in the H_2O_2 group, the protein expression levels of HO-1, the ratio of p-AKT to AKT, and Nrf2 nuclear to cytosolic translocation were increased in the RK1 group (Fig. 4A-D). The Nrf2 translocation in the PIG1 cell line following treatment with RK1 (0.4 mM) was subsequently investigated using immunofluorescence staining. As expected, RK1 promoted nuclear translocation of Nrf2 (Fig. 4E). These results indicated that H_2O_2 treatment inhibited the activation of the AKT/Nrf2/HO-1 signaling pathway and promoted Nrf2 translocation from the nucleus to the cytoplasm, while RK1 pretreatment could reverse this effect.

LY294002 reverses the protective effect of RK1 on H_2O_2-induced PIG1 cells. To clarify whether the PI3K/AKT signaling pathway is involved in the protective effects of RK1 in the PIG1 cell line against H_2O_2-induced damage, the PI3K inhibitor LY294002 was used to treat the melanocytes. As shown in Fig. 5A, pretreatment with LY294002 significantly decreased the effects of RK1 on the protein expression levels of p-AKT, Nrf2 and HO-1. In addition, LY294002 also reversed the nuclear translocation of Nrf2 induced by RK1 (Fig. 5C). The findings suggested that the protective effect of RK1 against H_2O_2-induced downregulation of the Nrf2/HO-1 signaling pathway and Nrf2 nuclear translocation was mediated by the PI3K/AKT signaling pathway in the PIG1 cell line.

The effect of LY294002 on the cell viability of the PIG1 cell line was subsequently investigated. As shown in Fig. 5B, LY294002 significantly reduced the protective effect of RK1 on the cell viability of the PIG1 cell line. Then, the effect of LY294002 on apoptosis in the PIG1 cell line exposed to H_2O_2 was investigated. As shown in Fig. 5D and E, RK1 treatment significantly decreased the apoptotic rate of the PIG1 cell line exposed to H_2O_2, while the pretreatment of LY294002 significantly inhibited the protective effect of RK1 on the PIG1 cell line. These results demonstrated that pretreatment with RK1 increased the viability of the PIG1 cell line and resulted in a significant decrease of apoptotic cells via the PI3K/AKT signaling pathway.

LY294002 reverses the protective effect of RK1 against H_2O_2-induced OS in the PIG1 cell line. To verify whether RK1 had a protective effect on H_2O_2-induced OS via the PI3K/AKT signaling pathway, the PIG1 cell line was treated with LY294002. As shown in Fig. 6A-C, treatment with RK1 significantly increased the activity level of SOD, CAT and GSH-Px in the PIG1 cell line exposed to H_2O_2, whereas LY294002 could significantly reverse this effect. These data indicated that RK1 alleviated the oxidative damage of the PIG1 cell line induced by H_2O_2 via the PI3K/AKT signaling pathway.

Discussion

In the present study, the protective effect of RK1 against H_2O_2-induced OS in human melanocytes was initially determined and the underlying molecular mechanism was elucidated. It was revealed that RK1 could effectively protect the PIG1 cell line by promoting cell viability, inhibiting cell apoptosis, and increasing the activity levels of SOD, CAT and GSH-Px. Notably, RK1 could protect the melanocytes against OS by increasing Nrf2 and HO-1 protein expression levels, and increasing Nrf2 translocation from the cytoplasm to the nucleus. In addition, it was verified that RK1 could significantly activate the PI3K/AKT signaling pathway. Notably, LY294002 could inhibit the protective effect of RK1 on melanocytes.

