ARTICLE
Journal of Cellular Biochemistry 114:2346–2355 (2013)

Journal of Cellular Biochemistry

Protective Effect of Melatonin on Bone Marrow Mesenchymal Stem Cells Against Hydrogen Peroxide-Induced Apoptosis In Vitro

Fu-Wu Wang,[1] Zhen Wang,[1] Yan-Min Zhang,[1] Zhao-Xia Du,[1] Xiao-Li Zhang,[1] Qian Liu,[1] Yu-Ji Guo,[1] Xin-Gang Li,[2*] and Ai-Jun Hao[1**]

[1]*Department of Histology and Embryology, Key Laboratory of the Ministry of Education for Experimental Teratology, Shandong Provincial Key Laboratory of Mental Disorders, Shandong University School of Medicine, No. 44 Wenhua Xi Road, 250012 Jinan, PR China*

[2]*Department of Neurosurgery, Qilu Hospital of Shandong University and Brain Science Research Institute, Shandong University, No. 107 Wenhua Xi Road, 250012 Jinan, PR China*

ABSTRACT

Bone marrow mesenchymal stem cells (MSCs) transplantation has shown great promises for treating various central nervous system (CNS) diseases. However, poor viability of transplanted MSCs in injured CNS has limited the therapeutic efficiency. Oxidative stress is one of major mechanisms underlying the pathogenesis of CNS diseases and has a negative impact on the survival of transplanted MSCs. Melatonin has recently been reported to have the antioxidant and anti-apoptotic properties in serial of cells. This study was designed to investigate the protective effect and potential mechanisms of melatonin against hydrogen peroxide (H_2O_2)-induced apoptosis of MSCs. MSCs were pretreated with melatonin (1, 10, and 100 nM, respectively) for 30 min, followed by exposure to 400 μM H_2O_2 and melatonin together for 12 h. The present study reports that melatonin pretreatment significantly attenuated H_2O_2-induced MSC apoptosis in a dose-dependent manner. Consistently, melatonin effectively suppressed the generation of intracellular ROS, expression ratio of Bax/Bcl-2, activation of caspase-3 and expression of phospho-P38MAPK in H_2O_2-induced MSCs. Luzindole, a nonselective melatonin receptor antagonist, significantly counteracted melatonin's promotion effect on cell survival, indicating that melatonin exerts its protective effect on MSCs, at least in part, through the activation of melatonin receptors. The findings suggest that melatonin may be an effectively protective agent against oxidative stress-induced MSC apoptosis. J. Cell. Biochem. 114: 2346–2355, 2013.

KEY WORDS: BONE MARROW MESENCHYMAL STEM CELLS; OXIDATIVE STRESS; MELATONIN; APOPTOSIS

Large numbers of studies have demonstrated that bone marrow mesenchymal stem cells (MSCs) have great promises in regenerating and repopulating the damaged neural cells, restoring its function and are considered to be an effective strategy for treating various central nervous system (CNS) diseases and injures in recent years [Parr et al., 2007; Park et al., 2008]. However, the clinical exploitation of MSCs is hampered by the fact that transplanted stem cells do not survive efficiently within the injured CNS [Swanger et al., 2005; Liu et al., 2009, 2012]. The low survival rate of MSCs transplanted into damaged CNS conditions implies that the injured microenvironment may not be conducive to MSC survival. Therefore, promoting the survival of implanted MSCs and protecting the cells from apoptosis may be crucial for their successful utilization in cell transplantation therapy.

Conflict of interest: None.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81271451, 81071057; Grant sponsor: Natural Science Foundation of Shandong Province; Grant numbers: ZR2012HM004, 2012GSF11842; Grant sponsor: Independent Innovation Foundation of Shandong University; Grant numbers: 2012TS118, 2012JC0006.

*Correspondence to: Xin-Gang Li, Department of Neurosurgery, Qilu Hospital of Shandong University and Brain Science Research Institute, Shandong University, No. 107 Wenhua Xi Road, 250012 Jinan, China. E-mail: lixg@sdu.edu.cn

**Correspondence to: Ai-Jun Hao, Department of Histology and Embryology, Key Laboratory of the Ministry of Education for Experimental Teratology, Shandong Provincial Key Laboratory of Mental Disorders, Shandong University School of Medicine, No. 44 Wenhua Xi Road, 250012 Jinan, China. E-mail: aijunhao@sdu.edu.cn

Manuscript Received: 19 March 2013; Manuscript Accepted: 23 April 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 4 July 2013

DOI 10.1002/jcb.24582 • © 2013 Wiley Periodicals, Inc.

Previous studies proved that oxidative stress was implicated in the pathogenesis of various CNS diseases such as trauma, ischemia/hypoxia, Alzheimer's disease, Parkinson's disease and so on [Federico et al., 2012]. And the reactive oxidative species (ROS) generated abundantly in serial of CNS diseases, maintained at a high level for a long period of time, which promoted the cell apoptosis [Jung et al., 2007; Das et al., 2010]. This demonstrated that excessive oxidative stress microenvironment would have an extremely negative impact on the survival of transplanted cells. Therefore, it is pivotal to improve the survival of donor stem cells under conditions of oxidative stress.

Melatonin or *N*-acetyl-5-methoxytryptamine, is an endogenous antioxidant secreted mainly from the pineal gland in mammals [Reiter, 1991]. Melatonin possesses a variety of important physiological functions, including regulation of circadian rhythms, reproductive and neuroendocrine actions [Dubocovich, 2007; Hardeland, 2008]. Recently, increasing evidences imply that melatonin acts as a potent free radical scavenger and antioxidant in a variety of cells. Melatonin is considered the most effective antioxidant, specifically as a powerful scavenger of superoxide anion and stimulator of the synthesis of antioxidant enzymes [Reiter, 1998]. Melatonin can detoxify many ROS generation and still exhibits antioxidative properties when converted to metabolites [Reiter et al., 2007]. And melatonin and its metabolites can reduce oxidative stress via several mechanisms [Hardeland, 2010]. Furthermore, melatonin has been shown to have the anti-apoptotic properties in numerous neurological disorders such as Alzheimer's disease, Parkinson's disease, and ischemic brain injures [Singhal et al., 2011; Hashimoto et al., 2012]. Our previous studies demonstrated that melatonin could protect the neural stem cells and astrocytes subjected to the cytotoxicity injures in vitro, respectively [Fu et al., 2011; Wang et al., 2012]. However, it is unclear whether melatonin protect the transplantated MSCs from oxidative stress injures.

To attain the objective, we established a cell model in vitro to mimic partially the injured microenvironment in vivo to unravel the role of melatonin on MSCs induced by oxidative stress, the subsequent signal transduction pathways and possible molecular mechanisms involved in the process. In this study, hydrogen peroxide (H_2O_2), a well-established model to study oxidative stress-induced apoptosis because of its relatively long half-life and solubility in both lipids and aqueous media and other unique biochemical properties, was used to induce the apoptosis of MSCs [Droge, 2002; Ryter et al., 2007]. We report here that melatonin has a powerful protective effect against H_2O_2-induced apoptosis of MSCs through modulating cell survival and apoptosis in vitro.

MATERIALS AND METHODS

CELL CULTURE

Primary MSCs were prepared from adult Kunming mice (KM strain) as described before with some modifications [Wang et al., 2009]. Briefly, the tibias and femurs of the mice (20–25 g) were dissected out and the marrow was flushed out by using a needle and syringe. The cell suspension was plated in low glucose-DMEM medium plus 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Non-adherent cells were removed by replacing the medium every 2 days. After 10 days of incubation, the cell culture was trypsinized and replated. Cells were used at passage 3–5 for the experiments.

In the handling and care of the animals, the International Guiding Principles for Animal Research, as stipulated by the World Health Organization (1985) and as adopted by the Laboratory Animal Center, Shandong University, were followed. During the study, the number of mice used and their suffering were minimized.

