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MiR-200a-3p Accelerated Hypoxia/Reoxygenation Injury in HCM Cells by Enhancing IGF2R via Wnt/β-catenin Signalling Pathway

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ABSTRACT The present study examined functions of miR-200a-3p accelerated progressions of HCM cells via IGF2R and Wnt/β-catenin signalling pathway after hypoxia/reoxygenation treatment in vitro. CCK-8 showed that cell viability of HCM was inhibited while apoptosis rates detected by flow cytometry were promoted in a time dependent manner after H/R (12 hours and 24 hours). Beyond that, Bcl-2 and c-IAP1 were decreased but Bax and caspase-3 were upregulated by H/R treatment. IL-1β, IL-6, TNF-α and NLRP3 were also increased after treatment. RT-qPCR showed increased expressions of miR-200a-3p by H/R treatment while its inhibitor elevated cell viability but depressed apoptosis rate and pro-inflammatory cytokines' expressions. IGF2R was upregulated after H/R treatment and its downregulation magnified effects of suppressed miR-200a-3p. HIF-1α/Wnt/β-catenin signalling pathway was activated by miR-200a-3p and IGF2R while IWP-2 treatment abolished the activation of Wnt3a and β-catenin, causing decreased apoptosis and pro-inflammatory cytokines' expressions but accelerated the cell viability.

INTRODUCTION

Myocardial ischemia reperfusion injury is a pathophysiological process that occurs with blood flow recanalization after cardiac ischemia, which has been demonstrated to cause myocardial cells apoptosis and myocardial fibrosis (Yu et al. 2020). Myocardial fibrosis is an important pathologic change causing heart failure due to continuous developments of various cardiovascular diseases (Kwiecinski et al. 2020; Prabhu and Frangogiannis 2016). However, the exact mechanism of myocardial fibrosis is still indistinct. Several aspects have been connected with pathogenesis of myocardial fibrosis. Renin-Angiotensin-Aldosterone System (RAAS) was the main regulatory system accelerating myocardial hypertrophy promotion and myocardial fibroblasts proliferation in the development of myocardial fibrosis (Böckmann et al. 2019; Rodriguez-Gonzalez et al. 2020; Somanna et al. 2015). Hypoxia/reoxygenation has also been demonstrated to induce oxidative stress (OS) in several kinds of diseases (McGarry et al. 2018; Ming et al. 2019). OS also participated in pathological developments of cardiovascular diseases including atherosclerosis, hypertension and heart failure and so on (Ayoub et al. 2017; Guzik and Touyz 2017; Kattoor et al. 2017). OS were also determined to facilitate development of myocardial fibrosis (Zhong et al. 2020).

MicroRNA is an endogenous non-coding RNA with 16-22 nucleotides in length and the first one was discovered by Lee and his co-workers through studying fruit flies and named Lin-4 (Lee et al. 1993). Based on research, thousands of miRNAs are expressed in the human heart, of which about sixty percent to seventy percent are tied to the occurrence and development of fibrosis in clinical heart diseases (Yang et al. 2014). MiRNA can bind 3'UTR of targeted mRNA to regulate proliferation, differentiation, apoptosis and other biological processes through suppressing the translation of mRNA (Liu et al. 2009). MiR-181c-5p was upregulated by H/R treatment in H9C2 cells and rat cardiac I/RI models and overexpression of miR-181c-5p aggravated damages and apoptosis of cells through

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targeting PTPN4 (Ge et al. 2019). MiR-15b was elevated by H/R treatment and its upregulation induced apoptosis of H9c2 cells through downregulating expressions of Bcl-2 and MAPK3 (Liu et al. 2018). In contrast, miR-221 significantly reduced H/R injuries caused by autophagy suppression through inhibiting Ddit4 and Tp53inp1 (Chen et al. 2016). According to previous studies, miR-200a-3p was found to be notably upregulated in hypoxic heart of rats by regulating DOR (Zhi et al. 2016). Nevertheless, deep molecular mechanisms of miR-200a-3p were unclear. Therefore, this study would figure out functions of miR-200a-3p and related factors in H/R treatment.