The precise etiology remains to be investigated; however, accumulating evidence has demonstrated that OS plays a crucial role in the pathological changes in the onset and progression of vitiligo, as it can directly induce the loss or complete destruction of functioning melanocytes (26). Several studies have also confirmed that the enzyme activity levels of CAT, SOD and GSH-Px in patients with vitiligo were reduced (26-29). RK1 has been reported to protect human keratinocytes by reducing ROS levels to attenuate OS, which reflects its antioxidant ability (30). Furthermore, the pro-oxidative effect of RK1 was reported in triple-negative breast cancer cells (31). Thus, the pro-oxidative and antioxidative activities of RK1 may be associated with different cell types. In the present study, it was revealed that RK1 could improve cell viability, protect the change in cell morphology, and increase the activity levels of SOD, CAT and GSH-Px in melanocytes. Apoptosis, a mode of cell death, is crucial for the initiation of vitiligo pathologies (32). Cellular apoptosis causes cell shrinkage, chromatin condensation and DNA fragmentation. Previous studies have reported that the accumulation of ROS could promote normal human epidermal melanocyte apoptosis (33-36), while RK1 could inhibit hepatocyte apoptosis induced by

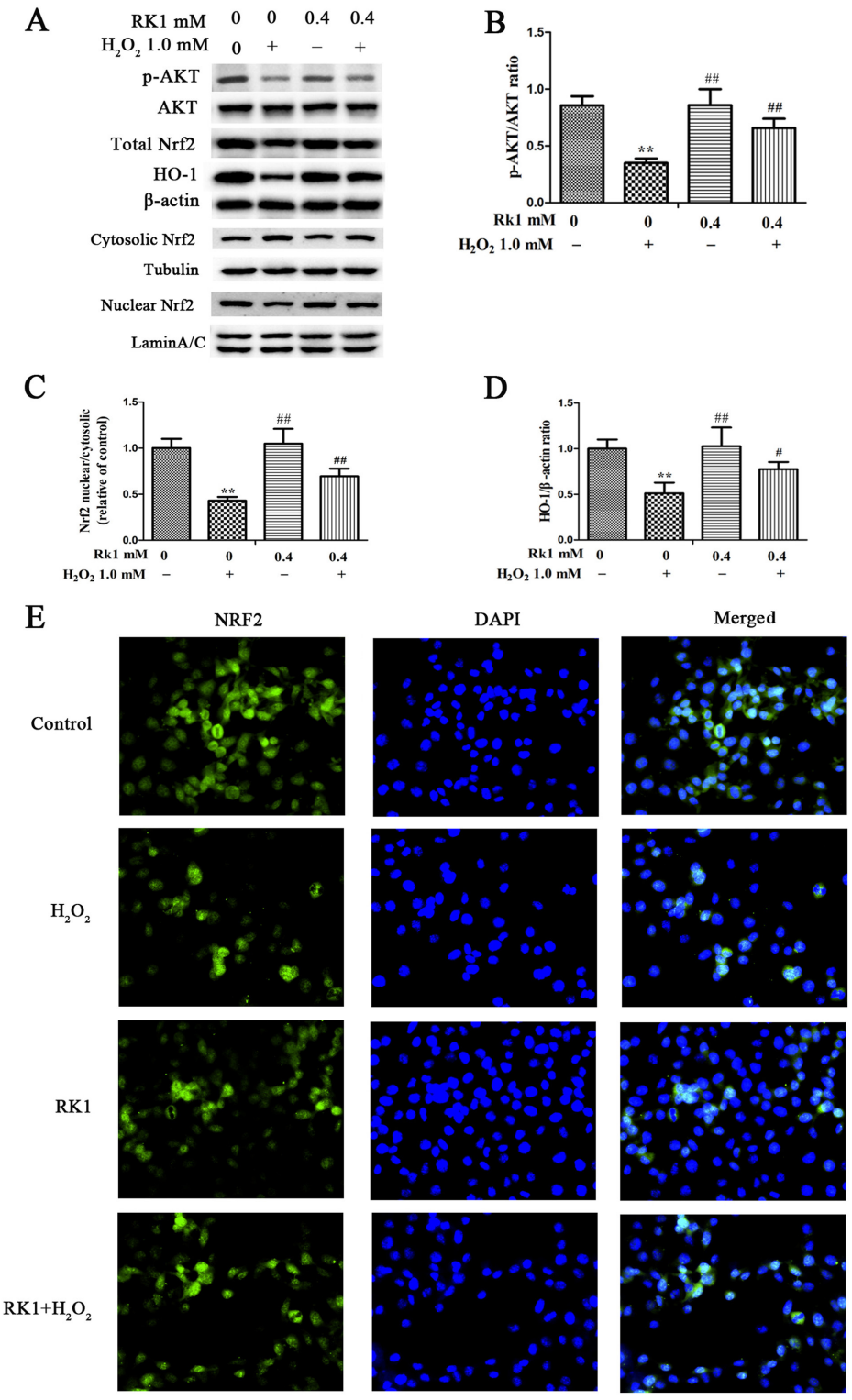

