

Research Article

miR-21 Reduces Hydrogen Peroxide-Induced Apoptosis in c-kit⁺ Cardiac Stem Cells In Vitro through PTEN/PI3K/Akt Signaling

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The low survival rate of cardiac stem cells (CSCs) in the infarcted myocardium hampers cell therapy for ischemic cardiomyopathy. MicroRNA-21 (miR-21) and one of its target proteins, PTEN, contribute to the survival and proliferation of many cell types, but their pro-survival effects in c-kit⁺ CSC remain unclear. Thus, we hypothesized that miR-21 reduces hydrogen peroxide (H₂O₂) induced apoptosis in c-kit⁺ CSC and estimated the contribution of PTEN/PI3K/Akt signaling to this oxidative circumstance. miR-21 mimics efficiently reduced H₂O₂-induced apoptosis in c-kit⁺ CSC, as evidenced by the downregulation of the proapoptosis proteins caspase-3 and Bax and upregulation of the antiapoptotic Bcl-2. In addition, the gain of function of miR-21 in c-kit⁺ CSC downregulated the protein level of PTEN although its mRNA level changed slightly; in the meantime, miR-21 overexpression also increased phospho-Akt (p-Akt). The antiapoptotic effects of miR-21 were comparable with Phn (bpV), the selective inhibitor of PTEN, while miR-21 inhibitor or PI3K's inhibitor LY294002 efficiently attenuated the antiapoptotic effect of miR-21. Taken together, these results indicate that the anti-H₂O₂-induced apoptosis effect of miR-21 in c-kit⁺ CSC is contributed by PTEN/PI3K/Akt signaling. miR-21 could be a potential molecule to facilitate the c-kit⁺ CSC therapy in ischemic myocardium.

1. Introduction

Ischemic heart disease is still the leading cause of deaths worldwide. Despite advances in medicine, such as the catheter-based therapies, the 5-year mortality rate for myocardial infarction remains as high as 50% [1]. Alternative strategies, such as stem cell-based therapies, are urgently needed [2].

Stem cell-based therapies are promising in repairing cardiac damage due to ischemia-reperfusion (I/R) injury [3, 4]. Among various types of stem cells being investigated, c-kit⁺ cardiac stem cells (CSCs) appeared to be particularly promising because they are capable of differentiating into cardiomyocytes, smooth muscle cells, and endothelial cells [5]. In the past decade, studies demonstrated the abilities of human and rodent c-kit⁺ CSCs to promote cardiac regeneration and

attenuate myocardial infarction (MI) induced heart dysfunction and remodeling in various animal models [6–13]. A recent report showed the beneficial effects of c-kit⁺ CSCs on ventricular remodeling and dysfunction sustained for more than one year in rats [7].

CSCs treatment of MI has demonstrated efficacy in the SCIPIO human Phase I trial [14]. After receiving CSCs, MI patients showed smaller infarct scars six months later. Despite the minimal cardiomyogenic potential of CSCs [7, 15], many researches have displayed their potential of promoting angiogenesis as well as decreasing apoptosis and necrosis in vivo, either via differentiation towards vascular lineages [16] or by secretion of growth factors [17] and/or extracellular microRNAs (miRNAs) [18].

However, poor engraftment and viability of CSCs minimize the percentage of cell survival and hamper functional

improvements and cardiac outcomes [19]. The very poor survival of donor cells is one of the challenges that need to be overcome before CSC-based therapies become a clinical reality. It is reported that >90% of transplanted CSCs die within a week and >95% within 5 weeks in mice with MI [20, 21]. The massive loss of CSCs limits their effectiveness as a therapy. Strategies to enhance cell survival after adoptive transfer would produce notable therapeutic implications in post-MI patients. Strategies to increase cell survival include preconditioning the cells with a variety of techniques, including heat shock of the cells prior to transplantation, forced expression of survival factors in the donor cells, and exposure of cells to prosurvival factors [22–24]. Hu et al. improved the engraftment of transplanted CSCs and therapeutic efficacy for treatment of ischemic heart disease using a miRNA prosurvival cocktail, which contained miR-21, miR-24, and miR-221 [19].

miRNAs are small noncoding RNAs, which inhibit translation or promote mRNA degradation of their target genes [25, 26]. Accumulating evidence indicates that miR-21 plays important roles in tumor growth [27], vascular smooth muscle cell survival, and cardiac cell growth [28]. miRNAs also play critical roles in cardiogenesis and cardiac regeneration [29–32]. Gain-of-function studies indicated that miR-21 reduces cardiomyocyte apoptosis under oxidative stress [33, 34]. Importantly, the miRNA expression is capable of controlling CSCs fate and holds the potential of enhancing clinical efficacy of cellular therapy [19, 31]. It is reported that miRNAs also contribute to CSC differentiation [35–37]. For example, miR-21 not only modulates the immunoregulatory function of bone marrow mesenchymal stem cells (BMSCs) through the PTEN/Akt/TGF- β 1 pathway [38], but also enhances human multipotent cardiovascular progenitors therapeutic effects via PTEN/HIF-1 α /VEGF-A signaling [39].

The phosphatase and tensin homolog deleted on chromosome ten (PTEN), which was found as a tumor suppressor gene, participates in growth, apoptosis, adhesion, invasion, and migration [40, 41]. Silencing of PTEN can promote cell proliferation [42]. Pharmacological inhibition of PTEN limits myocardial infarct size and improves left ventricular function after MI [43]. PTEN works partially through the prosurvival pathway by inhibiting phosphorylation of Akt (p-Akt) which is the active form of Akt [41]. The overexpression of PTEN increases apoptosis in cardiomyocytes, whereas the inactivation of PTEN activates the Akt signaling, reduces apoptosis, and increases survival [44–47]. It is well documented that PTEN is one of miR-21's target genes [38, 48–50]. Accumulating evidence indicates that miR-21 promotes cell proliferation via PTEN-dependent PI3K/Akt activation in cancer cells [51–55]. Gain of function of miR-21 can efficiently reduce I/R injury by downregulation of the expression of PTEN [56, 57].

In this study, we provide evidence that miR-21 may protect c-kit⁺ CSCs against H₂O₂-induced apoptosis through the PI3K/PTEN/Akt signaling. This suggests that miR-21 possesses the ability to protect c-kit⁺ CSCs from oxidative injury and miR-21 could be a potential molecule to facilitate stem cell treatment of ischemic myocardium.

2. Materials and Methods

2.1. Animals. Sprague-Dawley rats (male, about 3 weeks old, 45–60 g) were purchased from the Third Military Medical University (Chongqing, China) and maintained in Zunyi Medical College. They were kept in 12 h light/dark (with 8:00 a.m. to 8:00 p.m. light on) cycles and were given free access to rat chow and water. All experimental procedures were performed according to the *Guide for the Care and Use of Laboratory Animals* in China and approved by the local Experimental Animal Care and Use Committee.

2.2. Materials. PE conjugated anti-CD34 and anti-CD45 primary antibodies were from BioLegend (USA). Collagenase type II was from Sigma (USA). Ham's/F-12 medium and fetal bovine serum (FBS) were purchased from HyClone (USA). Trypsin was purchased from Gibco (USA). Penicillin and streptomycin were from Solarbio (China). Fibroblast growth factor was from Peprotech (USA). Leukocyte inhibitory factor was a product of Gibco (USA). Rabbit anti-rat c-kit⁺ primary antibody was supplied by Biorbyt (UK). M-280 beads conjugated with sheep anti-rabbit secondary antibody were from Dynal Biotech (Norway). miR-21 mimics, miR-21 inhibitor, and their scrambles were synthesized by RIBOBIO (China). Lipofectamine 2000 was from Invitrogen (USA). Primers, miRNA reverse transcription kit, and qRT-PCR kit were from Sangon Biotech (China). Anti- β -actin, anti-Bcl-2, anti-Bax, anti-caspase-3, anti-PTEN, anti-p-Akt, and anti-Akt primary antibody and other secondary antibodies were obtained from Boster (China). Annexin V-FITC apoptosis detecting kit was from Solarbio (China). LY294002 (PI3K inhibitor) was from Beyotime Technology (China). Phen (bpV, PTEN inhibitor) was a product of Merck (Germany). The unlisted reagents were of analytical grade.