Objectives

This study aimed to analyse changes of cell viability, apoptosis and expressions of miR-200a-3p, IGF2R, Bcl-2, caspase-3, Bax, c-IAP1, IL-6, TNF-α, IL-1², NLRP3, HIF-1α, Wnt3a and β-catenin after hypoxia/reoxygenation treatment and suppression of miR-200a-3p and IGF2R. This study also aimed at examining changes in cell viability, apoptosis and protein expressions after treated by IWP-2.

Experimental

Main Reagents

The main reagents used were HCM (#6200, Sciencell, USA), DMEM medium (Gibco, USA), FBS (Gibco, USA), penicillin/streptomycin (Invitrogen, USA), TRIzol reagent (Invitrogen, USA), High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, USA), SYBR Green PCR Master Mix (Applied Biosystems, USA), CCK-8 (96992, Sigma-Aldrich, USA), Annexin V-FITC Apoptosis Detection Kit (Invitrogen, USA), Cell lysis buffer for Western and IP (Beyotime, Shanghai, China), BCA Proteins Assay Kit (Beyotime, Shanghai, China), SDS-PAGE (Beyotime, Shanghai, China), anti-Bax (ab32503, Abcam, UK), anti-caspase-3 (ab197202), anti-Bcl-2 (ab185002), anti-c-IAP1 (ab25939), anti-IL-1β (ab9722), anti-IL-6 (ab6672), anti-TNF-α (ab1793), anti-NLRP3 (ab263899), anti-HIF-1α (ab1), anti-Wnt3a (ab81614), anti-β-catenin (ab32572), anti-GAPDH (ab181602), Goat Anti-Mouse IgG H&L (HRP) (ab205719), and BeyoECL Moon (Beyotime, Shanghai, China).

Ethical Statement

All the cellular experiments were carried out in accordance with the regulations of Hongze District People Hospital

METHODOLOGY

Cell Culture and H/R Treatment

Human myocardial cell line, HCM was obtained from Sciencell (#6200, USA). Cells then were incubated in DMEM medium (Gibco, USA) containing ten percent FBS (Gibco) and 100μg/ml penicillin/streptomycin (Invitrogen, USA) at 37^0C, five percent CO_2. After incubation, cells were grouped into the normal group and H/R group (12 hours and 24 hours). Cells in H/R groups were first incubated in incubators with ninety-five percent N_{2-} and five percent CO_2 at 37^0C for 10 hours and then incubated in normal condition.

RT-qPCR

Strictly in accordance with the manufacturer's instructions, total RNA was segregated from normal HCMs and H/R HCMs using TRIzol reagent (Invitrogen, USA). After that, cDNA was compounded using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) for reverse transcription of IGF2R and TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems) was applied for reverse transcription of miR-200a-3p. Next, primers' sequences were listed for PCR as, miR-200a-3p: F 5'- GGCTAACACTGTCTGGTAACGATG -3', R, 5'—GTGCAGGGTCCGAGGT-3'; U6: F, 5'-CTCGCTTCGGCAGCACA-3', R, 5'-AACGCTTCACGAATTTGCGT-3' (Zhang et al. 2017), IGF2R: F, 5'-GAGGGAAGAGGCAGGAAAG-3', R, 5'- TGTGGCAGGCATACTCAG-3' (Harris et al. 2011), and GAPDH: F, 5'-ACCCAGAAGACTGTGGATGG-3', R, 5'-TCAGCTCAGGGATGACCTTG-3'. With ProFlex™ PCR System (Applied Biosystems, USA), SYBR Green PCR Master Mix (Applied Biosystems) was performed in PCR. Amplification conditions of PCR were list-

ed as follows, that is, denaturation was at 95^0C for 30 seconds, renaturation was at 60^0C for 30 seconds, and extension was at 72^0C for 45 seconds, 40 cycles. Relative expressions of miR-200a-3p and IGF2R were calculated using the $2^{-\ddot{A}\ddot{A}Ct}$ method. GAPDH and U6 were internal controls of IGF2R and miR-200a-3p, respectively.