CELL TREATMENT

Before experiment, the MSCs were incubated with serum-free DMEM medium for 1 h. After this, the medium was replaced with serum-free DMEM containing different concentration of H_2O_2 (100–600 μM, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) for 12 h and the cell apoptosis was detected. The cells cultured in complete medium (containing 10% FBS) were used as the normal control. For melatonin protection experiments, the MSCs were pretreated with melatonin (0.001–10 μM, Sigma–Aldrich, St. Louis, MO) for 30 min and followed by H_2O_2 and melatonin exposure together for 12 h. And the morphologic observation of the MSCs treated differently was performed using the inverted phase contrast microscope (IX71, Olympus, Tokyo, Japan).

For antagonist or inhibitor studies, the cells were preincubated with a melatonin receptors antagonist Luzindole (Tocris Bioscience, Ellisville, MO) or a P38MAPK (mitogen-activated protein kinase) inhibitor SB203580 (Sigma–Aldrich) for 30 min prior to the administration of 100 nM melatonin or 400 μM H_2O_2, respectively.

MTT ASSAY

Cell viability was determined by the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich) assay. The MSCs were plated into 96-well culture plates (Corning Costar) at density of 1×10^4 cells/ml with 200 μl culture medium per well. When reaching 80% confluence, the cells were treated with 400 μM H_2O_2 for 12 h. Then, 20 μl MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 4 h. The medium was aspirated and 200 μl dimethylsulfoxide was added. The absorbance value was measured in a multi-well spectrophotometer (BioRad) at 490 nm. Three independent experiments were conducted.

ANNEXIN V/PROPIDIUM IODIDE (PI) STAINING ASSAY

Cell apoptosis was determined using an Annexin V-FITC/PI Apoptosis Detection Kit (Abcam, Cambridge, MA) according to the manufacturer's protocols. Briefly, the differently treated cells were washed with cold phosphate buffered saline (PBS) and resuspended in 200 μl of binding buffer. Then 10 μl Annexin V stock solution was added to the cells and incubated for 30 min at 4°C in the dark. This was followed by a further incubation with propidium iodide solution (5 μl, containing RNase) and then immediately detected by bivariate flow cytometry (FC500, Beckman Coulter) to measure the cell apoptosis. Approximately 10,000 cells were analyzed in each of the samples. Three independent experiments were conducted.

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED DUTP NICK-END LABELING (TUNEL) ASSAY

The TUNEL assay was performed by using the in situ cell detection kit (FITC) following the manufacturer's instructions (Chemicon, Temecula, CA). Briefly, The MSCs grown on glass cover slips were fixed in 4% paraformaldehyde for 20 min at room temperature. Cover slips were then washed with PBS and incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. Then, 50 μl of TUNEL reaction mixture was added on cover slips and incubated in a humidified chamber for 1 h at 37°C in the dark. Then the reaction was stopped and the cells were counterstained with 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI) at room temperature for 10 min. Images of TUNEL-positive cells were captured with a fluorescence microscope (IX71, Olympus, Tokyo, Japan), and counted in 10 random fields from each experimental condition. The proportion of TUNEL-positive cells was expressed as a percentage of the total cells counted.

ROS ASSAY

Intracellular ROS levels were measured by 2′,7′-dichlorodihydro-fluorescein diacetate (H_2DCFDA) and dihydroethidium (DHE) assays, respectively. H_2DCFDA or DHE is a membrane-permeable dye that is oxidized by intracellular H_2O_2 or O_2^- to the fluorescent product DCF or ethidium, respectively. Briefly, cells treated with various reagents were washed three times with PBS and incubated with either 10 μM H_2DCFDA or 2 μM DHE (Molecular Probes, Eugene, OR) for 20 min at 37°C. The cells were counterstained by DAPI. The images were taken with a fluorescence microscope (IX71, Olympus) and the fluorescence was measured with a fluorescence plate reader (Fluroskan Ascent II, Labsystems, Helsinki, Finland). Values were expressed relative to the fluorescence signal of the control.

IMMUNOCYTOCHEMISTRY

MSCs were plated on poly-L-lysine (PLL)-treated cover slips. Following various treatments, the cells were fixed in 4% paraformaldehyde for 20 min and blocked with 10% goat serum in PBS. Slides were incubated overnight in a humidified chamber at 4°C with the primary antibodies: anti-melatonin receptor 1 (MT1, 1:100, rabbit polyclonal, Santa Cruz), anti-MT2 (1:100, rabbit polyclonal, Santa Cruz), and anti-cleaved caspase-3 (1:200, rabbit polyclonal, Cell Signaling Tech.). After primary antibody incubation, samples were washed again and incubated in the appropriate fluorescent-conjugated secondary antibody (goat anti-rabbit IgG, 1:200, Sigma–Aldrich) for 1 h. The cells were counterstained by DAPI and the images were captured with a fluorescence microscope.

WESTERN BLOT

Cells were rinsed with cold PBS and lysed in ice-cold RIPA buffer containing protein inhibitors. Cell lysates were incubated at 4°C for 20 min. The sample was centrifuged at 11,000*g* for 10 min at 4°C, and protein concentration in the supernatants of cell extract was determined using a BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL). Equal amount of total proteins was loaded onto a 4–20% gradient polyacrylamide gel, electrophoretically transferred to polyvinylidene difluoride membrane, and probed with primary antibodies: rabbit anti-Bcl-2 (1:1,500, Santa Cruz), rabbit anti-Bax (1:2,000, Santa Cruz), rabbit anti-cleaved caspase-3 (1:1,000, Cell Signaling Tech.), rabbit anti-total caspase-3 (1:1,000, Cell Signaling Tech.), rabbit anti-P38MAPK (1:1,000, Cell Signaling Tech.) and rabbit anti-phospho-P38 MAPK (1:1,000, Cell Signaling Tech.), respectively. Monoclonal anti-β-actin (1:1,000, Sigma–Aldrich) was used as an internal control. Secondary antibodies were horseradish peroxidase conjugated to either goat anti-rabbit IgG or anti-mouse IgG (1:8,000, Sigma–Aldrich). The membranes were developed using an enhanced chemiluminescence detection system (Pierce, Rockford, IL). The intensity of bands was determined using the Image-Pro Plus 6.0 software.

STATISTICAL ANALYSIS

Quantitative data were presented as the mean ± SD of at least three independent experiments. Statistical analysis of data was carried out by Student's *t*-test or by one-way ANOVA using Dunnett's test in multiple comparisons of means. Differences were considered statistically significant if the *P*-value was <0.05.

RESULTS

MELATONIN IMPROVES THE CELL VIABILITY OF MSCs TREATED WITH H_2O_2

Initially, we estimated the cytotoxic effect of H_2O_2 on MSCs using MTT assay. Treatment with H_2O_2 for 12 h reduced the cell viability in a concentration-dependent manner. As shown in Figure 1A, 400 μM H_2O_2 significantly decreased the cell viability of MSCs compared with the control (53.8 ± 3.6%, $P < 0.01$), so we used this concentration in the subsequent experiments. To evaluate the protection of melatonin, the MSCs were pretreated with increasing concentrations of

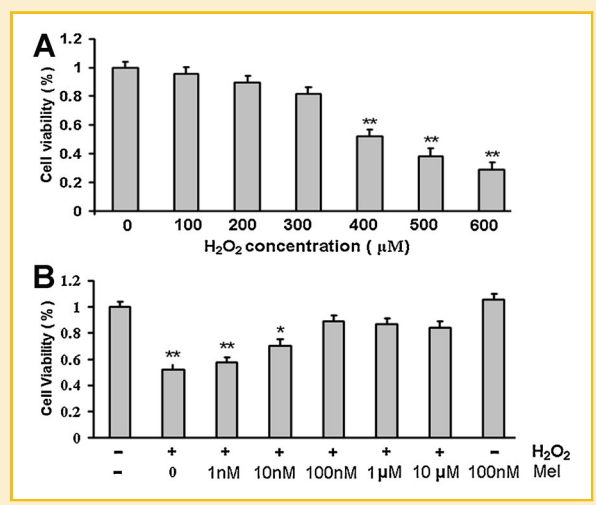

Fig. 1. Promotion of melatonin on MSCs viability from H_2O_2-induced oxidative stress in a dose-dependent manner. MSCs were treated with different concentrations of H_2O_2 (A) or pretreated with different concentrations of melatonin (Mel) followed by stimulation of 400 μM H_2O_2 (B) for 12 h, and cell viability was assessed by MTT assay. Data represent mean ± SD of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, compared with control.