Figure 4. Effects of RK1 on the protein expression levels of p-AKT, AKT, HO-1 and Nrf2, and the translocation of Nrf2 in human melanocytes. Human melanocytes were pretreated with different concentrations of RK1 for 2 h, then exposed to 1.0 mM H_2O_2 for 24 h. (A) Protein expression levels of p-AKT, AKT, HO-1, total Nrf2, cytosolic Nrf2 and nuclear Nrf2 in the PIG1 cell line were measured using western blot analysis. (B) Ratio of p-AKT to AKT shown as a bar graph. (C) Ratio of Nrf2 nuclear to cytosolic translocation shown as a bar graph. (D) Protein expression levels of HO-1 shown as a bar graph. (E) Nrf2 distribution in nucleus/cytoplasm was observed using immunofluorescence assay (magnification, x400). Nrf2 was stained with Nrf2-specific antibody (green staining), while the nucleus was stained with DAPI (blue staining). All the data are presented as the mean ± SD. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H_2O_2 group. RK1, ginsenoside Rk1; H_2O_2, hydrogen peroxide; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; p, phosphorylated.

OS (14). In the present study, it was revealed that treatment with RK1 decreased the cell apoptotic rate, indicating that RK1 prevented apoptosis in human melanocytes. It has been hypothesized that there are three distinct signaling pathways for apoptosis: The mitochondrial pathway, the death receptor pathway and the endoplasmic reticulum stress pathway (37). Activation of caspase-3 plays a crucial role in these pathways, which further drive the terminal events of apoptosis (38). In