Cell Transfection

HCM cells in growth period were collected and cultured onto 6-well plates (1×10^5 cells per well). Transfection was performed 24 hours after incubation. Small interfering RNA of IFG2R was obtained from GenePharma (Suzhou, China) named siIGF2R. Inhibitor of miR-200a-3p (miR-200a-3p inhibitor) was obtained from Invitrogen (Thermo Fisher, USA). Negative controls of them were named siNC and NC inhibitor, respectively. Thereafter, Lipofectamine 3000 (Invitrogen, USA) was to transfect siNC, siIGF2R, NC inhibitor, miR-200a-3p inhibitor and miR-200a-3p inhibitor with siIGF2R into HCMs, severally. Thereafter, cells were cultured for 24 hours after transfection, and expressions of transfected genes were quantified by RT-qPCR.

Cell Counting Kit-8

HCM after H/R (12 hours and 24 hours), suppression of miR-200a-3p and IGF2R and IWP-2 treatment were digested with 0.25 percent trypsin and cultured in 96-well plates with 1×10^4 cells per well. Thereafter, 10μl CCK-8 (96992, Sigma-Aldrich, USA) was added at 24 hours, 48 hours and 72 hours. Thereafter, HCMs were incubated for another 2 hours. Finally, Multiskan™ FC Microplate Readers (Thermo Scientific, USA) was performed to check optical density (OD) values at 450nm wavelength.

Flow Cytometry

HCMs were trypsinized and incubated for 48 hours. Cells were digested to single cells and rinsed with PBS twice and apoptosis were then checked by Annexin V-FITC Apoptosis Detection Kit (Invitrogen, USA). HCMs were first resuspended in 195μl binding buffer and cell density was regulated to $2x10^5$ cells/ml. later 5μl Annexin V-FITC was mixed with cell suspension and cultured for 10 minutes at 25^0C. HCMs were then rinsed and resuspended in 190μl binding buffer. 10μl PI (20μg/μl) was applied to stain cells for 15 minutes in darkness. Apoptosis rates of cells were analysed by Attune NxT Flow Cytometer (Invitrogen, USA).

Western Blot

Total protein in myocardial cells was isolated using Cell lysis buffer for Western and IP (Beyotime, Shanghai, China). Thereafter, 30μg of total protein was quantified with BCA Proteins Assay Kit (Beyotime) and segregated by 10 percent SDS-PAGE (Beyotime) and shifted onto PVDF membranes (Beyotime). Next, membranes were blocked by five percent non-fat milk powder and cultured with listed primary antibodies, namely, anti-Bax (1:1000; ab32503, Abcam, UK), anti-caspase-3 (1:1000; ab197202), anti-Bcl-2 (1:1000; ab185002), anti-c-IAP1 (1:1000; ab25939), anti-IL-1β (1:1000; ab9722), anti-IL-6 (1:1000; ab6672), anti-TNF-α (1:1000; ab1793), anti-NLRP3 (1:1000; ab263899), anti-HIF-1α(1:1000; ab1), anti-Wnt3a (1:1000; ab81614), anti-β-catenin (1:1000; 32572) and anti-GAPDH (1:2000; ab181602) overnight at 4^0C. Then, added secondary antibody, Goat Anti-Mouse IgG H&L (HRP) (1:900; ab205719) was applied to culture with PVDF membranes at room temperature for 2 hours. Finally, image developing was carried out by BeyoECL Moon (Beyotime) and ImageJ (National Institutes of Health, USA) was applied to analyse grey values of proteins.

Statistical Analysis

Experiments were all repeated three times independently. Data were shown as mean±SD and analysed using SPSS 19.0 (USA) and GraphPad Prism 7 (GraphPad Software, USA). Comparison between the two groups were examined through the Student's t-test. Comparisons in H/R treatment and comparisons among inhibitor of NC or miR-200a-3p and miR-200a-3p inhibitor with siIGF2R groups were analysed by one-way ANOVA. Differences were considered to have statistical significances with $^{**}P<0.05$.

