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Original Paper

Pre-Treatment with Melatonin Enhances Therapeutic Efficacy of Cardiac Progenitor Cells for Myocardial Infarction

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Key Words
Melatonin • MicroRNAs • Cardiac Progenitor Cells • STAT3 • H_2O_2 • MI

Abstract
Background/Aims: Melatonin possesses many biological activities such as antioxidant and anti-aging. Cardiac progenitor cells (CPCs) have emerged as a promising therapeutic strategy for myocardial infarction (MI). However, the low survival of transplanted CPCs in infarcted myocardium limits the successful use in treating MI. In the present study, we aimed to investigate if melatonin protects against oxidative stress-induced CPCs damage and enhances its therapeutic efficacy for MI. ***Methods:*** TUNEL assay and EdU assay were used to detect the effects of melatonin and miR-98 on H_2O_2-induced apoptosis and proliferation. MI model was used to evaluate the potential cardioprotective effects of melatonin and miR-98. ***Results:*** Melatonin attenuated H_2O_2-induced the proliferation reduction and apoptosis of c-kit$^+$ CPCs *in vitro*, and CPCs which pretreated with melatonin significantly improved the functions of post-infarct hearts compared with CPCs alone *in vivo*. Melatonin was capable to inhibit the increase of miR-98 level by H_2O_2 in CPCs. The proliferation reduction and apoptosis of CPCs induced by H_2O_2 was aggravated by miR-98. *In vivo*, transplantation of CPCs with miR-98 silencing caused the more significant improvement of cardiac functions in MI than CPCs. MiR-98 targets at the signal transducer and activator of the transcription 3 (STAT3), and thus aggravated H_2O_2-induced the reduction of Bcl-2 protein. ***Conclusions:*** Pre-treatment with melatonin protects c-kit+ CPCs against oxidative stress-induced damage via downregulation of miR-98 and thereby increasing STAT3, representing a potentially new strategy to improve CPC-based therapy for MI.

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Introduction

Ischemic heart diseases (IHD) remain a leading cause of deaths in humans worldwide due to its high morbidity and mortality. Although coronary intervention and drug treatments may relieve the symptoms and improve cardiac function in patients with myocardial infarction (MI), it is unable to regenerate damaged myocardium and revert the loss of cardiomyocytes following MI. Lately, stem cell transplantation-based therapies are emerging as a novel therapeutic strategy for IHD [1-5]. For instance, it was reported that injection of Sca-1$^+$/CD45$^+$/CD31$^+$ mouse bone mesenchymal stem cells (BMSCs) could express specific cardiac markers, promote cardiac microvascular formation, reduce infarct size, and improve left ventricular (LV) function in a mouse MI model [6]. Intramyocardial injection of embryonic stem cells (ESCs) is capable to improve cardiac function of infarcted animals 6 weeks after transplantation [7]. Lately, cardiac progenitor cells (CPCs) transplantation is becoming a novel promising approach for the treatment of IHD. The transplantation of c-kit$^+$ CPCs attenuates LV dysfunction and promotes cardiac regeneration in animal model of MI [8]. However, the low survival ratio of transplanted CPCs after MI has limited its curative effect. Thus, how to improve therapeutic efficacy is important for promoting clinical application of CPCs in the treatment of MI.

Melatonin (N-acetyl-5-methoxytryptamine, Mel) is an amine hormone secreted by the pineal gland of mammals. It is well known that melatonin acts as a mediator of circadian rhythms, immune activity and sleep, and also has potential to treat cancer, osteoporosis and neurodegenerative diseases. Recently, it was reported that melatonin also plays an important role in the regulation of biological activities of stem cells. Melatonin increases the proliferation of induced pluripotent stem cells (iPSCs) via ERK1/2 signaling pathway [9]. Melatonin also promotes the differentiation of mouse embryonic stem cells by inhibiting hypoxia inducible factors (HIF) [10].And melatonin plays a protective role in iron overload osteogenic differentiation dysfunction and senescense through blocking ROS accumulation and P53/ERK/P38 activation [11]. However, whether melatonin is able to inhibit apoptosis of CPCs and enhance therapeutic efficacy of CPCs transplantation in MI is still not clear.

MicroRNAs (miRNAs) are small RNAs containing about 22 nucleotides which do not encode proteins. MiRNAs regulate gene expression by inhibiting target mRNA translation or promoting its degradation. Growing evidence has shown that miRNAs play an important role in the regulation of transplanted stem cells. MiR-133a protects CPCs against death by targeting proapoptotic genes Bim and Bmf, and therefore transplantation of CPCs engineered by miR-133a could improve cardiac function by increasing vascularization, promoting cardiomyocyte proliferation, reducing fibrosis and inhibiting hypertrophy [12]. MiR-21 promotes the proliferation of CPCs via PTEN/PI3K/Akt pathway, which indicates that modification of miRNA expression in CPCs could increase cardiac functions after modified CPCs transplantation [13]. All these studies suggest miRNAs as a key target for enhancing therapeutic efficacy of CPCs. In the present study, we aimed to investigate if pre-treatment with melatonin protects against oxidative stress-induced apoptosis of CPCs and further explores the underlying mechanism of miRNAs involved in this process.