2.3. c-kit⁺ Cells Isolation, Purification, and Identification. CSCs were isolated [58] and purified [59] using previously published methods, with some modifications. Rats were deeply anesthetized with sevoflurane, and the atrial appendage was sliced and digested with 0.1% collagenase type II (Sigma, USA). After about 40 min digestion at 37°C, cells were collected by sedimentation at 1200 rpm for 5 minutes (min). Then, cells from the atrial appendage were incubated in a humidity chamber in Ham's F12 medium containing 10% FBS, 1% penicillin and streptomycin, 1% L-glutamine, 20 ng/mL human recombinant fibroblast growth factor, 20 ng/mL leukocyte inhibitory factor, and 10 ng/mL epidermal growth factor (EGF). When cells confluence reached >90%, they were suspended by trypsinization. Then, CSCs were incubated with rabbit anti-c-kit antibody (1:250 in F12 medium) for 1 hour (h) and sorted out with anti-rabbit secondary antibody conjugated 2.8 μ m magnetic beads (Dynal Biotech, Norway) in 30 min as instructed by the manufacturer's protocols. The purified c-kit⁺ CSCs were cultured in the aforementioned F12 medium. Flow cytometry was used to confirm the expression patterns of CSCs markers. Cells were incubated with fluorochrome-conjugated primary antibodies: anti-CD34-PE, anti-CD45-PE, and anti-c-kit

primary antibody and anti-c-kit IgG-allophycocyanin (APC) secondary antibody (all from BioLegend, USA).

2.4. H_2O_2 -Induced Oxidative Stress Model and Its Effect on Apoptosis in CSCs. Harvested CSCs were incubated in serum-free F12 medium for 24 h before being treated with H_2O_2 (0, 50, 100, and 200 μM) for 2 h. Early apoptosis and necrosis of CSCs were determined by flow cytometry using Annexin V-FITC/PI staining assay as reported elsewhere [34]. The phosphatidylserine level on CSC surface was estimated with Annexin V-FITC and propidium iodide (PI) apoptosis detection kit (Solarbio, China) according to the manufacturer's instructions. Apoptosis and necrosis of c-kit⁺ CSCs were analyzed in a FACSCalibur flow cytometer (BD Biosciences, USA). Results were expressed as the percentage of apoptotic or necrotic cells from total cells. Flow cytometry was performed thrice using CSCs from three independent experiments.

2.5. Reverse Transcription and Real-Time PCR of miR-21 and PTEN. mRNA and miRNA levels were determined by using quantitative RT-PCR as previously reported [60, 61]. Briefly, RNAs from CSCs were isolated by the TRIzol (Invitrogen, USA) method. RT-PCR was performed on cDNA generated from 3 μg of total RNA with a cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's protocol. RT-qPCR was performed with the CFX Connect Real-Time system (Bio-Rad, USA) using a SYBR Green PrimeScript RT kit (TaKaRa, Japan) based on the manufacturer's instructions. The PCR conditions included pre-denaturation at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 10 s and combined annealing/extension at 58°C for 30 s. All the mRNA expression levels were calculated based on the comparative quantification method ($2^{-\Delta\Delta CT}$). U6 and β -actin were used as internal controls for miR-21 and PTEN mRNA quantitation, respectively.

2.6. miR-21 Mimics and Inhibitor Transfection and the Detection of miR-21's Effects on Apoptosis in CSCs. For the miR-21 gain-of-function and loss-of-function experiments, miR-21 mimics, miR-21 inhibitor, and their control scrambles were added in 1.5 mL F12 medium in 6-well plates with 5 μL transfection reagent Lipofectamine 2000 (Invitrogen, USA) and then incubated with c-kit⁺ CSCs for 48 h according to the manufacturer's instructions. Early apoptosis and necrosis of CSCs were determined by flow cytometry using Annexin V-FITC/PI staining assay as previously mentioned. Flow cytometry was performed twice using c-kit⁺ CSCs from three independent experiments.

2.7. Immunofluorescence of Caspase-3. To characterize purity of isolated CSCs, immunocytochemistry was used to verify c-kit expression on purified cells as reported elsewhere [62]. Cells were fixed with 4% paraformaldehyde and then blocked with 10% goat serum before being incubated with anti-caspase-3 antibody. c-kit⁺ CSCs were subsequently incubated with FITC-conjugated secondary antibody. After washing,

the nuclei were counterstained with DAPI. The immunofluorescence photos were taken with a fluorescence microscope (Olympus, Japan).

2.8. Western Blot. Western blot analysis of total protein from c-kit⁺ cell lysis was performed as described previously [63]. The protein extracts were separated by SDS-polyacrylamide gels electrophoresis (SDS-PAGE) and transferred to PVDF membranes. After overnight blocking in nonfat milk solution, membranes were probed with anti-PTEN, anti-phospho-Akt, anti-Akt, anti-caspase-3, anti-Bax, anti-Bcl-2, anti- β -actin, or anti-GAPDH primary antibodies. PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and then enhanced chemiluminescence (Amersham Biosciences, USA). Immunoreactivity was visualized by a ChemiDoc MP system (Bio-Rad, USA). Protein levels were normalized to β -actin or GAPDH.

2.9. Statistical Analysis. Data are presented as mean \pm SD. All data were analyzed by Student's *t*-test or by one-way ANOVA followed by LSD or Dunnett's T3 post hoc test for multiple comparisons. A *P* value of less than 0.05 was considered to be statistically significant. Data analyses were carried out using SPSS (v.19.0, IBM, USA).

3. Results

3.1. Isolated c-kit⁺ CSCs. c-kit⁺ CSCs were isolated from rat atrial appendage and purified using anti-rabbit secondary antibody conjugated magnetic beads. Flow cytometry showed that 90.2% of cells were c-kit positive after the purification. Purified cells were stained with anti-c-kit antibody and counterstained with DAPI to visualize the nuclei. The immunofluorescence staining also showed a high percentage of double staining of c-kit⁺ and DAPI (Figure 1(a)).

3.2. H_2O_2 Induces Apoptosis and miR-21 Downregulation in CSCs. To establish an in vitro model of CSC apoptosis, H_2O_2 (50, 100, and 200 μM) was selected to stimulate CSCs for 2 h. Flow cytometry results indicated that, after 2 h incubation, control showed no change, and 50 μM and 100 μM H_2O_2 induced 51.8% and 74.9% early apoptosis, respectively, compared with control (both *P* < 0.05). 100 μM H_2O_2 challenge resulted in 74.9% apoptosis in CSCs (*P* < 0.05 compared with control or 100 μM H_2O_2 group; Figures 1(b) and 1(c)), so we chose 100 μM H_2O_2 to induce apoptosis in the subsequent experiments. Compared with control, 100 μM H_2O_2 also induced the upregulation of proapoptotic proteins caspase-3 and Bax and the downregulation of antiapoptotic protein Bcl-2, as shown in Figures 3(a)–3(c). In addition, H_2O_2 significantly reduced miR-21 mRNA expression compared with control (*P* < 0.05, Figure 1(d)).

3.3. Transfection of CSCs with miR-21 Mimics or Antagomir-21 Changes miR-21 Expression. RT-PCR of miR-21 showed a significant increase of miR-21 when cells were transfected with miR-21 mimics 48 h later (*P* < 0.05 compared with control or miR-21 mimics scramble group; Figures 2(a) and 2(b)).