melatonin (0, 1, 10, and 100 nM, 1 and 10 μM) before H_2O_2 exposure. And MTT results revealed that melatonin significantly improved the cell viability of MSCs in a dose-dependent manner in comparison with cells not treated with melatonin (Fig. 1B). Furthermore, compared to the H_2O_2 treatment alone ($50.3 \pm 3.4\%$), 100 nM melatonin pretreatment yielded the optimal effect on cell viability ($90.7 \pm 4.1\%$, $P < 0.01$). The stimulatory effect of melatonin appeared to decline at a concentration higher than 100 nM. And there was no significant difference of cell viability between the control cells and those treated with melatonin (100 nM) alone. In view of this, we have used the three concentration of melatonin (1, 10, and 100 nM) in the following experiments.

MELATONIN PREVENTS MSCs FROM H_2O_2-INDUCED APOPTOSIS-LIKE MORPHOLOGICAL CHANGES

To test the protective effect of melatonin on H_2O_2-induced apoptosis, the morphology of MSCs treated with H_2O_2 (400 μM) in the absence or presence of different concentration of melatonin was assessed by light microscopy. We observed that almost no abnormal cells were observed in the control group, while a high number of cells displayed typical apoptosis-like morphological changes including detachment, irregular shape, nuclear shrinkage, and decrease in cell size in H_2O_2 treatment group (Fig. 2). However, in the melatonin-pretreated groups, the proportion of apoptosis-like cells obviously reduced, indicating the protective effect of melatonin. Furthermore, in the three melatonin-pretreated groups (1, 10, and 100 nM, respectively), the proportion of apoptotic cells decreased with increasing melatonin concentration, indicating a concentration-dependent protective effect of melatonin on H_2O_2-induced apoptosis.

MELATONIN PROTECTS MSCs FROM H_2O_2-INDUCED APOPTOSIS

To ascertain the apoptosis of H_2O_2-treated MSCs, TUNEL assay was carried out. As shown in Figure 3A, the frequency of apoptotic cells was increased from $5.9 \pm 2.2\%$ in control group to $42.3 \pm 2.4\%$ in H_2O_2 treatment alone group ($P < 0.01$). While, pretreatment with 1, 10 and 100 nM melatonin reduced the percentage of cell apoptosis induced by H_2O_2 to $27.5 \pm 2.6\%$, $19.6 \pm 2.5\%$, and $11.3 \pm 2.4\%$, respectively, in a dose-dependent manner. And no significant difference was found between the control and 100 nM melatonin treatment alone (Fig. 3A).

The apoptotic cell ratio was further analyzed with Annexin V-FITC/PI double staining methods by flow cytometry. In the present study, the Annexin V^+/PI^- cells were counted as the apoptotic cells. The results showed that the percentage of apoptotic cells in H_2O_2

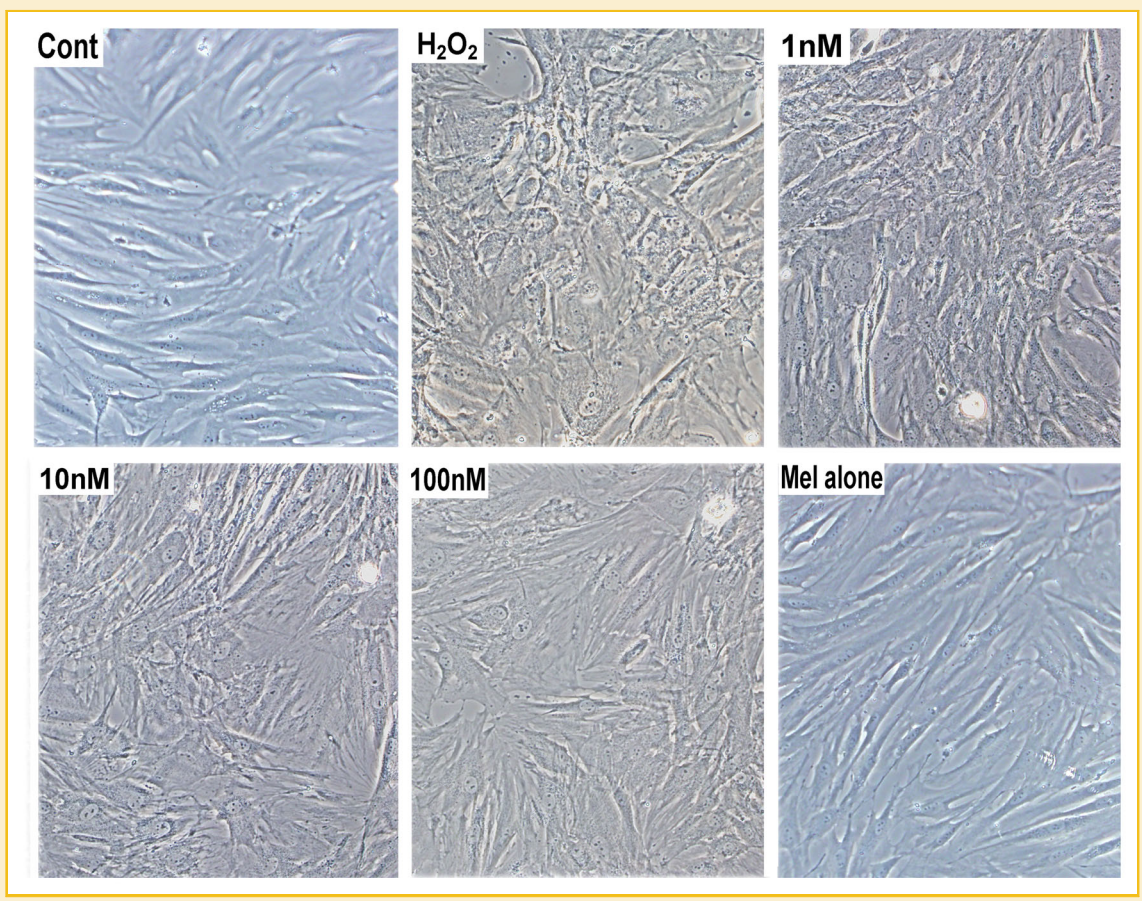

Fig. 2. Melatonin decreases the proportion of apoptosis-like morphologic MSCs induced by H_2O_2. Melatonin (Mel) at concentrations of 1, 10, and 100 nM was applied to MSCs 30 min before exposure to 400 μM H_2O_2. The anti-apoptotic effect of melatonin was then determined by detection of MSCs with morphological changes using inverted phase contrast microscope. Images are representative of triplicate sets.