Materials and Methods

Animals

The mice were purchased from the Experimental Animal Center of The Affiliated Second Hospital of Harbin Medical University (Harbin, China). All protocols were approved by the guidelines of the Animal Ethics Committee of Harbin Medical University. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Isolation and culture of CPCs

The hearts of 1-3 day-old neonatal C57BL/6 mice were harvested and minced into 1-2mm^3. The tissue fragments digested with 0.2% trypsin (Beyotime) and 0.1% collagenase II (GIBCO Sigma, Milan, Italy)

for 5 min at 37°C. The step of digestion was repeated three times. The supernatant containing cells was discarded, the remaining tissue fragments were cultured in cell culture flasks with culture medium (CEM: DMEM/high glucose, HyClone, supplemented with 10% fetal calf serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, 4 mmol/L L-glutamine, and 4500 mg/L Glucose) at 37°C and 5% CO_2 after washing with PBS three times. When the bottom of culture flask was covered with cells, 0.2% trypsin was used to digest the cells and the third passage was used in this study.

EdU incorporation assay

EdU incorporation assay was performed according to Cell-Light™ EdU Apollo®567 *In vitro* Imaging Kit [14]. Briefly, Cells were incubated with 5-Ethynyl-2'-deoxyuridine (EdU) for 2 hr. Then the cells were fixed with 4% paraformaldehyde for 15 min at 37°C. The cells were incubated with Apollo Staining reaction liquid for 30 min. DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to counter stain the nucleus for 15 min at room temperature.

TdT mediated dUTP nick end labelling (TUNEL)

Apoptotic cells were detected with an In Situ Cell Death Detection Kit (Roche) according to the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde at 37°C for 15 min. Blocking buffer (3% H_2O_2 in CH_3OH) was added to the wells and then cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were incubated with TUNEL reaction mixture for 1 hr at 37°C. DAPI was used to counter stain the nucleus.

Quantitative real-time PCR

The total RNA was extracted by TRIzol reagent (Invitrogen) and was reverse-transcribed with reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan). A 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time PCR (qRT-PCR) according to the manufacturer's instructions of GreenER Two-Step qRT-PCR Kit Universal (Invitrogen). The primer sequences of miR-98 are as follows:

RT primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACAAT

forward: GCGGCGGTGAGGTAGTAAGTTG.

reverse: ATCCAGTGCAGGGTCCGAGG. The expression of GAPDH was detected as a control.

Transfection

MiR-98 mimics, inhibitors and their corresponding controls were obtained from Genepharma (Shanghai, China). According to the manufacturer's instructions, they were mixed with Opti-MEMÒ I Reduced Serum Medium (Gibco, New York, NY, USA) for 5 min and then mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 20 min. MiR-98 inhibitor sequence is 5' -AACAAUACAACUUACUACCUCA-3', miR-98 mimics sequence is 5' -UGAGGUAGUAAGUUGUAUUGUU-3', 5'-CAAUACAACUUACUACCUCAUU -3'.

Western blot

CPCs were scraped in RIPA lysis buffer (Beyotime, Shenzhen, Guangdong, China). The lysates (50-70 μg) were separated by 10% SDS-polyacrylamide gel (10–15%) electrophoresis (SDS-PAGE). Proteins were electrotransferred onto the Pure Nitrocellulose Blotting membrane (Life Sciences) (Millipore, Bedford, MA, USA) and then blocked with 5% nonfat milk for 2 hr at room temperature. The membrane was washed with PBST and then incubated with primary antibody p-STAT3 (Cell Signaling Technology), Bcl-2 (Cell Signaling Technology) and β-actin (Abcam) at 4°C overnight. After washing with PBST, the membrane was incubated with secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hr at room temperature. Odyssey v1.2 software (LI-COR Biosciences, Lincoln, NE, USA) was used to quantify the bands. β-actin was used as an internal control.The values of control group were normalized to 1.

Myocardial infarction model

Male C57BL/6 mice(24-27g) were anesthetized with 0.3% pentobarbital sodium (90 $mg \cdot kg^{-1}$; Sigma-Aldrich, St. Louis, MO, USA). The left anterior descending coronary artery was ligated permanently by a 7-0 silk suture to establish MI model [15]. Animals that underwent thoracotomy were used as sham group.

Echocardiography

Cardiac function was evaluated by transthoracic echocardiography using a Vevo 1100 VisualSonics device (VisualSonics, Canada) for mice. Two-dimensional (2D) B-mode imaging was used to capture the long-axis projection with guided M-Mode images. Left ventricular ejection fraction (EF) and fractional shortening (FS) were determined using VisualSonics V1.3.8 software.

Transplantation of DAPI-labelled CPCs

The CPCs transfected with miR-98 inhibitor or NC for 48 hr were digested with 0.2% trypsin, and then incubated with DAPI for 5 min at room temperature. At five minutes post ligation, 1×10^5 DAPI-labelled CPCs were injected into three sites around infarct border zone.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software and experimental data are presented as mean±SEM. ANOVA and Student's t-test was used to observe any significant differences, and $p<0.05$ was considered significant.We used a one-way analysis of variance for multiple comparison.