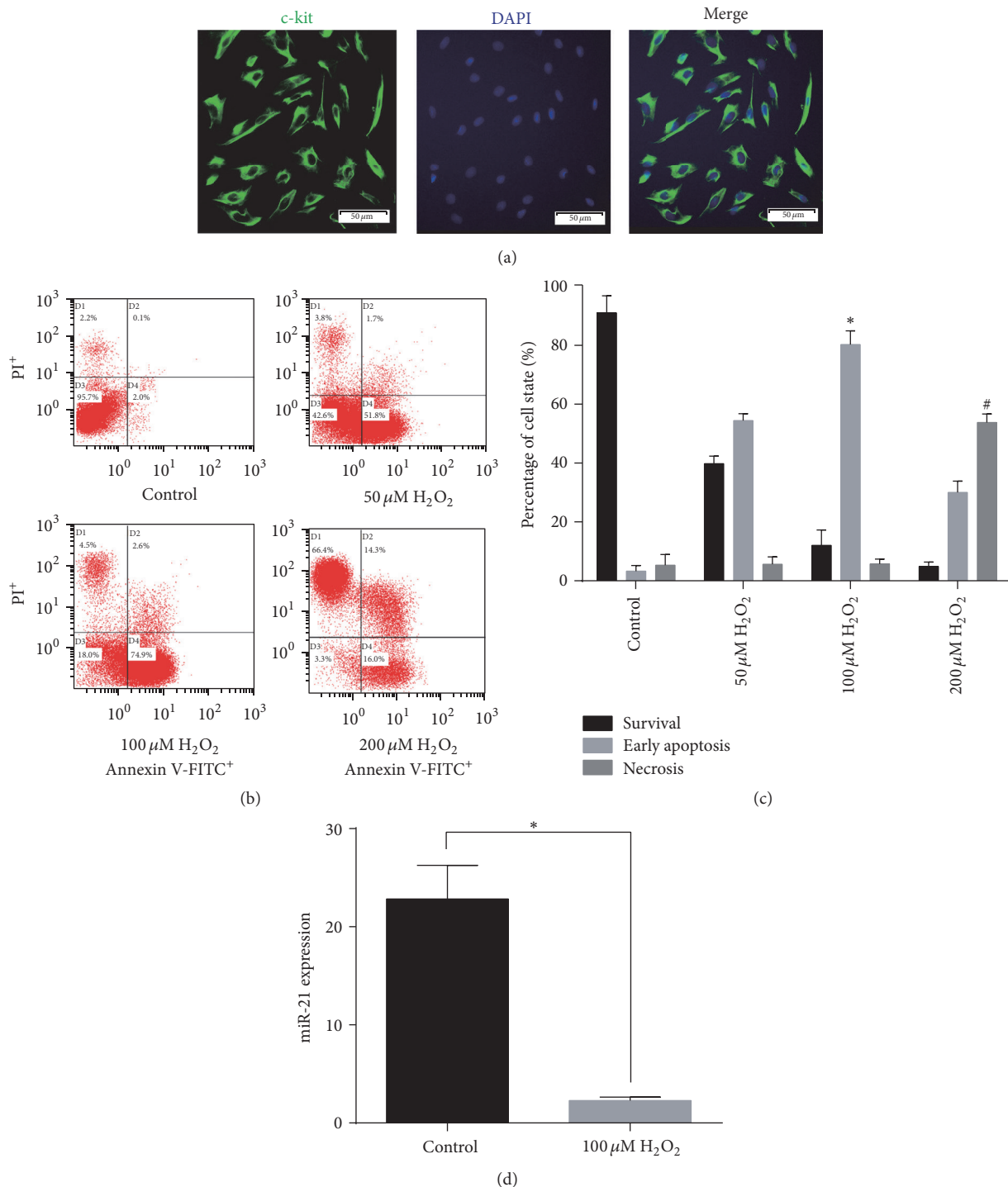
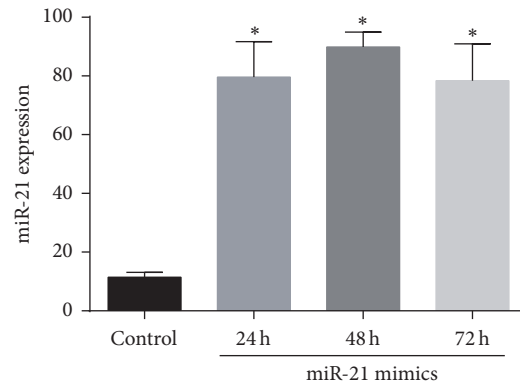
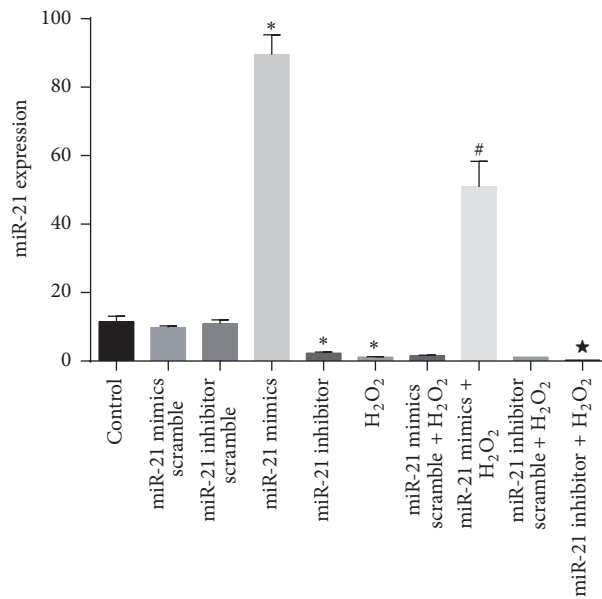