RESULTS

Hypoxia/reoxygenation Inhibited Cell Viabilities but Promoted Expressions of Proinflammatory Cytokines and Apoptosis in HCMs

After HCMs were treated with hypoxia and reaeration, cell viabilities of HCMs were detected,

showing that H/R treatment could downregulate cell viabilities time dependently compared to the normal one (Fig. 1A, **P<0.05 and ^^P<0.01). Meanwhile, detections of apoptosis revealed that hypoxia/reoxygenation significantly increased apoptosis rates of HCMs time dependently (Fig. 1B, **P<0.05 and ^^P<0.01). Moreover, Bax and caspase-3 protein expressions were greatly upregulated while levels of Bcl-2 and c-IAP1 were suppressed distinctly (Fig. 1C, **P<0.05 and ^^P<0.01). Besides that, expressions of factors in inflammation were examined, which indicated that H/R treatment significantly promoted protein levels of IL-6, TNF-β, IL-1β and NLRP3 (Fig. 1D, **P<0.05 and ^^P<0.01). Hence, H/R treatment has been found to inhibit cell viability but accelerated apoptosis and proinflammatory cytokines' expressions in HCMs cells.

Downregulated miR-200a-3p Mediated Cell Viabilities, Inflammation and Apoptosis in Hypoxia/Reoxygenation-treated HCMs

After regulation of H/R to HCMs were confirmed, levels of miR-200a-3p were validated in normal HCMs and H/R-treated HCMs. RT-qPCR indicated that miR-200a-3p was dramatically elevated in H/R-treated HCMs time-dependently (Fig. 2A,

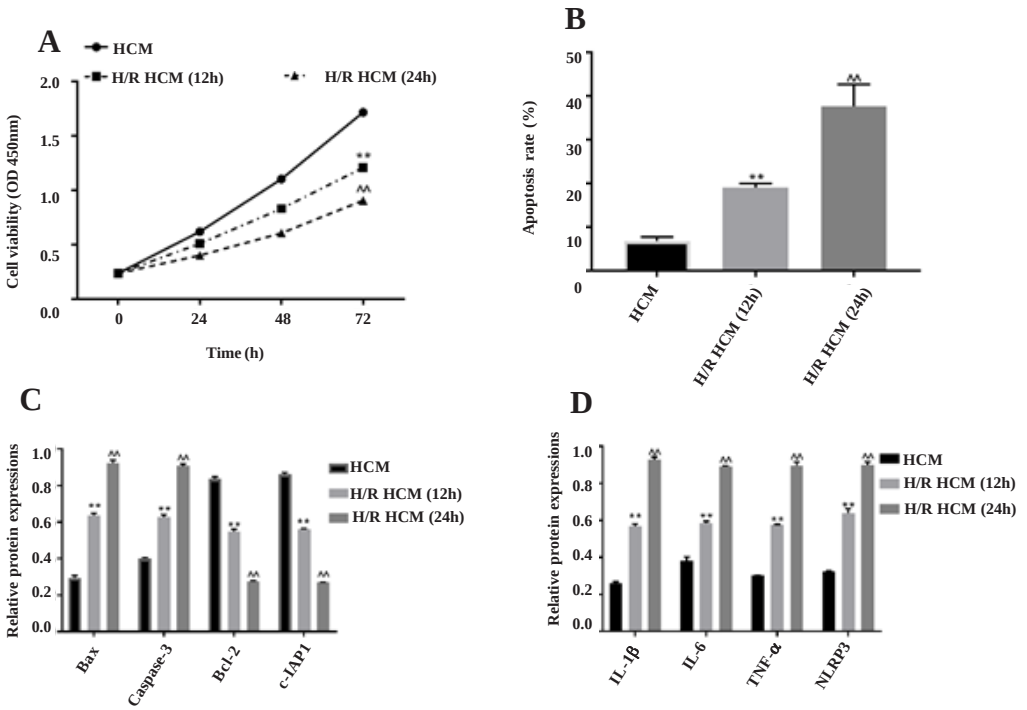

Fig. 1. Hypoxia/reoxygenation repressed cell viabilities but promoted apoptosis and inflammation of HCMs
A: CCK-8 was used for validating cell viabilities of HCMs after H/R treatment, **P<0.05 in comparison with HCM group and ^^P<0.01 in comparison with HCM group
B: Apoptosis rate of HCMs in normal condition and H/R treatment were detected using flow cytometry, **P<0.05 versus HCM group and ^^P<0.01 versus HCM group
C: Bax, caspase-3, Bcl-2 and c-IAP1 protein levels in HCM cells and H/R HCM cells were evaluated by western blot, **P<0.05 in comparison with HCM group and ^^P<0.01 versus HCM group
D: Western blot was to measure IL-1β, IL-6, TNF-α and NLRP3 protein levels, **P<0.05 in comparison with HCM group and ^^P<0.01 versus HCM group