Results

Melatonin inhibited apoptosis and promoted proliferation of CPCs in response to oxidative stress

To verify the protective effect of melatonin, the cultured CPCs were pre-treated with different concentrations of melatonin (10 nM, 100 nM, 1 μM, 10 μM, 100 μM) for 2 hr and then exposed to H_2O_2 150 μM in the serum-free medium for 24 hr. As shown in Fig. 1A, H_2O_2 significantly decreased the proliferation of CPCs, but pre-treatment with melatonin 10μM and 100μM could suppress the proliferation reduction of CPCs significantly as showed by the EdU incorporation assay. Further TUNEL assay showed that H_2O_2 treatment induced a significant increase of apoptotic CPCs, and pre-treatment with melatonin 10μM and 100μM inhibited H_2O_2-induced apoptosis in CPCs (Fig. 1B). However, there is no significant difference between 10μM and 100μM melatonin in the rate of proliferation and apoptosis, so melatonin 10μM was used for the subsequent experiments. This result suggests that melatonin enhances the resistance of CPCs to oxidative stress.

Pre-treatment with melatonin enhanced therapeutic efficacy of CPCs for MI

Then we further investigated whether melatonin may enhance CPCs transplantation therapy for MI. We established mouse MI model by the occlusion of the left anterior descending coronary artery, and then the cultured CPCs without any treatment and CPCs which were pre-treated with melatonin for 2 hr were injected into the myocardium around the ligature. Four weeks later, echocardiography results showed that CPCs improved heart function and attenuated ventricular remodeling following MI. Notably, CPCs pre-treated with melatonin further increased cardiac function than CPCs alone (Fig. 1C-F). It suggests that pre-treatment with melatonin increases therapeutic efficacy of CPCs in MI.

Melatonin protected CPCs via a MT membrane receptor dependent manner

It was previously reported that melatonin acts via melatonin dependent or independent manner. Thus, we further studied if the protective effect of melatonin on CPCs is dependent on MT membrane receptor. CPCs were treated with melatonin 10 μM or melatonin plus MT receptor blocker luzindole 10 μM for 2 hr, and then exposed to H_2O_2 150 μM for 24 hr. EdU incorporation assay showed that melatonin failed to induce the increase of CPCs proliferation after exposed to H_2O_2 in the presence of luzindole (Fig. 2A). Likewise, luzindole also antagonized the inhibitory effects of melatonin on H_2O_2-induced apoptosis of CPCs (Fig. 2B). These data indicate that melatonin produces protective effects on CPCs in a MT membrane receptor dependent manner.

Melatonin attenuated the increase of miR-98 level in response to oxidative stress

To clarify its underlying mechanism, CPCs were exposed to melatonin or melatonin plus luzindole for 2 hr and then followed by H_2O_2 for 24 hr. Several apoptosis or oxidative stress-associated miRNAs: miR-15a [16], miR-669b [17], miR-200c [18], miR-674 [19], miR-181a-2 [20] and miR-98 [21] in CPCs were detected by qRT-PCR. As showed in Fig. 4, we found that