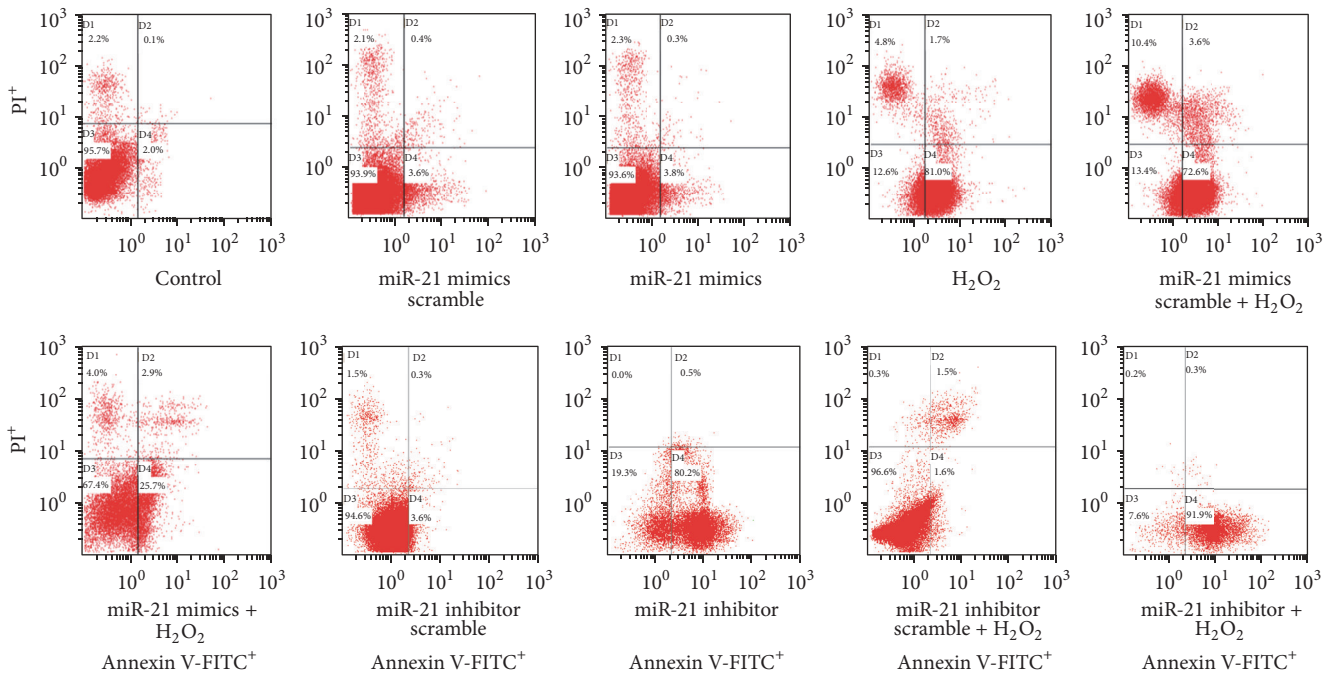
FIGURE 1: Effect of H₂O₂ (50, 100, and 200 μM) on CSCs. After isolation from rat atrial appendage, cells were purified by combined use of c-kit antibody and magnetic beads conjugated with secondary antibody. Flow cytometry showed that c-kit⁺ cells were more than 90%. (a) Purified cells were double stained by c-kit (green) and DAPI (blue) and observed under a fluorescence microscope (Olympus, Japan). (b) The apoptosis rate of CSCs exposed to different concentrations of H₂O₂ for 2 h was measured using the Annexin V-FITC/PI staining assay and statistically calculated with flow cytometry. PI: propidium iodide. (c) The statistics of the states of CSCs in (b). **P* < 0.05 compared with the apoptosis rate in other groups; #*P* < 0.05 compared with the necrosis rate in other groups. *n* = 3. (d) H₂O₂'s effects on miR-21 expression. **P* < 0.05. *n* = 3 for both groups.



(a)



(b)



(c)

FIGURE 2: Continued.

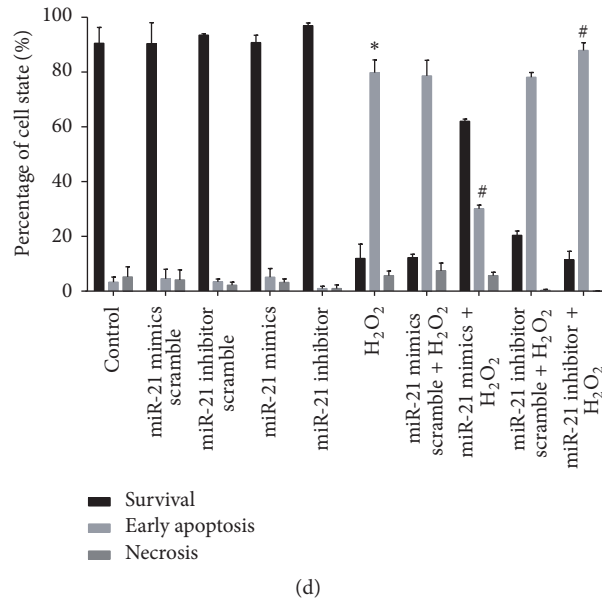


FIGURE 2: miR-21 mimics and inhibitor's effects on CSC apoptosis. (a) Cultured CSCs were treated with miR-21 mimics for 24, 48, or 72 h before miR-21 RT-PCR detection. miR-21 mimics significantly increased miR-21 but no difference was detected among the three time points. * $P < 0.05$ compared with control. $n = 3$ in each group. (b) CSCs were incubated with miR-21 mimics, miR-21 inhibitor, or their scrambles for 48 h or challenged by 2 h incubation with 100 μM H₂O₂ at the same time. miR-21 mimics significantly increased miR-21 levels, while miR-21 inhibitor significantly decreased miR-21 levels in normal or H₂O₂ stress condition. * $P < 0.05$ compared with control; # $P < 0.05$ compared with H₂O₂ group; * $P < 0.05$ compared with miR-21 inhibitor group. $n = 3$ in each group. (c) The apoptosis rate of *c-kit*⁺ CSCs treated with mimics for 48 h and/or 100 μM H₂O₂ for 2 h was measured using the Annexin V-FITC/PI staining assay. PI: propidium iodide. (d) The statistics of the states of CSCs in (c). * $P < 0.05$ compared with control; # $P < 0.05$ compared with H₂O₂ group.

The upregulation of miR-21 was stable in 72 h, and no difference was detected among 24, 48, and 72 h group (Figure 2(a)). We choose 48 h as the incubation time in the subsequent experiments. In addition, 48 h miR-21 inhibitor treatment decreased miR-21 expression compared with control or inhibitor scramble group (Figure 2(b)).

3.4. miR-21 Decreased H₂O₂-Induced Apoptosis in CSCs. The antiapoptotic effect of miR-21 was detected with flow cytometry using the Annexin V-FITC/PI staining assay. We found that miR-21 and its inhibitor have a little effect on apoptosis in normal cultured CSC, but miR-21 mimics significantly decreased CSC apoptosis after H₂O₂ insult (Figures 2(c) and 2(d)). Moreover, the caspase-3 and Bax expression was markedly decreased, while Bcl-2 apparently increased in the miR-21 mimics group, as determined by using immunofluorescence and western blot (Figure 3).

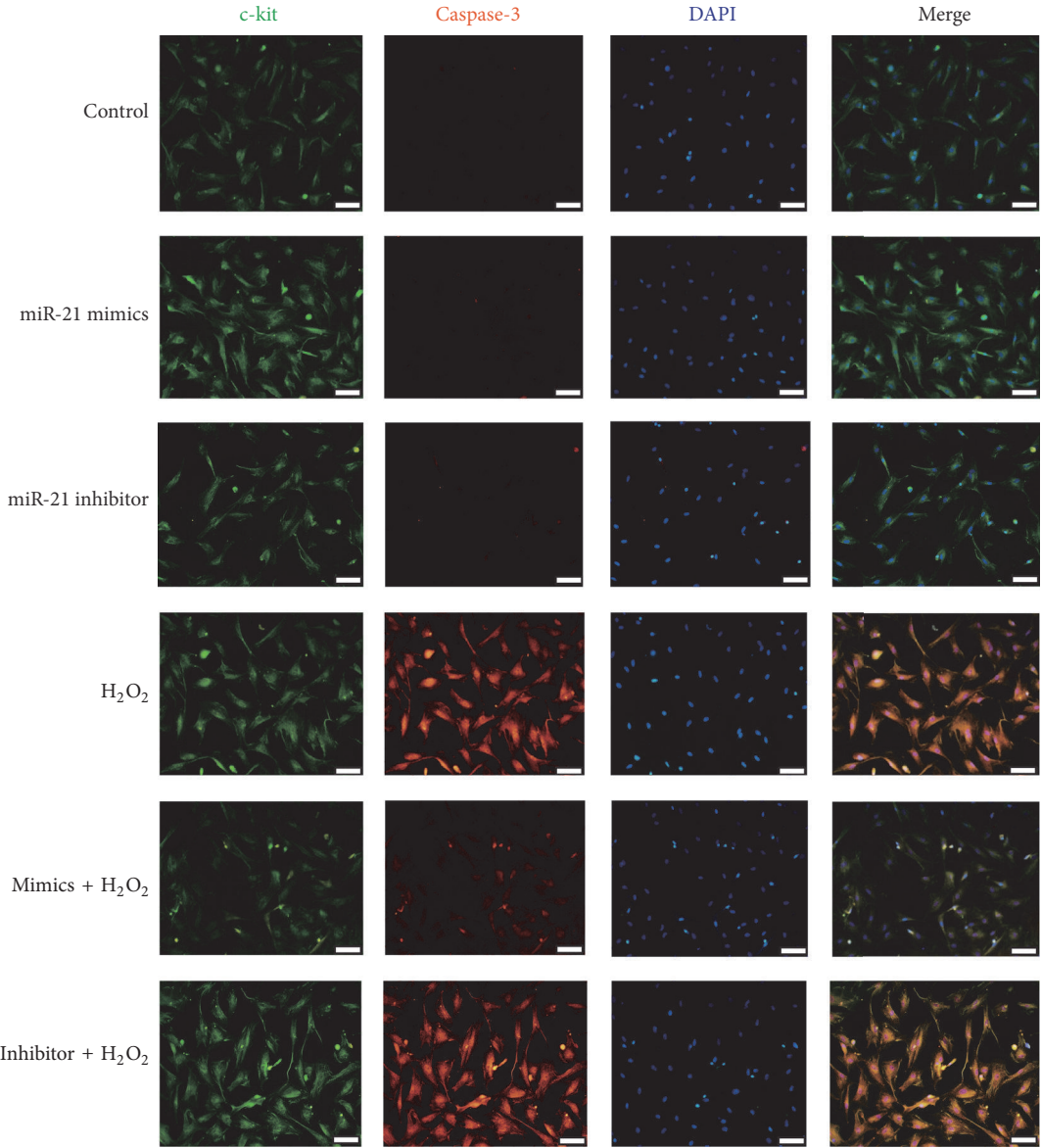
3.5. miR-21 Decreased PTEN Protein Expression. Although PTEN was extensively reported as one of miR-21's target genes in many cell types, western blot was employed to verify miR-mimic's effect on PTEN expression in *c-kit*⁺ CSCs. mRNA level of PTEN did not change (Figure 4(a)), while PTEN protein was significantly downregulated in mimics group as compared with control or miR-21 scramble group ($P < 0.05$, Figure 4(b)).