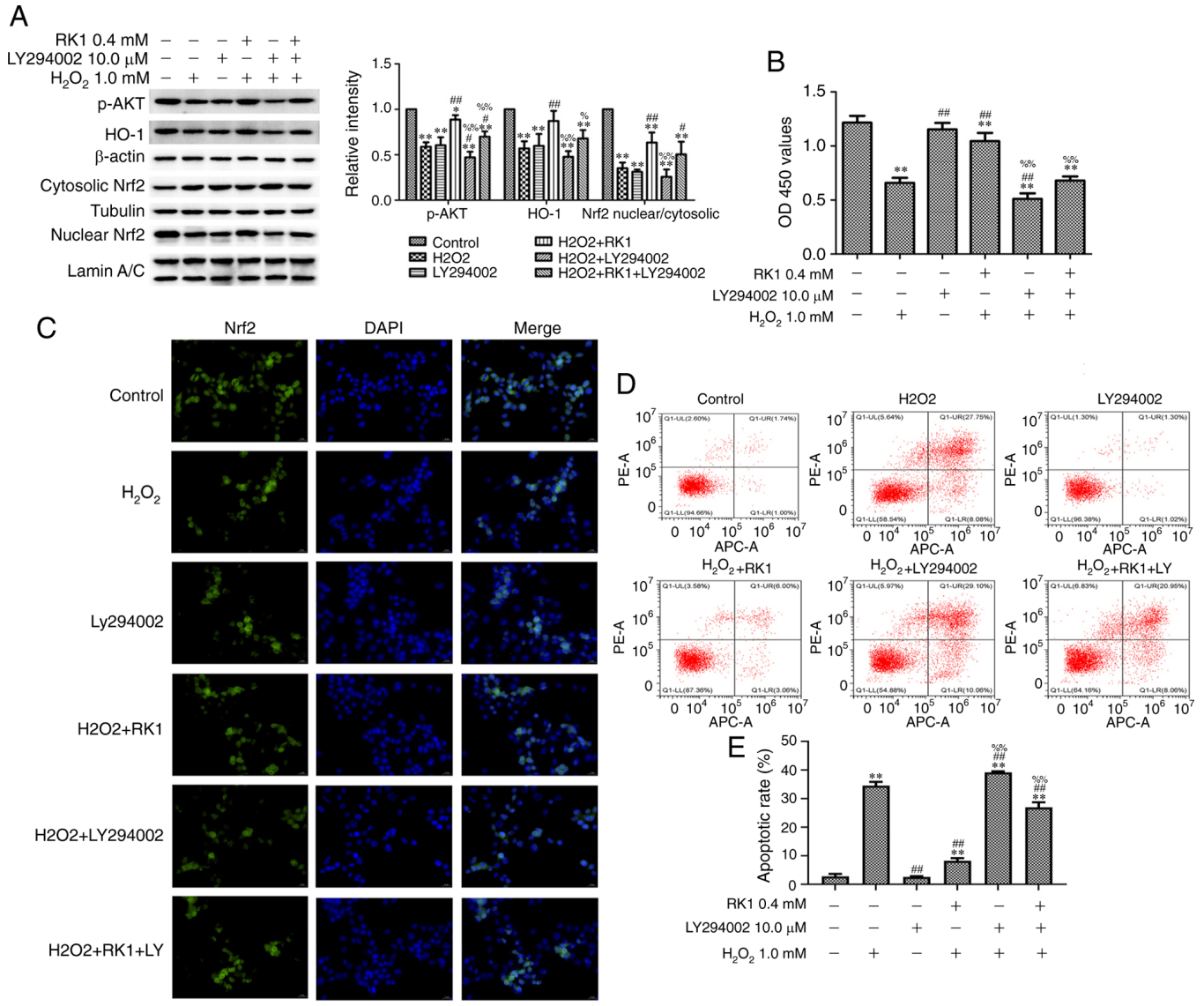

Figure 5. LY294002 reduces the protective effects of RK1 against H_2O_2-induced cell injury in human melanocytes. Human melanocytes were pretreated with or without RK1 (0.4 mM) or LY294002 (10.0 μM) for 2 h, then exposed to H_2O_2 (1.0 mM) for 24 h. (A) Protein expression levels of p-AKT, Nrf2 and HO-1 in the PIG1 cell line were measured using western blot analysis. (B) Cell viability was determined using a Cell Counting Kit-8 assay. (C) Nrf2 distribution in the nucleus/cytoplasm was observed using an immunofluorescence assay (magnification, x400). Nrf2 was stained with Nrf2-specific antibody (green staining), and the nucleus was stained with DAPI (blue staining). (D and E) Cell apoptosis was determined using flow cytometry. All the data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H_2O_2 group; $^{\%}$P<0.05 and $^{\%\%}$P<0.01 vs. RK1 + H_2O_2 group. RK1, ginsenoside Rk1; H_2O_2, hydrogen peroxide; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; p, phosphorylated.