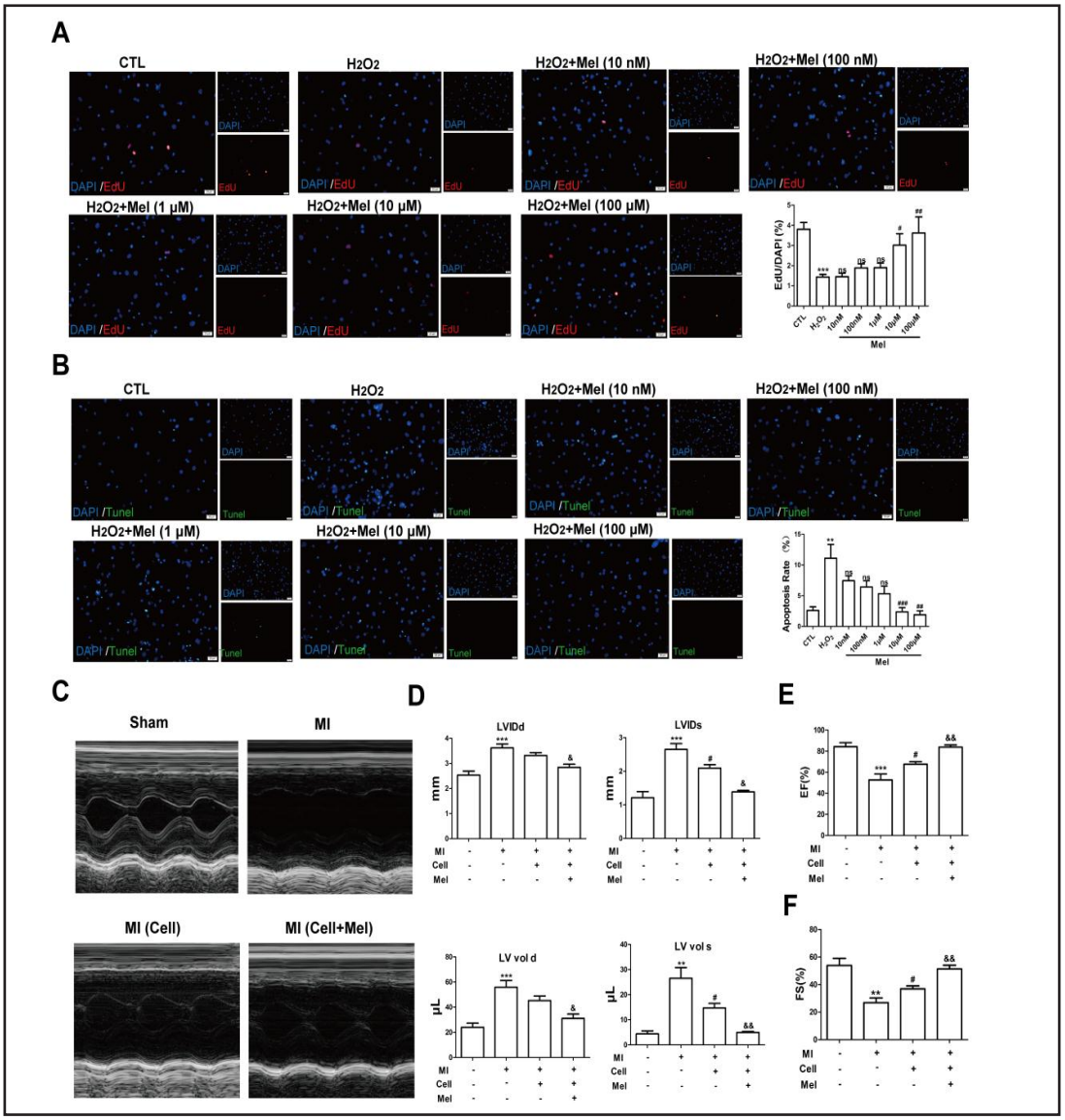

Fig. 1. Melatonin inhibited apoptosis and promoted proliferation of CPCs in response to oxidative stress in vitro and in vivo. CPCs were pretreated with different concentrations of melatonin (Mel) (10 nM, 100 nM, 1 μM, 10 μM, 100 μM) for 2 hr and then CPCs were exposed to H2O2 150 μM for 24 hr in serum-free media in the presence of melatonin. (A) The rate of proliferation in CPCs was determined by EdU incorporation assay. (B) TUNEL assay for apoptosis of CPCs. n=6, **p<0.01 vs. CTL (control), ***p<0.001 vs. CTL, #p<0.05 vs. H2O2, ##p<0.01 vs. H2O2, ###p<0.001 vs. H2O2. "(C-F) CPCs were pretreated with melatonin 10 μM, and then the CPCs were injected into three points around the ligature. Cardiac function was evaluated by left ventricular internal diastolic diameter (LVIDd), left ventricular internal dimension at end-systole (LVIDs), left ventricular end diastolic volume (LV vol d), left ventricular end-systolic volume (LV vol s), ejection fraction (EF) and fractional shortening (FS). n=6, **p<0.01 vs. Sham, ***p<0.001 vs. Sham, #p<0.05 vs. MI, &p<0.05 vs. MI (Cell), &&p<0.01 vs. MI (Cell).