3.6. miR-21 Prevented CSCs from H₂O₂-Induced Apoptosis via the PTEN/PI3K/Akt Pathway. To study the mechanisms

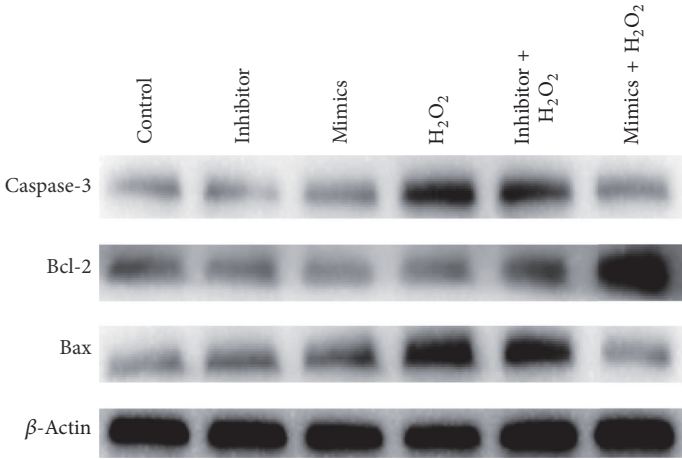
responsible for miR-21 mediated antiapoptotic effects in *c-kit*⁺ CSCs, we blocked PTEN and PI3K with their specific inhibitors Phen and LY294002, respectively. Molecular tests displayed that Phen significantly reduced the mRNA and protein expression of PTEN (Figures 5(a) and 5(b)). Both Phen and miR-21 mimics increased p-Akt level, while PI3K inhibitor LY294002 decreased p-Akt level dramatically (Figures 5(b) and 5(c)). Phen administration decreased apoptosis rate of CSCs under 100 μM H₂O₂-induced stress just like the antiapoptotic effect of miR-21 mimics, while LY294002 partially reversed effects of miR-21 mimics (all $P < 0.05$, Figures 6(a) and 6(b)). LY294002 reversed miR-21 mimics' effect on caspase-3, Bax, and Bcl-2 expression, which was evidenced by the increase of caspase-3 and Bax and the decrease of Bcl-2 (Figures 6(c)–6(e)).

4. Discussion

Stem cell therapy is promising for the prevention and treatment of ischemic cardiomyopathy [1]. *c-kit*⁺ CSCs have emerged as one of the most potential CSCs [64]. Nevertheless, poor engraftment and viability limit the percentage of injected stem cells that contribute to cardiac functional improvements [19]. miRNAs hold the potential of improving engraftment and functional outcomes of cardiac progenitor cell transplantation [19, 31]. Studies have shown that miR-21 protects the myocardium against ischemic injury [61]. miR-21 also protects cardiomyocytes and BMSCs from H₂O₂-induced cell apoptosis and death. However, it is unknown



(a)



(b)

FIGURE 3: Continued.

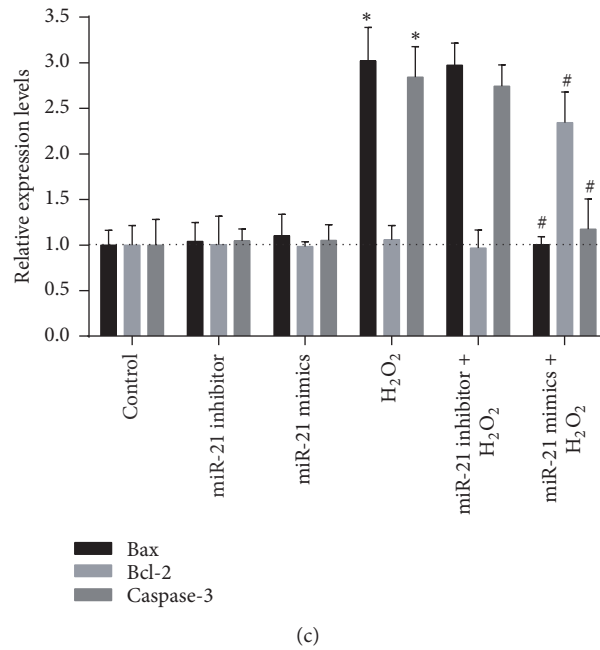


FIGURE 3: miR-21's effect on apoptosis related proteins in CSCs. Cultured CSCs were treated with miR-21 mimics, inhibitor, or scrambles for 48 h or challenged by 100 μ M H₂O₂ for 2 h at the same time. (a) c-kit⁺ CSCs were triple stained by c-kit (green), caspase (orange), and DAPI (blue) and observed under a fluorescence microscope (Olympus, Japan). Bar = 50 μ m. PI: propidium iodide. (b) miR-21 mimics' influences on Bax, Bcl-2, and caspase-3 detected with immune blotting. (c) Statistics of the relative expression level of proteins in (b). miR-21 mimics increased the expression of Bcl-2 and decreased Bax and caspase-3 compared with H₂O₂. * $P < 0.05$ compared with control; # $P < 0.05$ compared with H₂O₂ group, $n = 3$.