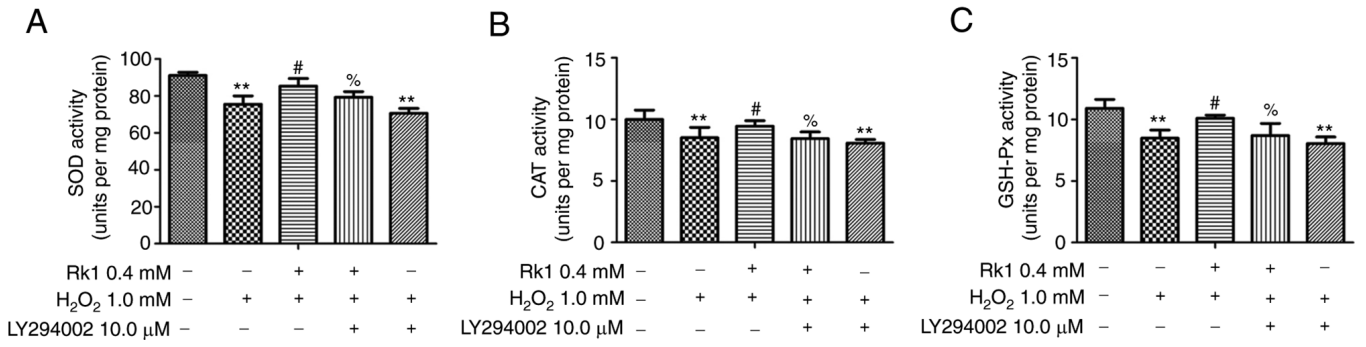

Figure 6. LY294002 reduces the protective effects of RK1 against H_2O_2-induced oxidative damage in human melanocytes. The human melanocytes were pretreated with or without RK1 (0.4 mM) or LY294002 (10.0 μM) for 2 h, then exposed to H_2O_2 (1.0 mM) for 24 h. (A) SOD, (B) CAT and (C) GSH-Px activity levels in each group. All the data are presented as the mean ± SD. **P<0.01 vs. control group; $^{\#}$P<0.05 vs. H_2O_2 group; $^{\%}$P<0.05 vs. RK1 + H_2O_2 group. RK1, ginsenoside Rk1; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, chloramphenicol acetyltransferase; GSH-Px, glutathione peroxidase.

addition, it was found to regulate the transfer and activation of the Bcl-2 family proteins (37). The Bcl-2 family, particularly the ratio of Bcl-2 to Bax, plays a critical role in the regulation of apoptosis. In the present study, it was found that pretreatment with RK1 significantly increased the protein expression levels of Bax and caspase-3, and reduced the protein expression level of Bcl-2. These data demonstrated that the anti-apoptotic effect of RK1 may be mediated via the caspase-dependent pathway in human melanocytes. Lu *et al* (39) reported that geniposide, an iridoid glycoside purified from the fruit of the herb *Gardenia jasminoides*, also protects melanocytes from H_2O_2-induced oxidative damage and apoptosis via the PI3K/AKT signaling pathway. Next, the present study focused on the effect of RK1 on Nrf2 gene expression and translocation.

A previous study revealed that Nrf2, as a transcription factor, plays an important role in regulating the mRNA expression of antioxidant enzymes in melanocytes, and is a major target for vitiligo treatment (40). Nrf2 translocates from the cytoplasm to the nucleus to activate HO-1. HO-1 is a stress-response protein, which removes oxygen free radicals, thus preventing oxidative injury in cells (41). The PI3K/AKT signaling pathway has been reported to be involved in regulating cell survival, metabolism and apoptosis (42). Previous studies have demonstrated that the PI3K/AKT signaling pathway could regulate Nrf2 expression and activity levels (43-45). H_2O_2 was found to decrease the PI3K/AKT signaling pathway in human epidermal melanocytes (46). In the present study, it was revealed that H_2O_2 inhibited the PI3K/AKT/Nrf2/HO-1 signaling pathway; however, RK1 could not only promote the protein expression levels of p-AKT, Nrf2 and HO-1, but could also promote the nuclear translocation of Nrf2. These results illustrated that the PI3K/AKT/Nrf2/HO-1 signaling pathway may be involved in the protective effect of RK1 on H_2O_2-induced oxidative injury in melanocytes. Unfortunately, the present study did not examine the influence of different incubation times of H_2O_2 on Nrf2 protein expression and Nrf2 nuclear translocation, which will be considered in future studies. To further investigate the role of the PI3K/AKT signaling pathway, LY294002 and RK1 were used to determine their combined effect on melanocytes. The results showed that the protective effect of RK1 on melanocytes was significantly inhibited following pretreatment with LY294002, indicating that Rk1 protected melanocytes from H_2O_2-induced oxidative injury by activating the PI3K/AKT signaling pathway. However, vitiligo is an autoimmune disease, and whether RK1 could inhibit the accumulation of melanocyte-specific $CD8^+$ T cells has not been clarified in the present study.

In conclusion, the present study showed that RK1 served a protective role against OS-related injury in melanocytes by activating the PI3K/AKT/Nrf2/HO-1 signaling pathway to enhance cell viability and attenuate apoptosis. Furthermore, RK1 may be a potential therapeutic strategy for the treatment of vitiligo; however, further investigation is required to clarify the specific underlying mechanism of RK1-mediated PI3K/AKT activation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author.

Authors' contributions

JX and JG conceived and designed the experiments. JX, JY and KY performed the experiments and analyzed the data. JX wrote the paper. JX and JG confirm the authenticity of all the raw data. JG reviewed and revised the manuscript. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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