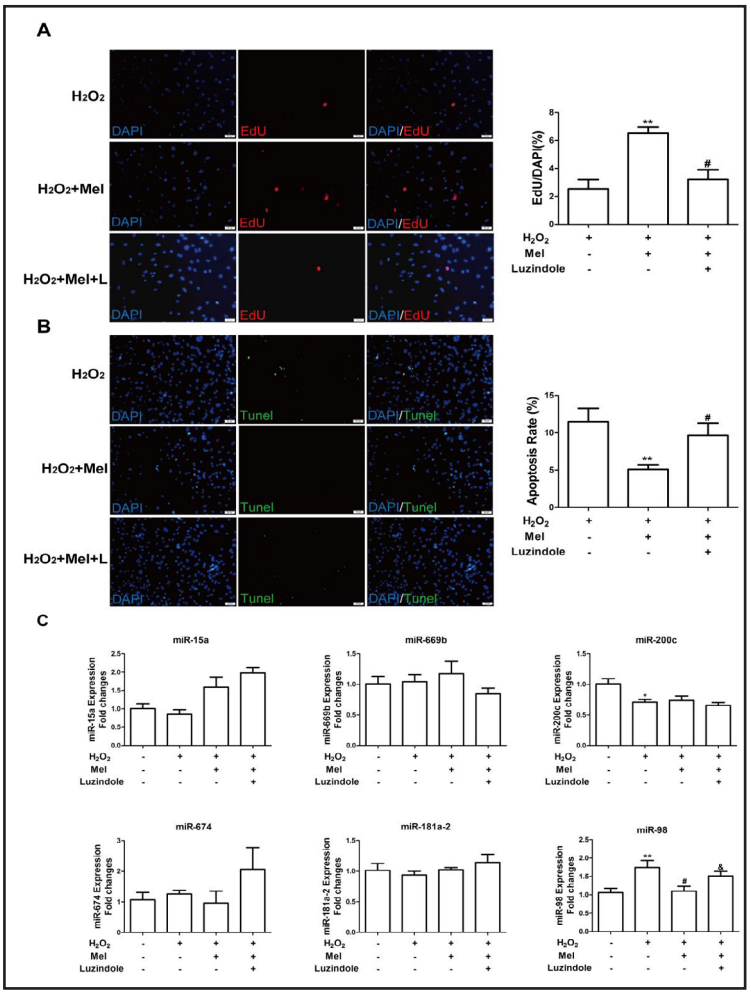

Fig. 2. Melatonin protected CPCs via a MT membrane receptor dependent manner. (A,B) CPCs were treated with MT membrane receptor luzindole (L) 10 μM, melatonin 10μM, and then exposed to H2O2 150 μM. Melatonin failed to attenuate H2O2 induced reduction of proliferation and increase of apoptosis in CPCs in the present of luzindole. CPCs were treated with melatonin or melatonin plus luzindole for 2 hr, and then exposed to H2O2 for 24 hr in the present of melatonin and luzindole. (C) qRT-PCR was used to measure the expression of miR-15a, miR-669b, miR-200c, miR-674, miR-181a-2, miR-98. n=6, *p<0.05 vs. CTL, **p<0.01 vs. CTL, #p<0.05 vs. H2O2, &p<0.05 vs. H2O2+Mel.

the level of miR-98 was significantly increased and the level of miR-200c was reduced by H_2O_2 in CPCs, but the upregulation of miR-98 by H_2O_2 was attenuated by melatonin treatment, and the reduction of miR-200c was not rescued by melatonin treatment (Fig. 2C). It indicates that miR-98 is involved in protective effects of melatonin against apoptosis of CPCs in response to oxidative stress.

MiR-98 regulated oxidative stress-induced apoptosis in CPCs

We further determined the effect of miR-98 on apoptosis in CPCs under oxidative stress, we transfected CPCs with miR-98 mimics and mimics-NC for 48 hr, and then exposed CPCs to H_2O_2 150 μM for 24 hr. Overexpression of miR-98 by its mimics could decreased the proliferation and promoted the apoptosis of CPCs after exposure to H_2O_2 for 24 hr (Fig. 3A, B). In order to further confirm the role of miR-98 in CPCs under oxidative stress, we knockdown miR-98 by its inhibitor for 48 hr followed by exposure to H_2O_2 150 μM for 24 hr. We found that miR-98 inhibitor could attenuate the proliferation reduction and apoptosis of CPCs in the presence of H_2O_2 (Fig. 3C, D). This result implies miR-98 plays a regulatory role in the apoptosis and proliferation of CPCs under oxidative stress.

Knockdown of miR-98 enhanced CPCs transplantation therapy for MI

We further explored the effects of miR-98 silencing in CPCs on cardiac functions after MI *in vivo*. CPCs transfected with miR-98 inhibitor for 48 hr were injected into mouse heart with MI. Four weeks after MI, CPCs transfected with miR-98 inhibitor showed a more remarkable

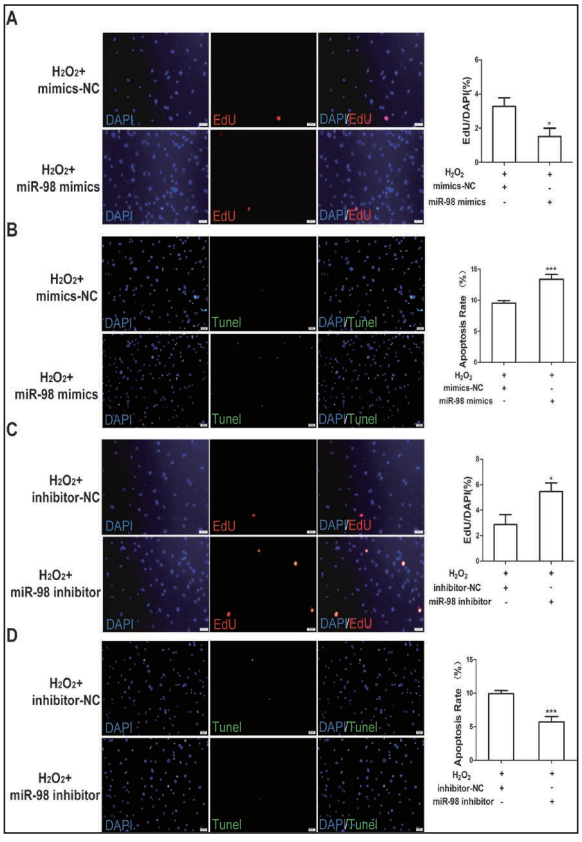

Fig. 3. MiR-98 regulated oxidative stress-mediated apoptosis in CPCs. (A-D) CPCs were transfected with miR-98 mimics, miR-98 inhibitor or scramble controls for 48 hr and treated with 150 μM H2O2 for 24 hr. miR-98 mimics inhibited the proliferation and increased the rate of apoptosis in CPCs. The miR-98 inhibitor promoted the proliferation and reduced the rate of apoptosis in CPCs. n=6. *p<0.05 vs. mimics-NC or inhibitor-NC, ***p<0.001 vs. mimics-NC or inhibitor-NC.