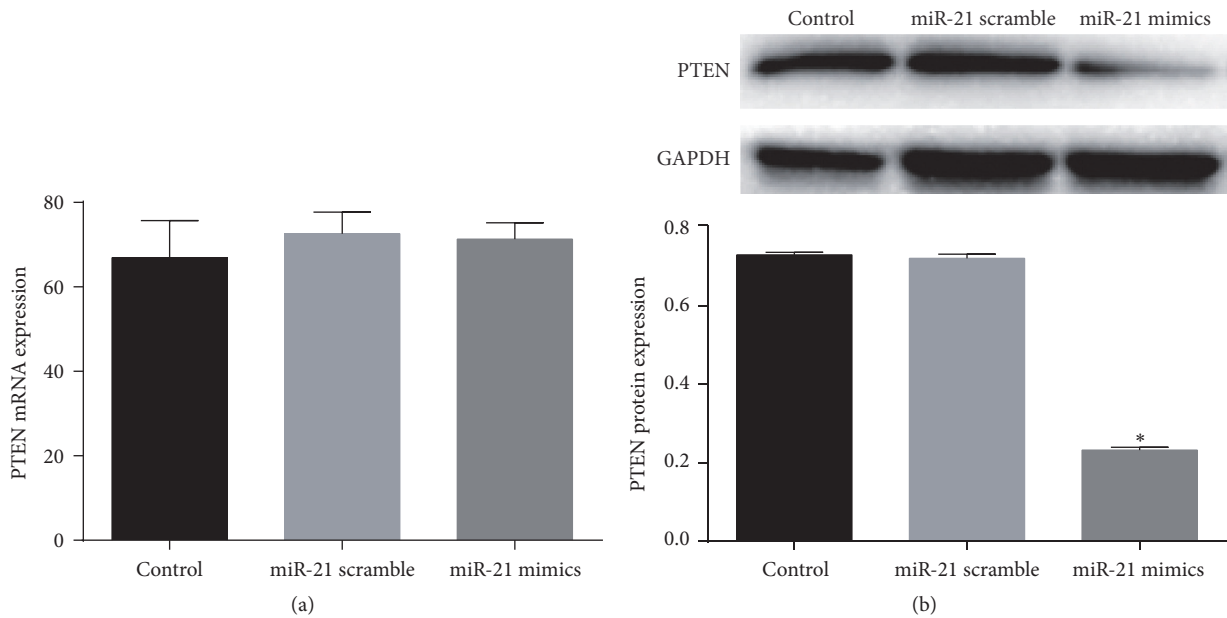


FIGURE 4: miR-21's effect on PTEN expression in CSCs under normal condition. Cultured CSCs were treated with miR-21 mimics or its negative control scramble for 48 h, and then cells were harvested and subjected to RT-PCR or western blot. PTEN mRNA of control, scramble treated, or miR-21 mimics treated cells showed no significant difference (a), but PTEN protein dramatically decreased after miR-21 mimics treatment (b). * $P < 0.05$ compared with the other two groups. $n = 3$ in each group.

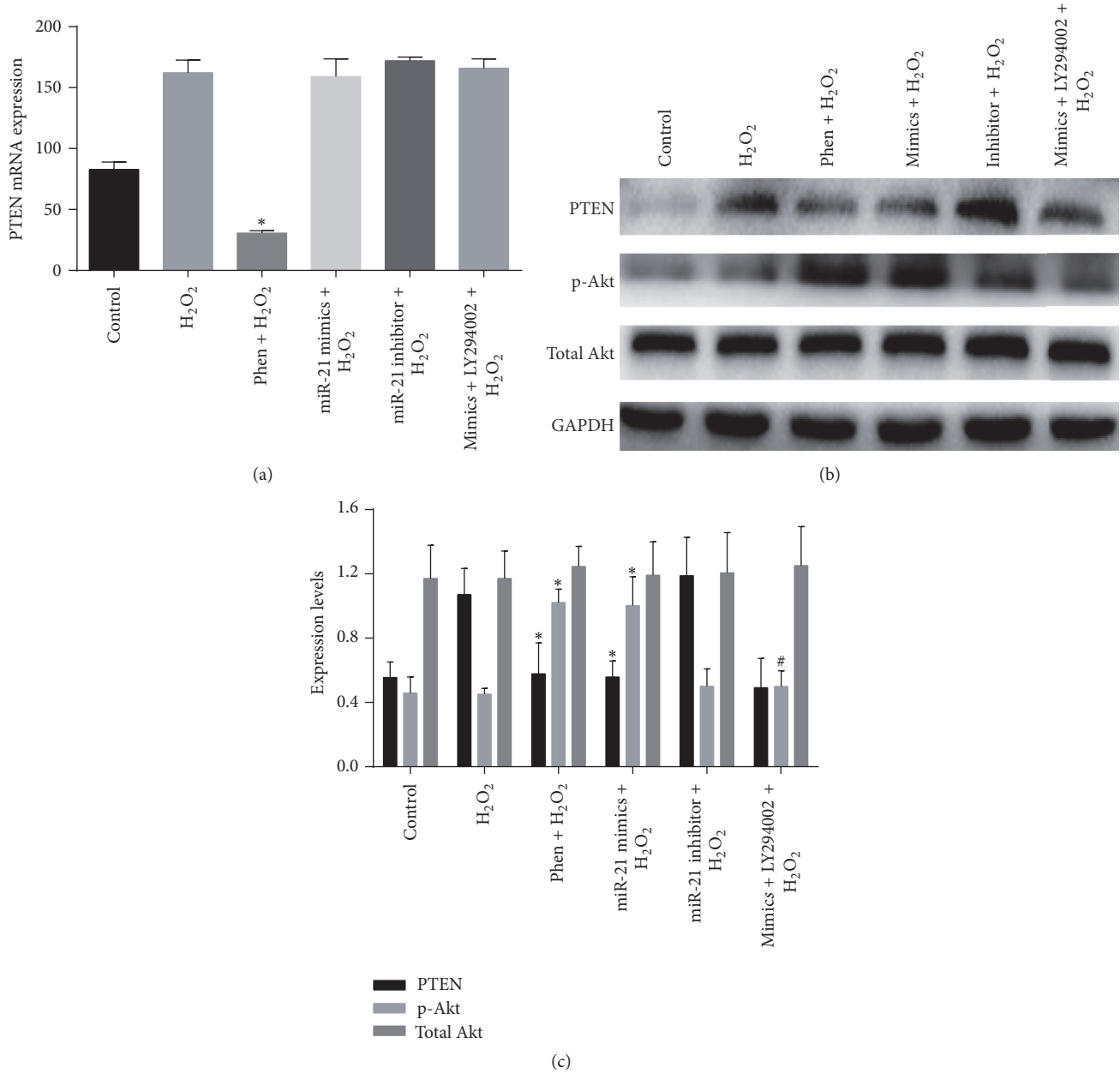


FIGURE 5: PTEN/PI3K/Akt pathway's contribution to miR-21 mimics' antiapoptotic effects. Cultured CSCs were treated with miR-21 mimics for 48 h before being subjected to 2 h H₂O₂ insult. PTEN and PI3K were inhibited with Phen or LY294002, respectively, before H₂O₂ insult. (a) RT-PCR was carried out to detect Phen, miR-21 mimics, and inhibitor's effects on PTEN expression. Phen significantly reduced the mRNA expression of PTEN. No change was detected among mimics + H₂O₂, inhibitor + H₂O₂, and H₂O₂ group. **P* < 0.05 compared with other groups. *n* = 3 in each group. (b-c) Western blot was carried out to detect Phen and miR-21 mimics' effects on PTEN and Akt protein expression. miR-21 mimics significantly downregulated PTEN protein in mimics + H₂O₂ group compared with H₂O₂ group. In addition, both Phen treatment and miR-21 mimics incubation increased p-Akt level, while PI3K inhibitor LY294002 decreased p-Akt level dramatically (*P* < 0.05). **P* < 0.05 compared with H₂O₂ group; #*P* < 0.05 compared with mimics + H₂O₂ group. *n* = 3 in each group. p-Akt: phospho-Akt.

whether miR-21 can influence CSCs survival under oxidative stress. Additionally, the underlying protective molecular mechanisms of miR-21 in CSCs need to be elucidated.