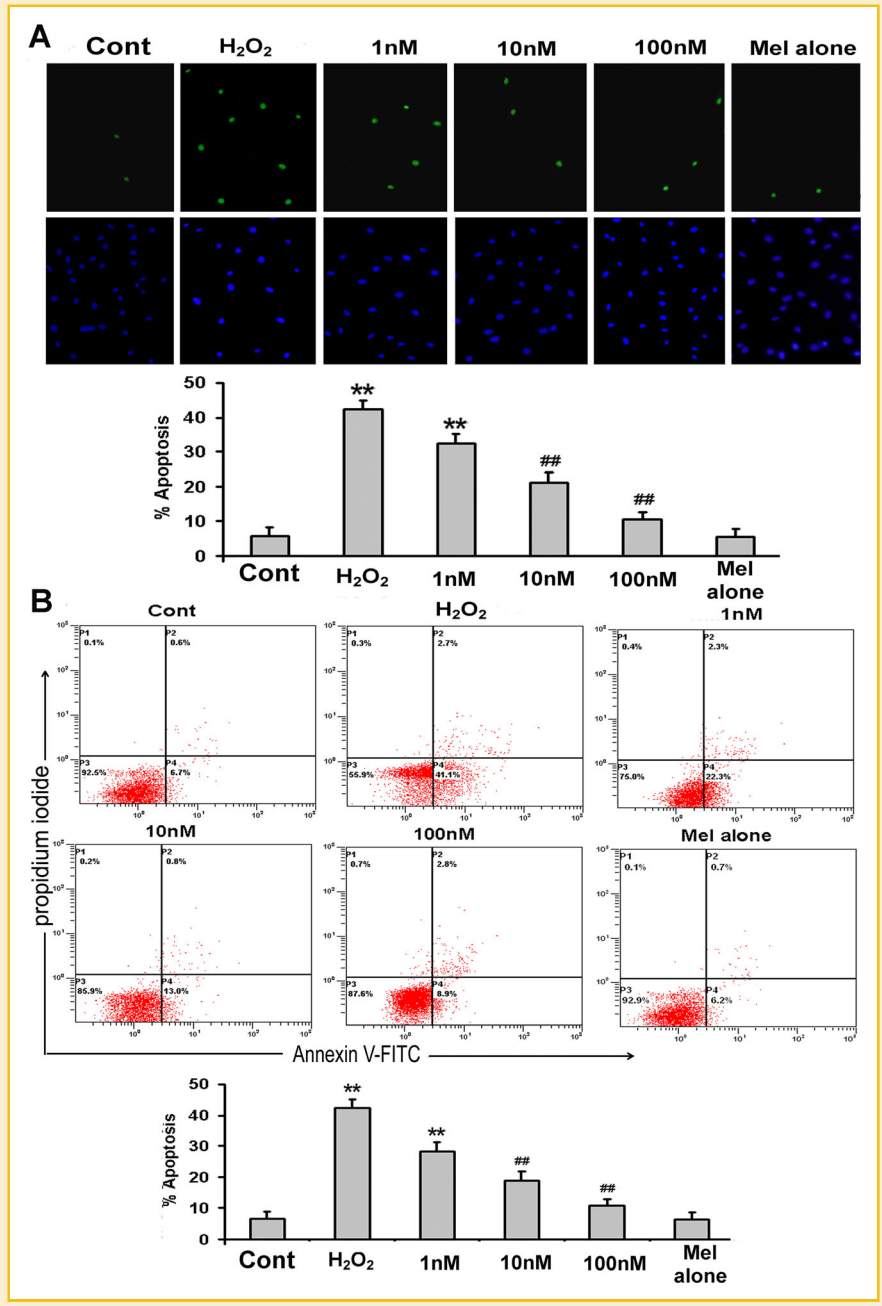

Fig. 3. Melatonin protects MSCs against H_2O_2-induced apoptosis. MSCs were treated with 400 μM H_2O_2, or pretreatment with melatonin (1, 10, and 100 nM, respectively) followed by H_2O_2 and melatonin together for 12 h. Some cells were subjected to TUNEL assay and the images were captured by fluorescence microscopy (A), while other cells were stained with Annexin V/PI and the apoptosis was quantified by flow cytometric analysis (B). Bar graphs indicate the percentage of apoptotic cells based on TUNEL (A) or FACS analysis (B), respectively. Data represent mean $\pm$ SD of three independent experiments. $^{**}P < 0.01$ versus control, $^{\#\#}P < 0.01$ versus H_2O_2 alone.

treatment alone group was remarkably increased ($40.3 \pm 3.3\%$) compared with that in control ($6.3 \pm 2.1\%$, $P < 0.01$, Fig. 3B). And pretreatment with different concentration of melatonin (1, 10, and 100 nM, respectively) significantly inhibited the H_2O_2-induced apoptosis ($26.3 \pm 2.8\%$, $15.1 \pm 2.6\%$, and $9.7 \pm 2.3\%$, respectively) compared to 400 μM H_2O_2-treated cells, which is consistent with the TUNEL results. It is noteworthy that 100 nM melatonin alone did not affect the rate of apoptotic cells supported by Annexin V-PI staining assay methods (Fig. 3B).

MELATONIN ATTENUATES THE GENERATION OF INTRACELLULAR ROS

To determine whether melatonin attenuate the cell death of MSCs by reducing the ROS generation in the present study, the intracellular ROS level was examined using the H_2DCFDA and DHE assay, respectively. Figure 4 shows that H_2O_2 significantly increased the intracellular ROS level in MSCs, and pretreatment with melatonin (1, 10, and 100 nM, respectively) markedly blocked the generation of ROS, respectively, in a concentration-dependent manner. The findings indicate that

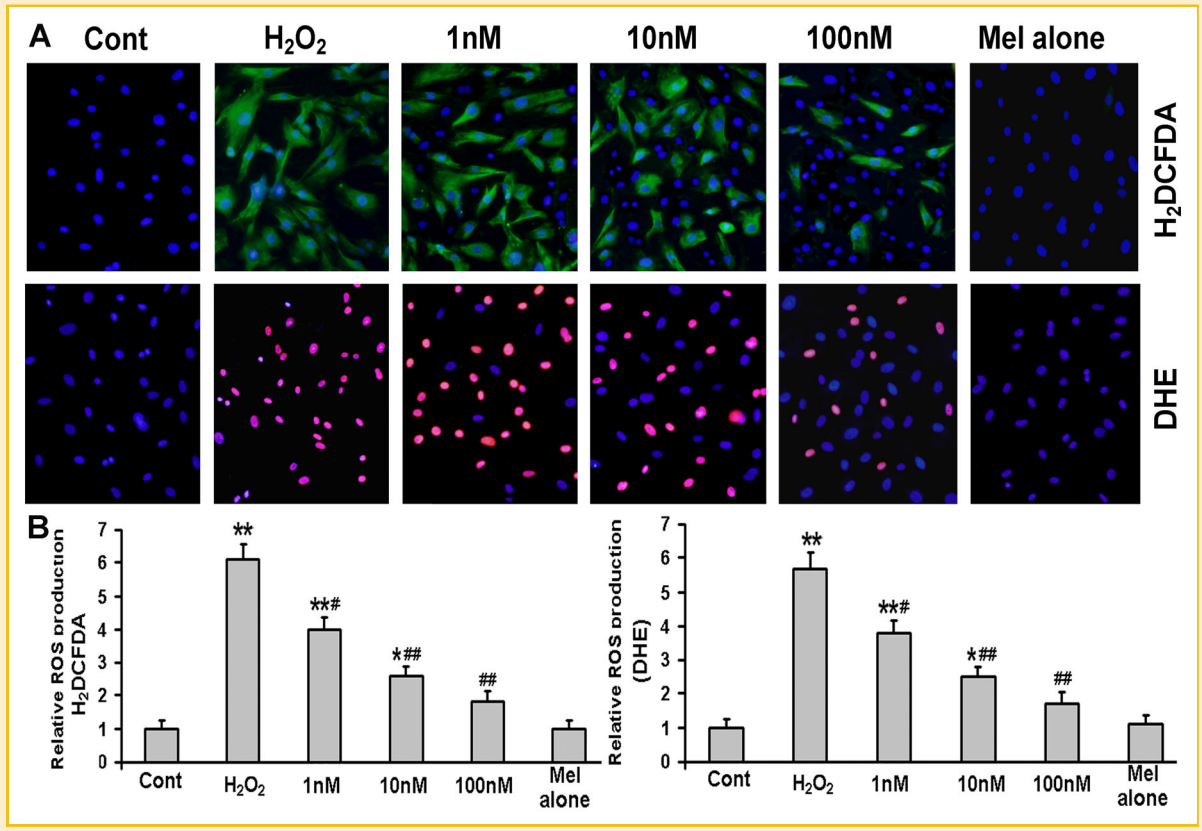

Fig. 4. Melatonin suppresses the generation of intracellular reactive oxygen species (ROS) in H_2O_2-induced MSCs. A: MSCs were stimulated with 400 μM H_2O_2, or pretreatment with melatonin (1, 10, and 100 nM, respectively) followed by H_2O_2 and melatonin together for 12 h. After washing with phosphate-buffered saline, the cells were treated with 10 μM H_2DCFDA (green) or 2 μM dihydroethidium (DHE, red), respectively and counterstained with DAPI (blue); the level of ROS was detected by using a fluorescence microscope. Images are representative of triplicate sets. B: Quantification of the ROS was determined by fluorescence plate reader. Values were expressed relative to the fluorescence signal of respective controls. Values represent the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus H_2O_2 alone.

melatonin protects the MSCs from oxidative stress injury, at least partially, by inhibiting the intracellular ROS generation.