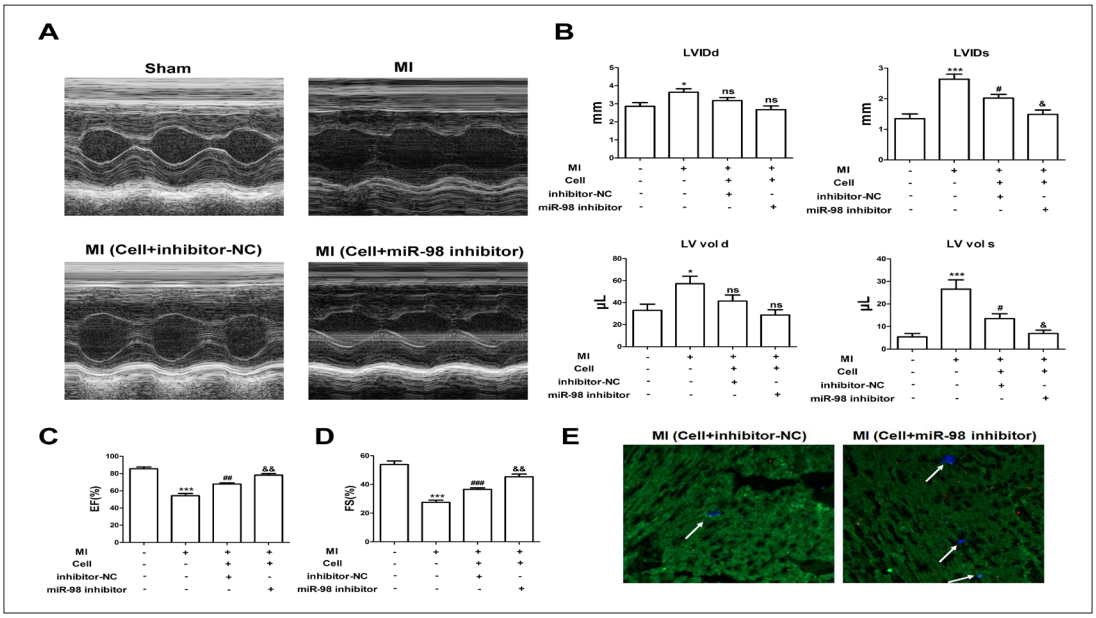

Fig. 4. Knockdown of miR-98 enhanced CPCs transplantation therapy for MI. (A-D) CPCs transfected with miR-98 inhibitor or inhibitor-NC were injected into the heart around the ligation point. After four weeks, cardiac function was quantified by LVIDd, LVIDs, LV vol d, LV vol s, EF and FS. n=6, *p<0.05 vs. Sham, ***p<0.001 vs. sham, #p<0.05 vs. MI, ##p<0.01 vs. MI, ###p<0.001 vs. MI, &p<0.05 vs. MI (cell+inhibitor-NC), &&p<0.01 vs. MI (cell+inhibitor-NC). (E) CPCs were transfected with miR-98 inhibitor or inhibitor-NC for 48 hr, and then labelled with DAPI. The labelled CPCs were injected into the hearts with MI induced by LAD ligation. Four weeks after injection, frozen slices of the hearts were immunostained with anti-α-actinin. Arrows indicate DAPI labelled cells.

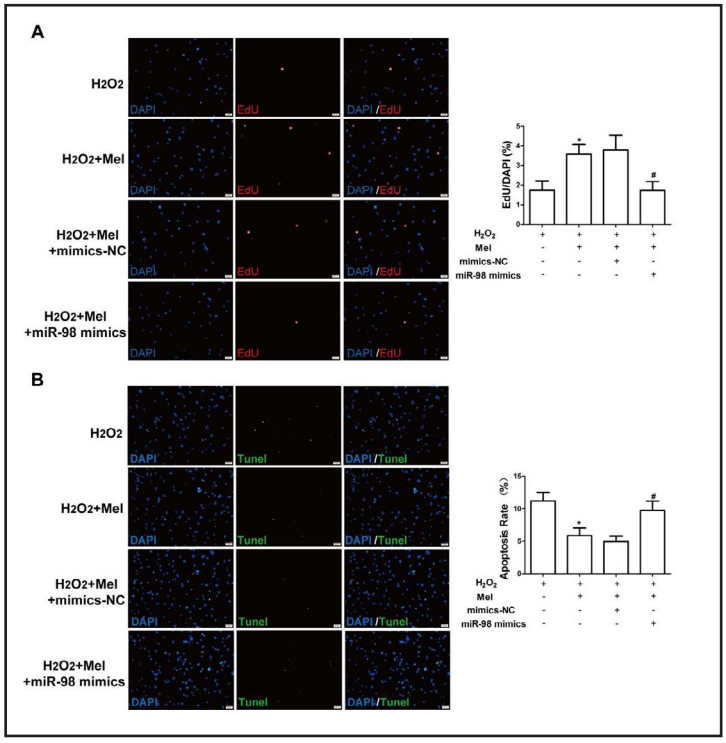

Fig. 5. Overexpression of miR-98 blocked the protective effect of melatonin in response to oxidative stress. (A-B) 24 hrs after transfection with miR-98 mimics or scramble control, the CPCs were expose to H2O2 for 24 hr in the presence of melatonin, and then EdU incorporation assay was used to detect the rate of proliferation . Apoptotic CPCs were detected by TUNEL assay. n=6, *p<0.05 vs. H_2O_2, **p<0.01 vs. H_2O_2, #p<0.05 vs. H_2O_2+Mel+mimics-NC.

increase in cardiac function compared with CPCs transfected with inhibitor-NC (Fig. 4A-D). These data suggest that miR-98 knockdown enhances the efficacy of CPCs transplantation in MI.