H₂O₂ has been widely used as an inducer of oxidative stress, which causes cell apoptosis [34]. In this study, we established an in vitro oxidative stress model with different concentration of H₂O₂ to simulate the microenvironment of

infarcted myocardium. Given that 100 μM H₂O₂ induced the highest apoptosis (up to 74.9%) and relatively low necrosis in c-kit⁺ CSCs, we chose 100 μM H₂O₂ to study miR-21's prosurvival effects and mechanisms. We found that H₂O₂ induced the upregulation of proapoptosis proteins, caspase-3 and Bax, and the downregulation of antiapoptotic protein Bcl-2. Interestingly, our results showed that H₂O₂ reduced

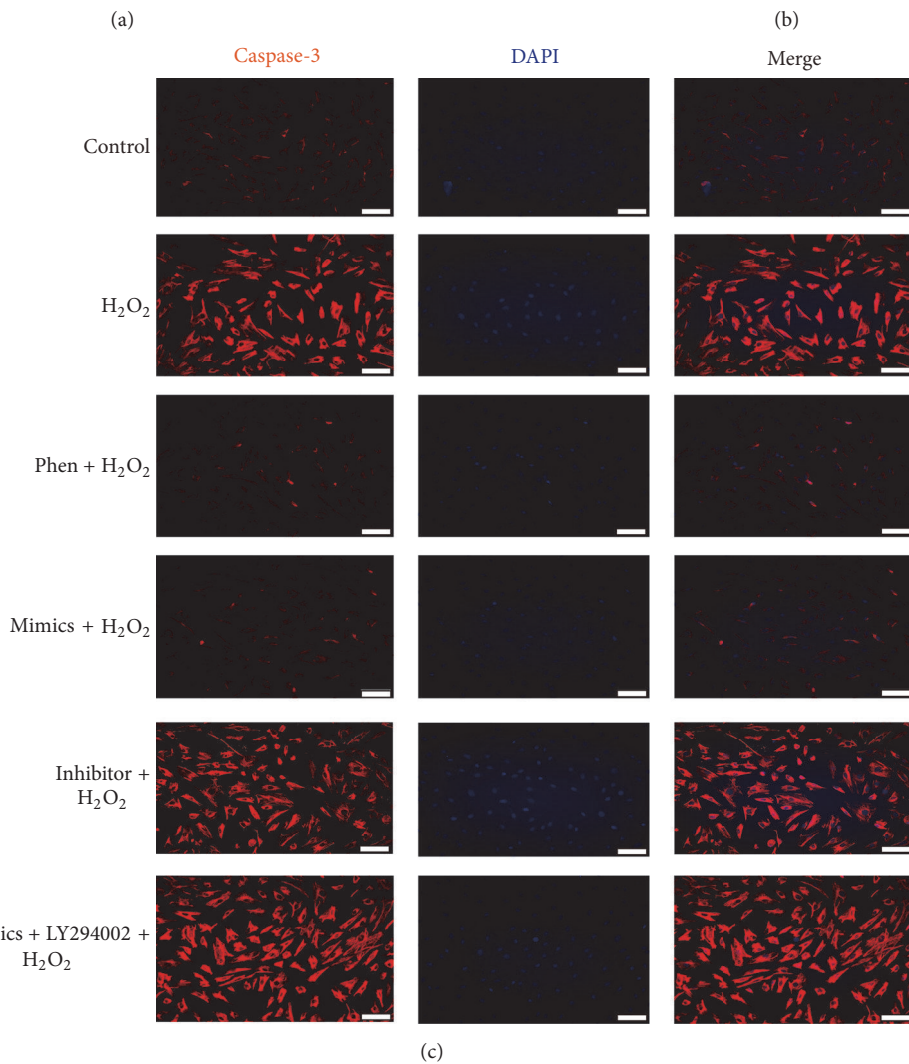
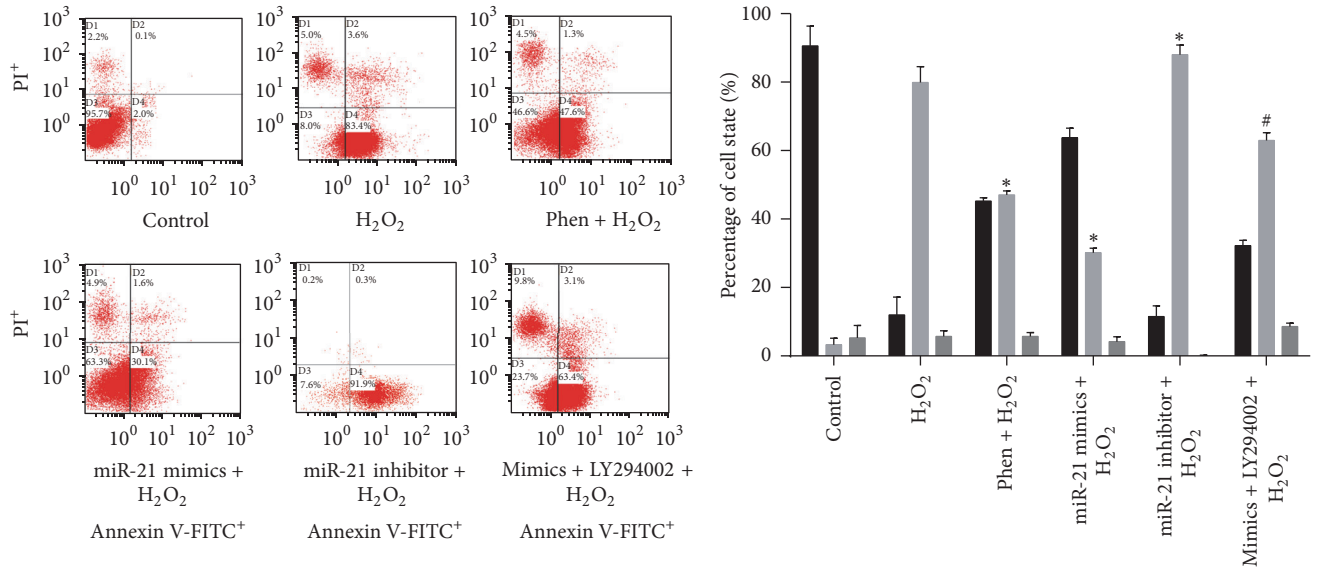


FIGURE 6: Continued.