MELATONIN REGULATES THE PROTEIN EXPRESSION OF BAX AND BCL-2 IN H_2O_2-INDUCED MSCs

Considering that Bcl-2 family proteins play a pivotal role in modulating cell life and death [Chetsawang et al., 2006]. We examined the expression of Bax and Bcl-2, two important apoptosis regulatory proteins of Bcl-2 family, at protein level. The results showed that H_2O_2 (400 μM) treatment significantly increased the expression of pro-apoptotic protein Bax and decreased the expression of anti-apoptotic protein Bcl-2, compared with the control. While melatonin pretreatment significantly attenuated the H_2O_2-induced down-regulation of Bcl-2 expression and up-regulation of Bax expression. And significant difference was observed in Bax/Bcl-2 ratio at protein level between the cells treated with H_2O_2 alone and those pretreated with 100 nM melatonin (2.8- to 3.6-folds, $P < 0.01$). Furthermore, melatonin pretreatment decreased the ratio of Bax/Bcl-2 protein expression in a dose-dependent manner. Melatonin (100 nM) treatment alone did not significantly alter the Bax/Bcl-2 ratio. Again, level of β-actin was monitored to ensure that equal amount of protein was loaded in each lane (Fig. 5).

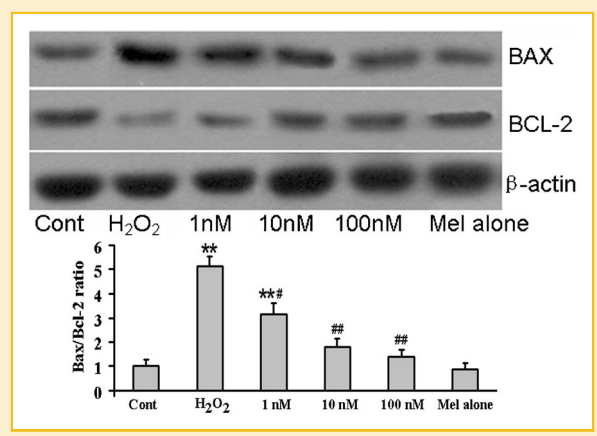

Fig. 5. Melatonin regulates the expression of Bax and Bcl-2 in H_2O_2-induced MSCs. A: MSCs were stimulated with 400 μM H_2O_2, or pretreatment with melatonin (1, 10, and 100 nM, respectively) followed by H_2O_2 and melatonin together for 12 h. The protein levels of Bax, Bcl-2, and β-actin were then analyzed by Western blot and β-actin serves as an internal control. Densitometric analysis shows the Bax/Bcl-2 expression ratio. Values represent the mean ± SD of three independent experiments. $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus H_2O_2 alone.

MELATONIN INHIBITS THE ACTIVATION OF CASPASE-3 IN H_2O_2-INDUCED MSCs

It is well known that caspases are downstream of Bcl-2 family in the apoptotic cascade and activated caspase-3 cleaved from pro-caspase-3 has been considered as a major executioner [Yao et al., 2007; Wisessmith et al., 2009]. We next tested the caspase-3 activity for further evaluating the protective effect of melatonin on MSCs. As shown in Figure 6A, the levels of cleaved products of caspase-3 were significantly increased in H_2O_2-treated cells by Western blot studies ($P < 0.01$), whereas total caspase-3 remained unchanged (used as loading control). As expected, pretreatment with melatonin significantly suppressed the caspase-3 activity and the inhibitory effect was positively correlated with the concentrations of melatonin (Fig. 6A). Melatonin treatment alone (100 nM) did not markedly alter the caspase-3 activity. The Western blot results of active caspase-3 was further supported by immunofluorescence study using cleaved caspase-3-specific antibodies (Fig. 6B). Taken together, the findings suggest that melatonin can inhibit the activation of caspase-3 in H_2O_2-induced MSCs.

THE PROTECTIVE EFFECT OF MELATONIN ON H_2O_2-INDUCED MSC APOPTOSIS IS MELATONIN RECEPTORS DEPENDENT

It is documented that melatonin is a neurohormone that regulates target cells through binding to specific high affinity plasma membrane receptors, MT1 and MT2 [Fu et al., 2011; Wang et al., 2012]. So, the expression of MT1 and MT2 in MSCs used in this experiment was detected by immunocytochemistry, respectively. The results showed that both MT1 and MT2 were expressed in the present study (Fig. 7A). To confirm the involvement of the two receptors in the protective effect of melatonin on MSCs exposed to H_2O_2, the cells were pretreated with 10 μM luzindole, a melatonin receptor antagonist, for 30 min prior to the administration of 100 nM melatonin. TUNEL assay showed that pretreatment with luzindole significantly counteracted the melatonin's effect in inhibiting the H_2O_2-induced MSC apoptosis, while the cell apoptosis in the presence of 10 μm luzindole alone was comparable to the control group (Fig. 7B). Moreover, luzindole pretreatment also significantly attenuated the inhibitory effect of melatonin on the activation of caspase-3 in H_2O_2-induced MSCs (Fig. 7C). These results indicate the