We further explored whether the improvement of cardiac functions results from increased survival rate of CPCs. CPCs were transfected with miR-98 inhibitor or inhibitor-NC for 48 hr, and then labelled with DAPI. The DAPI-labelled CPCs were injected into the hearts of mouse with MI. Four weeks after MI, more DAPI-labelled CPCs were observed in miR-98 inhibitor group than inhibitor-NC group. It indicates that miR-98 inhibitor could increase the survival rate of transplanted CPCs (Fig. 4E).

Overexpression of miR-98 abrogated the protective effects of melatonin on CPCs

We further investigated whether melatonin plays a protective role in response to oxidative stress via miR-98. The CPCs were treated with melatonin and then exposed to H_2O_2 150 μM after transfection with miR-98 mimics or mimics-NC. We found that melatonin failed to increase the proliferation and decrease the apoptosis after overexpression of miR-98, which indicates that melatonin inhibits the damage of CPCs induced by oxidative stress by downregulating miR-98 (Fig. 5A, B).

MiR-98 targeted at STAT3 in CPCs

We further elucidated the underlying mechanism of miR-98 regulating apoptosis of CPCs. Previous studies have demonstrated that has-miR-98 targets at STAT3 3'UTR, and bioinformatics prediction showed that mmu-miR-98 has binding sites at the 3'UTR of STAT3 gene (Fig. 6A). Interestingly, mmu-miR-98 has exactly the same binding sites as has-miR-98 [22], which indicates that mmu-miR-98 may target at STAT3 by binding to its 3' UTR. Consistently, western blot showed that miR-98 could inhibit the level of phosphorylated STAT3 (p-STAT3) protein (Fig. 6B, C). The expression of STAT3 downstream apoptosis-related protein Bcl-2 was also detected by western blot. The result showed that miR-98 mimics decreased the expression of Bcl-2 protein (Fig. 6D), and miR-98 inhibitor increased the level of Bcl-2 protein (Fig. 6E). These data indicate that miR-98 mediates apoptosis of CPCs by targeting STAT3.

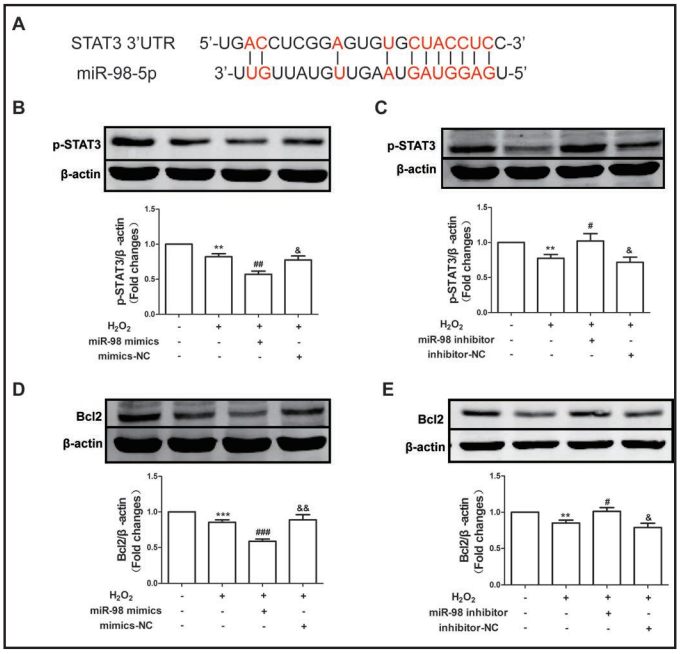

Fig. 6. MiR-98 targeted at STAT3 in CPCs. (A) The possible binding site between STAT3 and miR-98. (B,C) Western blot showing the expression of p-STAT3 in CPCs in response to H2O2 after transfection with miR-98 mimics, inhibitor and their scramble controls for 48 hr. (D, E) Western blot showing the level of Bcl-2 in the presence of H2O2 with miR-98 overexpression or knockdown. n=5. **p<0.01 vs. CTL, ***p<0.001 vs. CTL, #p<0.05 vs. H_2O_2, ##p<0.01 vs. H_2O_2, ###p<0.001 vs. H_2O_2, &p<0.01 vs. H_2O_2+miR-98 mimics or H_2O_2+miR-98 inhibitor, &&p<0.01 vs. H_2O_2+miR-98 mimics.

Discussion

Here, for the first time, we found that pre-treatment with melatonin antagonizes apoptosis of CPCs in response to oxidative stress by regulating miR-98/STAT3 signaling pathway. It provides the key evidence for clinical application of melatonin to protect CPCs against apoptosis.