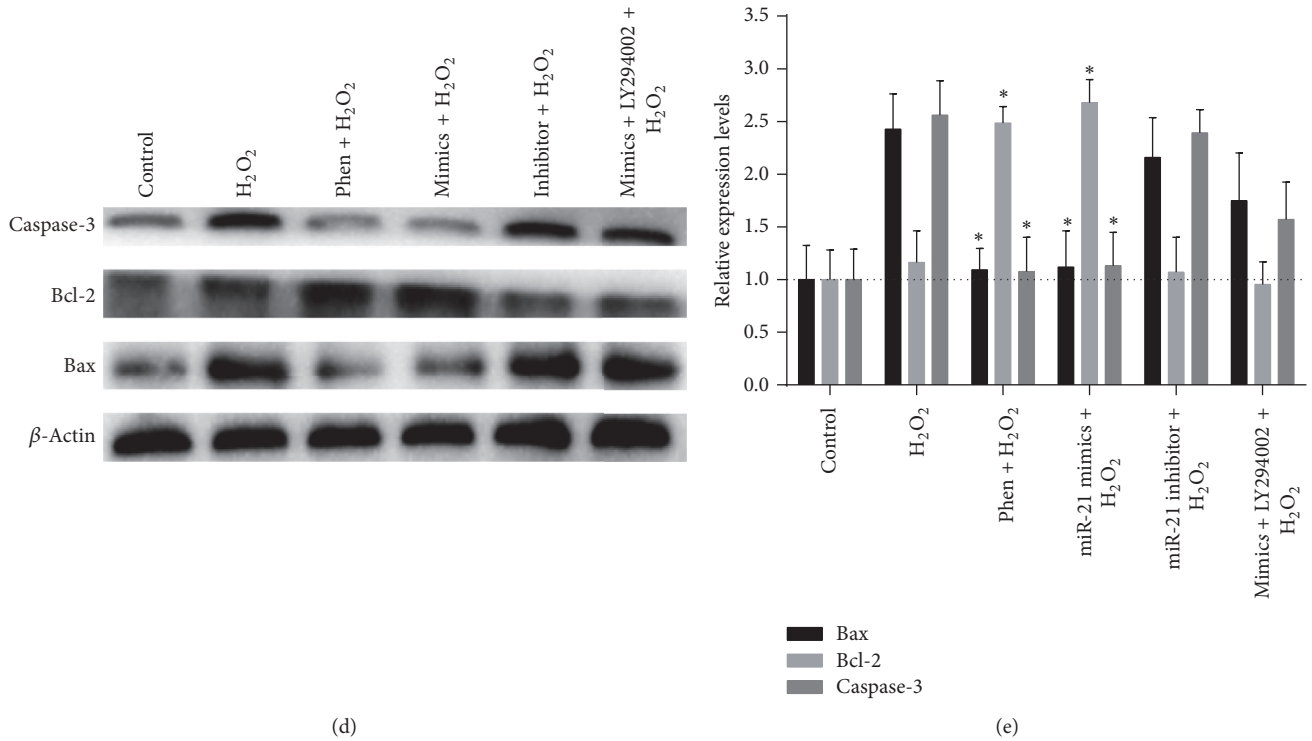


FIGURE 6: PTEN/PI3K/Akt pathway's contribution to H_2O_2 -induced apoptosis in $c\text{-kit}^+$ CSCs. Cultured CSCs were treated with miR-21 mimics or inhibitor for 48 h before being subjected to 2 h H_2O_2 insult. PTEN and PI3K were inhibited with Phen or LY294002, respectively, before H_2O_2 insult. (a) Flow cytometry was employed to detect necrosis and early apoptosis in CSCs that underwent different treatments. PI: propidium iodide. (b) Statistics of Annexin V-FITC/PI staining assay result in (a). When PTEN was inhibited by Phen, there was notably a decrease of necrosis and apoptosis in CSCs compared with H_2O_2 group. When PI3K was inhibited by LY294002, there was notably an increase of necrosis and apoptosis in mimics + LY294002 + H_2O_2 group compared with mimics + H_2O_2 group in CSCs. * $P < 0.05$ compared with H_2O_2 group; # $P < 0.05$ compared with miR-21 mimics group. This trend was further confirmed by caspase-3 immunofluorescence tests (c) and caspase-3, Bax, and Bcl-2 immunoblotting tests (d-e). LY294002 reversed miR-21 mimics' effect on caspase-3, Bax, and Bcl-2, which is evidenced by the increase of caspase-3 (c-e) and Bax and the decrease of Bcl-2 (d-e). * $P < 0.05$ compared with H_2O_2 group. $n = 3$ in each group. Bar = 50 μm . PI: propidium iodide.

miR-21 expression in $c\text{-kit}^+$ CSCs too. Although miR-21 was found to be downregulated by H_2O_2 treatment in H9C2 cell line [65] and BMSCs [27], most of the cardiac (myocytes, fibroblasts) and vascular cells (smooth muscle cells) treated with H_2O_2 showed increased miR-21 level [61, 66]. These results indicate that miR-21 expression patterns after H_2O_2 insult are cell specific. In addition, we should realize that miR-21 is a double-edged sword in ischemia-reperfusion injury, such as inflammation and angiogenesis [67]. This negative correlation between apoptosis and miR-21 expression indicates that miR-21 downregulation may aggravate apoptosis. This interaction was further confirmed by the gain-of-function analyses of miR-21, in which miR-21 significantly decreased $c\text{-kit}^+$ CSC apoptosis, as well as the caspase-3 and Bax expression, and meanwhile increased Bcl-2 protein expression (Figures 3(a)–3(c)). These results indicate that miR-21 may be an antiapoptotic factor in $c\text{-kit}^+$ CSCs under oxidative stress.

Although PTEN has been extensively reported as one of miR-21's target genes, it is not confirmed in CSCs to our knowledge. In many cell types, such as hepatocytes, cardiomyocytes, and cancer cells, miR-21 mediates

the expression of PTEN [27, 49, 57]. We hypothesized that PTEN is a target gene of miR-21 in CSCs too. PTEN expression was directly calculated (Figure 3) after upregulation of miR-21 and the results confirmed our assumption. miR-21 upregulation caused significant downregulation of PTEN protein expression although the mRNA level of PTEN did not change much (Figures 3(a)–3(c)).

The PI3K/Akt pathway constitutes an approach to inhibit apoptosis and promote cell proliferation [68]. The activation of Akt significantly protects cells from H_2O_2 -induced cell apoptosis [69, 70]. It has been reported that miR-21 affects the PI3K/Akt pathway through the expression of PTEN [49], but this effect has not been investigated in $c\text{-kit}^+$ CSCs. To study whether the PTEN/PI3K/Akt signaling is responsible for miR-21 mediated antiapoptotic effect, we blocked PTEN and PI3K with their inhibitors Phen and LY294002, respectively, and then tested phosphorylation of Akt. Just like the pro-survival effects of miR-21, Phen administration decreased H_2O_2 -induced apoptosis in $c\text{-kit}^+$ CSCs (Figures 4(a) and 4(b)). For PI3K blocker LY294002, it partially reversed antiapoptotic effects of miR-21 mimics; at the same time, it attenuated miR-21's suppression of caspase-3 and Bax and the upregulation

of Bcl-2 (Figures 6(c)–6(e)). Furthermore, both Phen and miR-21 mimics increased p-Akt level, while PI3K inhibitor LY294002 decreased p-Akt level drastically, which suggests that Akt is downstream of PI3K and PTEN. Although most literatures reported that Phen only downregulated the activity of PTEN but not its expression, some found that the mRNA and/or the protein expression was decreased after Phen administration [71, 72] as our results showed. We deem that the time window and the dose could be two influence factors. There may exist some feedback between PTEN activity and PTEN expression.

We found that the PTEN and PI3K inhibitors did not completely offset the prosurvival effects of miR-21. This is reasonable because miR-21 targets more than one gene, and, besides PTEN and PI3K mediated pathway, there could be other survival pathways involved. For example, miR-21 protects cardiac myocytes from the H₂O₂-induced injury by targeting PDCD4 gene, which is upstream of activator protein 1 (AP-1) [61]. AP-1 has been proved to be a key signaling molecule that determines life or death cell fates in response to extracellular stimuli including ROS. In addition, miR-21 promotes glioma invasion by targeting RECK and TIMP3 genes, which are suppressors of malignancy and inhibitors of matrix metalloproteinases [73]. PTEN also enhances human multipotent cardiovascular progenitors therapeutic effects via miR-21 initiated PTEN/HIF-1 α /VEGF-A signaling. miR-21 in hESC-derived stage-specific embryonic antigen 1 (SSEA-1) positive cells inhibited phosphatase and tensin homolog (PTEN), which resulted in the activation of HIF-1 α and the systemic release of VEGF-A [39].

In conclusion, our data reveal that miR-21 prevents CSCs from H₂O₂-induced apoptosis partially through the PTEN/PI3K/Akt pathway. The present study demonstrates that miR-21 is a prosurvival molecule for stressed c-kit⁺ CSCs. It also indicates that modification on miRNA expression may be able to enhance the clinical efficacy of cellular therapy.

We must confess some shortcomings of this study. In vivo studies are warranted to further confirm miR-21 and PTEN/PI3K/Akt pathway's effects on survival of c-kit⁺ CSCs. Besides, the luciferase assay could be a more direct way to confirm that PTEN is the target gene of miR-21.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Wenwen Deng and Yan Wang contributed equally to this study.

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