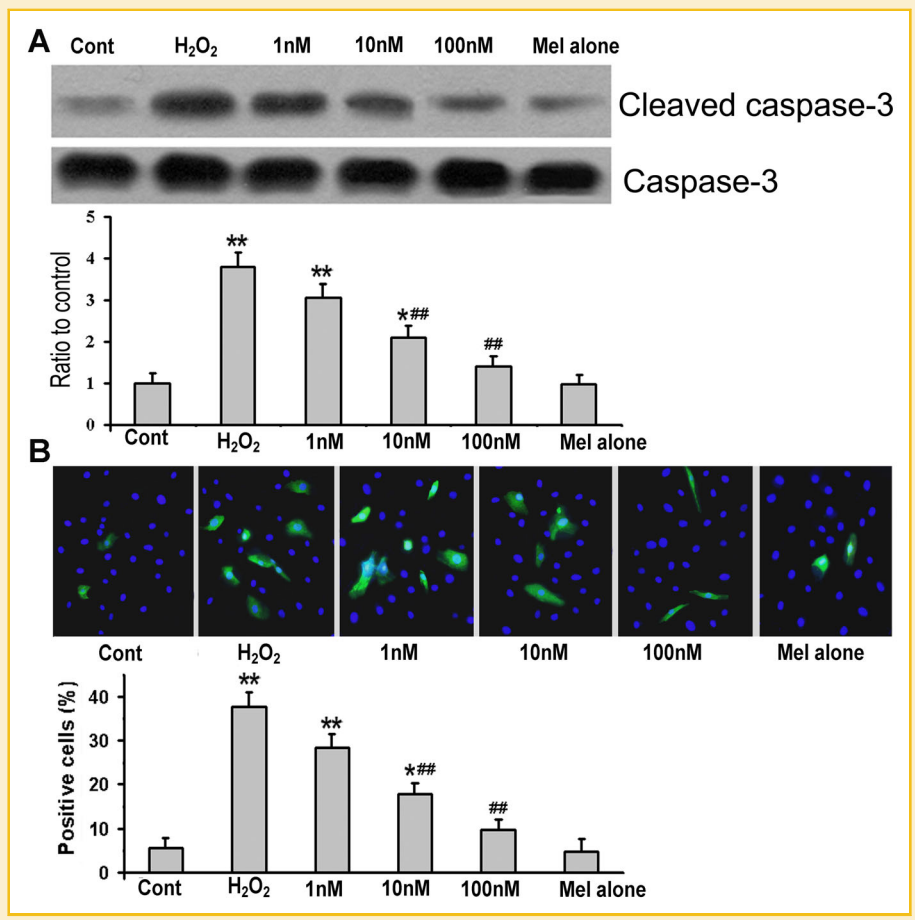

Fig. 6. Melatonin inhibits the activation of caspase-3 in H_2O_2-induced MSCs. MSCs were stimulated with 400 μM H_2O_2, or pretreatment with melatonin (1, 10, and 100 nM, respectively) followed by H_2O_2 and melatonin together for 12 h. The cells were then processed to investigate the activation of caspase-3 by Western blot and immunofluorescence analysis, respectively. A: Immunoblotting results showed the protein expression of cleaved and total caspase-3 in differently treated MSCs. Blots were probed for total caspase-3 as an internal loading control. Results were expressed as cleaved caspase-3/caspase-3 ratio. B: The representative immunofluorescence images of cleaved caspase-3-positive cells (green) were shown and the cells were counterstained with DAPI (blue). Quantification of cleaved caspase-3-positive cells over the total MSCs was shown in B. Data were represented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$ versus H_2O_2 alone.

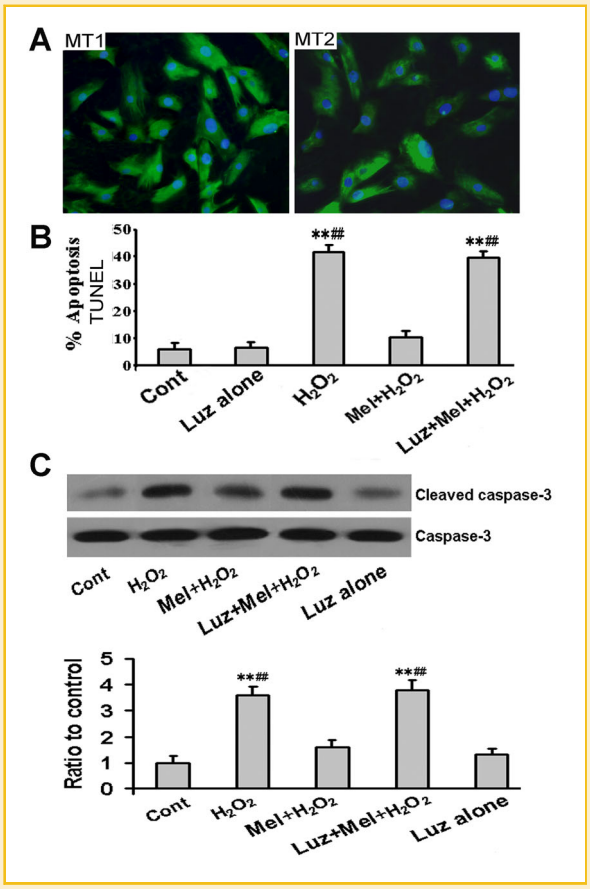

Fig. 7. Protection of melatonin on H_2O_2-induced MSC apoptosis is receptors dependent. A: Melatonin receptor MT1 and MT2 protein were analyzed by immunocytochemistry (green) and the nuclei were counterstained with DAPI (blue). Representative immunofluorescence images of MT1- and MT2-positive MSCs were shown in A, respectively. B–C: MSCs were stimulated with 400 μM H_2O_2, or in combination with preadministration of melatonin (1, 10, and 100 nM, respectively) or luzindole (Luz, 10 μM) for 12 h. Cells were subjected to TUNEL assay (B) or were processed to detect the activation of caspase-3 by Western blot (C). B: The proportion of TUNEL-positive cells was expressed as a percentage of the total cells counted. Values represent the mean ± SD of three independent experiment. C: The Western blot results showed luzindole significantly neutralized the inhibitory effect of melatonin on the activation of caspase-3 in H_2O_2-induced MSCs. $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$ versus Mel + H_2O_2.

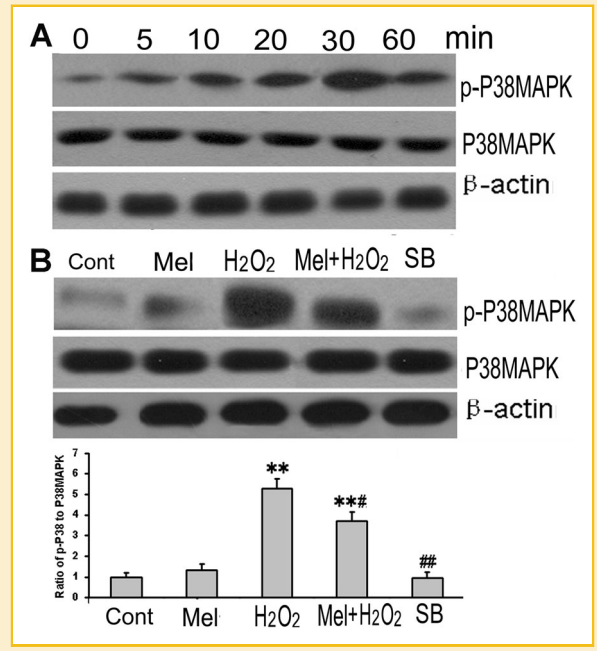

Fig. 8. Melatonin inhibits the activation of P38MAPK in H_2O_2-induced MSCs. A: MSCs were stimulated with 400 μM H_2O_2 for different period of time and cell lysates were obtained and subjected to Western blot to detect the activation of P38MAPK. B: MSCs were pretreated with P38MAPK specific inhibitor, SB203580 (SB, 10 μM), or melatonin (100 nM), respectively, for 30 min followed by 400 μM H_2O_2 treatment for 30 min. Then the cells were processed to investigate the expression of phospho-P38MAPK (p-P38MAPK). Densitometric analysis shows the p-P38MAPK/P38MAPK ratio. Data represent the mean ± SD of three independent experiments. $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus H_2O_2 alone.

protective action of melatonin is mediated, at least in part, through the activation of melatonin receptor in H_2O_2-induced MSCs.

MELATONIN INHIBITS THE ACTIVATION OF P38MAPK IN H_2O_2-INDUCED MSCs

Previous studies reported that the activation of P38MAPK was responsible for oxidative stress-induced apoptosis in serial cells [Wei et al., 2010; Xu et al., 2010]. Then the activity of P38MAPK in MSCs was determined in the experiment. The MSCs were stimulated with H_2O_2 (400 μM) and harvested at 0, 5, 10, 20, 30, and 60 min, respectively for Western blot analysis. Figure 8A shows that the expression of phosphorylation of P38MAPK (p-P38MAPK) was increased within 5 min of initiating treatment, and peaked at 30 min. While total P38MAPK remained unchanged along the time course. As expected, pretreatment with SB203580 (28.6 ± 2.5%) partially reversed the H_2O_2-induced apoptosis of MSCs compared with H_2O_2 treatment alone (39.5 ± 2.4%) via TUNEL analysis. Furthermore, melatonin pretreatment (100 nM) also significantly reduced the expression of p-P38MAPK in H_2O_2-induced MSCs, while 100 nM melatonin alone did not significantly altered the expression of p-P38MAPK (Fig. 8B). The level of β-actin was monitored to ensure that equal amount of protein was loaded in each lane. These findings suggest that melatonin can reduce the H_2O_2-induced apoptosis partially by inhibiting the activation of P38MAPK.