Recently, increasing evidence demonstrated that CPCs transplantation has emerged as a promising therapeutic strategy for ischemic heart diseases. It was reported that CPCs in the heart may home to damaged regions to promote the formation of functionally coronary vasculature by differentiating into endothelial cells and smooth muscle cells in MI [23]. Thus, transplantation of CPCs has emerged as a promising way to restore cardiac function. However, the transplanted CPCs seem to remain in an oxidative environment, and oxidative stress induces apoptosis and cell cycle arrest which will lead to a reduction in transplantation efficiency. Apoptosis is a predominant reason for cell death of CPCs in MI. Thus, how to suppress the apoptosis of CPCs and enhance the survival of CPCs is critical for improving therapeutic efficacy of CPCs transplantation in MI. Melatonin, secreted by the pineal gland, has been reported as an important hormone in a variety of cells. It is well documented that melatonin has the antioxidant and anti-ischemia effects [24]. In multiple types of stem cells, melatonin plays an important role in the survival, proliferation, apoptosis and differentiation by certain signaling pathways. For example, melatonin protected mesenchymal stem cells (MSCs) against oxidative stress-induced apoptosis and attenuated intracellular ROS generation to increase cell viability and improve bone remodeling [25]. It was reported that melatonin inhibited NO production, promoted the expression of SOX2 and activated the PI3K/Akt/Nrf2 signaling to improve neural stem cells (NSCs) survival and proliferation [26]. Melatonin also has anti-apoptotic effects in ESCs by extracellular signal-regulated kinase and protein kinase B (ERK/PKB) pathway [27]. Melatonin could enhance induced pluripotent stem cells (iPSCs) generation by inhibiting the p53-mediated apoptotic pathway [28]. Our previous study has shown that melatonin mediated CPCs premature senescence via H19/miR-675/USP10 signaling pathway [14]. However, the role of melatonin in CPCs apoptosis remains unclear. In this study, we investigated whether melatonin antagonized apoptosis of CPCs.

Previous studies have reported that treatment with H_2O_2 150 μM could induce damage in a variety of cell types [29, 30]. Consistently, in this study, we also found that treatment with

H_2O_2 150 μM for 24 hr could inhibit the proliferation and promote the apoptosis in CPCs. More importantly, our results showed that melatonin at the concentration of 10μM and 100μM could antagonize H_2O_2-induced reduction in the proliferation and the increase in apoptosis of CPCs. It suggests that melatonin enhances the resistance of CPCs to oxidative stress-induced damage. Likewise, melatonin has been shown to protect MSCs against oxidative stress [31] . Furthermore, we verified the protective effects of melatonin on CPCs *in vivo*, and the results showed that melatonin pre-treated CPCs caused a significant increase of cardiac functions than CPCs alone. Thus, both *in vitro* and *in vivo* studies supported that pre-treatment with melatonin may increase the survival of CPCs and enhance therapeutic efficacy of CPCs for MI.

Lots of studies have indicated that direct free radical scavenging of melatonin is independent on MT receptor, but indirect anti-oxidative function of melatonin was mediated by MT receptor [32]. Increasing evidence has presented that the MT receptor blocker luzindole abolishes cardioprotection actions of melatonin [33]. In this study, we found that the protective effect of melatonin on CPCs was also blocked by luzindole, which indicated that melatonin increases the viability of CPCs in a melatonin receptor-dependent manner.

MicroRNAs contains about 22 nucleotides without protein-coding potential, and plays an important role in the repair of injured organs, especially in the liver, kidney and heart. It was recently reported that miR-98 was involved in cellular proliferation, apoptosis and differentiation in several cell lines [34]. In this study, we found that overexpression of miR-98 suppressed cell proliferation and promoted cell apoptosis, and downregulation of miR-98 increased the rate of cell proliferation and reduced the rate of cell apoptosis. Consistently, recent studies showed that miR-98 inhibited the proliferation of human ovarian cancer stem cells [35]. Our study further found that miR-98 was downregulated by melatonin treatment, but the reduction was blocked by luzindole. This indicates miR-98 is involved in the protective reaction of melatonin in response to H_2O_2.

We also found that miR-98 mimics aggravated the reduction of proliferation and increase of apoptosis induced by H_2O_2 .To further verify the function of miR-98, we blocked miR-98 by its inhibitor. We found that miR-98 inhibitor further attenuated H_2O_2-induced decrease of proliferation and upregulation of apoptosis. This suggests that miR-98 mediates the viability and apoptosis of CPCs when CPCs are exposed to H_2O_2. In agreement, our *in vivo* study showed that the transplantation of CPCs transfected with miR-98 inhibitor promoted cardiac function in MI, and found more survival CPCs in infracted myocardium. Our data indicate that melatonin inhibits miR-98 to protect against apoptosis of CPCs and further enhances therapeutic efficiency in MI.

The signal transducer and activator of the transcription 3 (STAT3) is a well-known key regulator of cell survival and proliferation. STAT3 has been shown to regulate apoptosis-related protein genes including Bcl-2 in a few of cells [36]. It was also reported that miR-98 could regulate cisplatin-induced A549 cell death by increasing the level of Bcl-2 [37]. So, we investigated whether the effect of miR-98 on CPCs inhibited by melatonin is through STAT3/Bcl-2 signaling pathway. We found that miR-98 could decrease the level of STAT3 and Bcl-2 proteins and then promote the apoptosis in CPCs. This indicates that the miR-98 silencing might play its protective role in CPCs through STAT3/Bcl-2 signaling pathway.

Conclusion

In summary, our findings uncovered that pre-treatment with melatonin protects CPCs against damage caused by oxidative stress and improves therapeutic efficacy of CPCs transplantation in MI via miR-98/STAT3 signaling pathways. It will provide a new understanding about protective effects of melatonin on CPCs and offer a new strategy to promote therapeutic efficacy of CPCs for MI.

Acknowledgements

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Disclosure Statement

The authors declare to have no conflict of interests.

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