DISCUSSION

The major finding of this study was that melatonin has a strong anti-apoptotic effect on H_2O_2-treated MSCs supported by changes in cell viability, Annexin V/PI staining assay and TUNEL analysis. The mechanism of this action was mediated through inhibiting the generation of intracellular ROS, expression ratio of Bax/Bcl-2, and activation of caspase-3. Luzindole, a non-selective melatonin receptor antagonist, significantly blocked the melatonin's protective effect on cell survival, suggesting a direct relationship between cytoprotection and melatonin receptor. We further showed that

P38MAPK signaling molecule was involved in melatonin-mediated anti-apoptotic effect in H_2O_2-induced MSCs.

Evidences demonstrated a burst of excessive production of reactive oxygen species (ROS) was generated in the pathogenesis of various CNS diseases such as trauma, ischemia/hypoxia and Alzheimer's disease, etc. [Federico et al., 2012]. And augmented ROS, among the numerous factors that cause cell loss and/or death, in the damaged CNS might be an important factor to induce apoptosis of transplanted MSCs [Zeng et al., 2012]. Therefore, it is essential to find ways to promote the survival of implanted MSCs and protect the cells from oxidative stress-induced apoptosis for the successful stem cell transplantation.

Recent studies revealed that melatonin can efficiently scavenge the free radicals, ROS and reactive nitrogen species, by enhancing the activity of anti-oxidative enzymes such as glutathione peroxidase, glutathione reductase, glutathione, *s*-transferase, and superoxide dismutase and so on [Antolín et al., 1996; Urata et al., 1999], and has considerable antioxidant activities in various cell types [Chetsawang et al., 2006]. And our previous studies confirmed that melatonin could protect the neural stem cells and astrocytes from cytotoxicity injures in vitro, respectively [Fu et al., 2011; Wang et al., 2012]. So the melatonin was used to investigate the protective role on MSCs exposed to H_2O_2. The present results showed that melatonin significantly suppressed the intracellular ROS generation in H_2O_2-treated MSCs and consequently inhibited the apoptosis of MSCs in a dose-dependent manner (Figs. 1–4), which further demonstrated that melatonin has a strong protective effect on oxidative stress-induced apoptosis of MSCs. The findings also provide an experimental basis for the successful utilization in MSC transplantation therapy.

Previous studies have proved that the proteins of Bcl-2 family are important regulators of apoptosis, with some members such as Bcl-2 inhibiting the cell apoptosis and others such as Bax inducing the cell death, and the ratio of protein expression in Bax/Bcl-2 regulates the release of cytochrome *c* and the downstream consequences for caspase activation [Kluck et al., 1997; Jürgensmeier et al., 1998; Lim et al., 2002]. Recently, some studies have demonstrated the relationship between melatonin, Bcl-2, and caspase-3 expression [Macías et al., 2003; Lin et al., 2007]. For example, studies by Wisessmith et al. [2009] reported the protective effect of melatonin against injured cerebral neurons is related to Bax and caspase-3 protein. In the present study, we found that 400 μM H_2O_2 strongly increased the expression of pro-apoptotic protein Bax and decreased the expression of anti-apoptotic protein Bcl-2. However, pretreatment with melatonin significantly attenuated the increase of Bax/Bcl-2 ratio (Fig. 5). Because caspases are downstream of Bcl-2 family in the apoptotic cascade and caspase-3 is one of the key effector of apoptosis [Yao et al., 2007; Wisessmith et al., 2009]. We further detected the activation of caspase-3 and found that melatonin pretreatment markedly reduced the caspase-3 activities compared with cells treated with H_2O_2 alone (Fig. 6). Therefore, apart from directly protecting the cells against ROS damage, melatonin may provide additional cytoprotection by inhibiting the rise in Bax/Bcl-2 ratio and mitochondria-dependent caspase cascades.

Considerable studies have shown that it was through binding to its membrane receptor on the surface of target cells that melatonin plays the antioxidant and anti-apoptosis roles [Dubocovich, 2007; Das et al., 2010]. Therefore, we tested the protein expression of two major subtypes of melatonin receptors, MT1 and MT2, in MSCs. The results of immunocytochemistry showed the positive presence of both MT1 and MT2 in MSCs in the present experiment (Fig. 7A), which was in agreement with previous results [Olivier et al., 2009; Wang et al., 2012]. Furthermore, the present study demonstrated that luzindole, a nonselective antagonist of melatonin receptors, significantly counteracted the protective effects of melatonin on MSCs exposed to H_2O_2 (Fig. 7B,C), which was consistent with our and other previous results [Radogna et al., 2007; Wang et al., 2012]. These findings support that melatonin exerts its protective property in H_2O_2-treated MSCs, at least in part, through the activation of its receptors.

It was reported that melatonin receptors could couple to multiple signaling pathways, which participated in the oxidative stress-induced apoptosis [Das et al., 2010; Rosen et al., 2012]. However, the mechanisms involved in the protection of melatonin have yet to be fully elucidated. In the study, we chose P38MAPK, an established component in apoptotic signaling pathways, to investigate the possible protective mechanism of melatonin on MSCs. The present results revealed that H_2O_2 treatment remarkably enhanced the expression of phosphorylation of P38MAPK (Fig. 8A) and the corresponding MSCs apoptosis. However, the preadministration of P38MAPK inhibitor SB203580 almost completely suppressed the expression of p-P38MAPK and partially reversed the apoptosis of MSCs by TUNEL analysis, which was consistent with previous reports that P38MAPK was responsible for oxidative stress-induced apoptosis [Porras et al., 2004; Wei et al., 2010; Xu et al., 2010]. Furthermore, melatonin pretreatment also significantly reduced the expression of p-P38MAPK in H_2O_2-induced MSCs (Fig. 8B) and neutralized the cell apoptosis in part (Figs. 2–3). The findings suggest that melatonin may exert its protective property in oxidative stress-induced MSCs apoptosis by inhibiting the activation of P38MAPK signaling molecule partially.

However, our present study also showed that the P38MAPK inhibitor did not completely block H_2O_2-induced cell apoptosis, which suggests apart from P38MAPK, other molecules in H_2O_2-induced MSCs were involved in the process. Accumulating data demonstrated that lots of other signaling molecules and pathways such as JNK, ERK1/2, PI3K/Akt, and NF-κB, also participated in oxidative stress-induced cell apoptosis [Wei et al., 2010; Zeng et al., 2012]. These results indicated that P38MAPK along with other molecules may act in synergism or antagonism to participate in the oxidative stress-induced apoptosis of MSCs, which need to be explored further in future studies.

In conclusion, the present study demonstrates that melatonin effectively prevents H_2O_2-mediated apoptosis in MSCs. The protective effect of melatonin was associated with the inhibition of the intracellular ROS generation, the expression ratio of Bax/Bcl-2, the caspase-3 activation and phospho-P38MAPK expression. Additionally, the protective role was melatonin membrane receptor-dependent. Our results imply that melatonin may have promising therapeutic potential for promoting MSCs survival and improving the efficiency of stem cell therapy after various CNS injures.

REFERENCES

Antolín I, Rodríguez C, Saínz RM, Mayo JC, Uría H, Kotler ML, Rodríguez-Colunga MJ, Tolivia D, Menéndez-Peláez A. 1996. Neurohormone melatonin prevents cell damage: Effect on gene expression for antioxidant enzymes. FASEB J 10:882–890.

Chetsawang B, Putthaprasart C, Phansuwan-Pujito P, Govitrapong P. 2006. Melatonin protects against hydrogen peroxide-induced cell death signaling in SH-SY5Y cultured cells: Involvement of nuclear factor kappa B, Bax and Bcl-2. J Pineal Res 41:116–123.

Das A, McDowell M, Pava MJ, Smith JA, Reiter RJ, Woodward JJ, Varma AK, Ray SK, Banik NL. 2010. The inhibition of apoptosis by melatonin in VSC4.1 motoneurons exposed to oxidative stress, glutamate excitotoxicity, or TNF-alpha toxicity involves membrane melatonin receptors. J Pineal Res 48:157–169.

Droge W. 2002. Free radicals in the physiological control of cell function. Physiol Rev 82:47–95.

Dubocovich ML. 2007. Melatonin receptors: Role on sleep and circadian rhythm regulation. Sleep Med 8:34–42.

Federico A, Cardaioli E, Da Pozzo P, Formichi P, Gallus GN, Radi E. 2012. Mitochondria, oxidative stress and neurodegeneration. J Neurol Sci 322: 254–262.

Fu J, Zhao SD, Liu HJ, Yuan QH, Liu SM, Zhang YM, Ling EA, Hao AJ. 2011. Melatonin promotes proliferation and differentiation of neural stem cells subjected to hypoxia in vitro. J Pineal Res 51:104–112.

Hardeland R. 2008. Melatonin, hormone of darkness and more: Occurrence, control mechanisms, actions and bioactive metabolites. Cell Mol Life Sci 65:2001–2018.

Hardeland R. 2010. Melatonin metabolism in the central nervous system. Curr Neuropharmacol 8:168–181.

Hashimoto K, Ueda S, Ehara A, Sakakibara S, Yoshimoto K, Hirata K. 2012. Neuroprotective effects of melatonin on the nigrostriatal dopamine system in the zitter rat. Neurosci Lett 506:79–83.

Jung TW, Lee JY, Shim WS, Kang ES, Kim SK, Ahn CW, Lee HC, Cha BS. 2007. Rosiglitazone protects human neuroblastoma SH-SY5Y cells against MPP+ induced cytotoxicity via inhibition of mitochondrial dysfunction and ROS production. J Neurol Sci 253:53–60.

Jürgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. 1998. Bax directly induces release of cytochrome *c* from isolated mitochondria. Proc Natl Acad Sci USA 95:4997–5002.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. 1997. The release of cytochrome *c* from mitochondria: A primary site for Bcl-2 regulation of apoptosis. Science 275:1132–1136.

Lim ML, Lum MG, Hansen TM, Roucou X, Nagley P. 2002. On the release of cytochrome *c* from mitochondria during cell death signaling. J Biomed Sci 9:488–506.

Lin AM, Fang SF, Chao PL, Yang CH. 2007. Melatonin attenuates arsenite-induced apoptosis in rat brain: Involvement of mitochondrial and endoplasmic reticulum pathways and aggregation of alpha-synuclein. J Pineal Res 43:163–171.

Liu NS, Du X, Lu J, He BP. 2012. Diva reduces cell death in response to oxidative stress and cytotoxicity. PLoS ONE 7:e43180.

Liu XB, Wang JA, Ogle ME, Wei L. 2009. Prolylhydroxylase inhibitor dimethyloxalylglycine enhances mesenchymal stem cell survival. J Cell Biochem 106:903–911.

Macías M, Escames G, Leon J, Coto A, Sbihi Y, Osuna A, Acuña-Castroviejo D. 2003. Calreticulin-melatonin. An unexpected relationship. Eur J Biochem 270:832–840.

Olivier P, Fontaine RH, Loron G, Van Steenwinckel J, Biran V, Massonneau V, Kaindl A, Dalous J, Charriaut-Marlangue C, Aigrot MS, Pansiot J, Verney C, Gressens P, Baud O. 2009. Melatonin promotes oligodendroglial maturation of injured white matter in neonatal rats. PLoS ONE 4:e7128.doi: 10.1371/journal.pone.0007128

Park HJ, Lee PH, Bang OY, Lee G, Ahn YH. 2008. Mesenchymal stem cells therapy exerts neuroprotection in a progressive animal model of Parkinson′s disease. J Neurochem 107:141–151.

Parr AM, Tator CH, Keating A. 2007. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. Bone Marrow Transplant 40:609–619.

Porras A, Zuluaga S, Black E, Valladares A, Alvarez AM, Ambrosino C, Benito M, Nebreda AR. 2004. P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. Mol Biol Cell 15:922–933.

Radogna F, Paternoster L, Albertini MC, Cerella C, Accorsi A, Bucchini A, Spadoni G, Diamantini G, Tarzia G, De Nicola M, D′Alessio M, Ghibelli L. 2007. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res 43:154–162.

Reiter RJ. 1991. Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. Endocr Rev 12:151–180.

Reiter RJ. 1998. Oxidative damage in the central nervous system: Protection by melatonin. Prog Neurobiol 56:359–384.

Reiter RJ, Tan DX, Terron MP, Flores LJ, Czarnocki Z. 2007. Melatonin and its metabolites: New findings regarding their production and their radical scavenging actions. Acta Biochim Pol 54:1–9.

Rosen RB, Hu DN, Chen M, McCormick SA, Walsh J, Roberts JE. 2012. Effects of melatonin and its receptor antagonist on retinal pigment epithelial cells against hydrogen peroxide damage. Mol Vis 18:1640–1648.

Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, Choi AM. 2007. Mechanisms of cell death in oxidative stress. Antioxid Redox Signal 9:49–89.

Singhal NK, Srivastava G, Patel DK, Jain SK, Singh MP. 2011. Melatonin or silymarin reduces maneb- and paraquat-induced Parkinson′s disease phenotype in the mouse. J Pineal Res 50:97–109.

Swanger SA, Neuhuber B, Himes BT, Bakshi A, Fischer I. 2005. Analysis of allogeneic and syngeneic bone marrow stromal cell graft survival in the spinal cord. Cell Transplant 14:775–786.

Urata Y, Honma S, Goto S, Todoroki S, Iida T, Cho S, Honma K, Kondo T. 1999. Melatonin induces gamma-glutamylcysteine synthetase mediated by activator protein-1 in human vascular endothelial cells. Free Radic Biol Med 27:838–847.

Wang FW, Jia DY, Du ZH, Fu J, Zhao SD, Liu SM, Zhang YM, Ling EA, Hao AJ. 2009. Roles of activated astrocytes in bone marrow stromal cell proliferation and differentiation. Neuroscience 160:319–329.

Wang Z, Liu D, Wang J, Liu S, Gao M, Ling EA, Hao A. 2012. Cytoprotective effects of melatonin on astroglial cells subjected to palmitic acid treatment in vitro. J Pineal Res 52:253–264.

Wei H, Li Z, Hu S, Chen X, Cong X. 2010. Apoptosis of mesenchymal stem cells induced by hydrogen peroxide concerns both endoplasmic reticulum stress and mitochondrial death pathway through regulation of caspases, p38 and JNK. J Cell Biochem 111:967–978.

Wisessmith W, Phansuwan-Pujito P, Govitrapong P, Chetsawang B. 2009. Melatonin reduces induction of Bax, caspase and cell death in methamphetamine-treated human neuroblastoma SH-SY5Y cultured cells. J Pineal Res 46:433–440.

Xu ZR, Hu L, Cheng LF, Qian Y, Yang YM. 2010. Dihydrotestosterone protects human vascular endothelial cells from $H_{(2)}O_{(2)}$-induced apoptosis through inhibition of caspase-3, caspase-9 and p38 MAPK. Eur J Pharmacol 643:254–259.

Yao H, Tang X, Shao X, Feng L, Wu N, Yao K. 2007. Parthenolide protects human lens epithelial cells from oxidative stress-induced apoptosis via inhibition of activation of caspase-3 and caspase-9. Cell Res 17:565–571.

Zeng X, Yu SP, Taylor T, Ogle M, Wei L. 2012. Protective effect of apelin on cultured rat bone marrow mesenchymal stem cells against apoptosis. Stem Cell Res 8:357–367.