**P<0.05 while ^^P<0.01). Beyond that, downregulated transfection repressed its RNA expression in H/R-treated HCMs (Fig. 2B, **P<0.05). Then, cell viabilities of HCMs (H/R 24 hours) with suppressed miR-200a-3p were greatly lower than the control group (Fig. 2C, **P<0.05). Meanwhile, apoptosis rates of HCMs were decreased largely after miR-200a-3p inhibition, which were reflected by decreased Bax and caspase-3 but promoted Bcl-2 and c-IAP1 (Fig. 2D, E, **P<0.05). Furthermore, knockdown of miR-200a-3p also significantly downregulated expressions of IL-6, TNF-α, IL-1β and NLRP3 (Fig. 2F, **P<0.05). Therefore, miR-200a-3p was demonstrated to accelerate apoptosis and expressions of proinflammatory cytokines with inhibiting cell viability after H/R treatment.

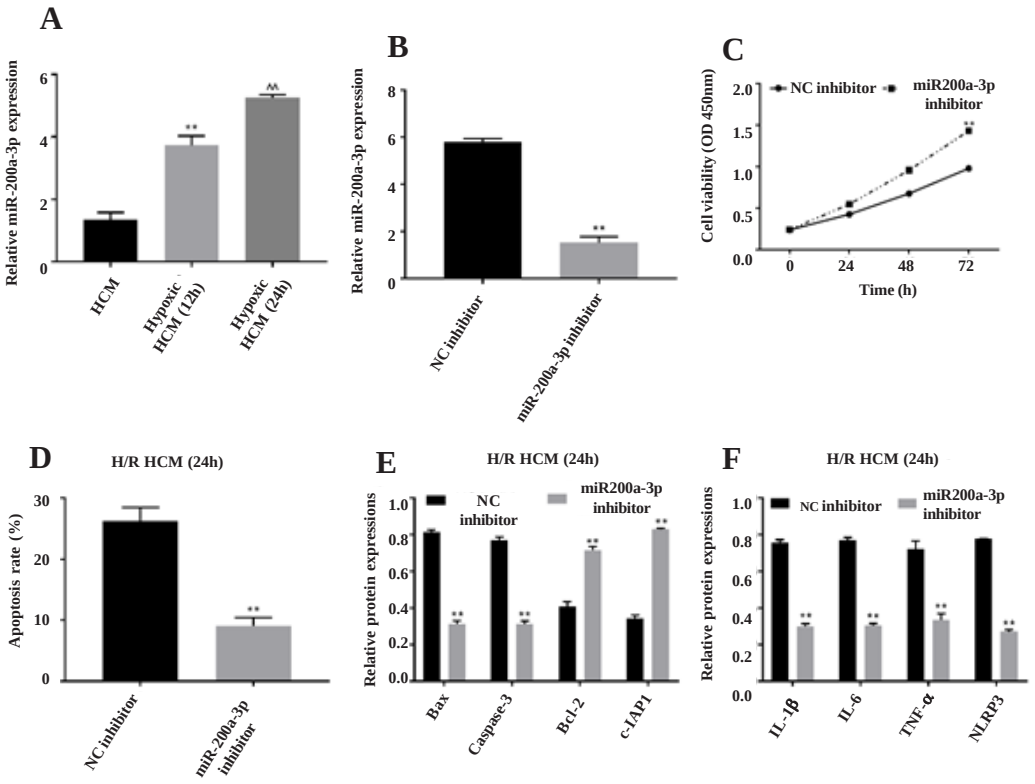

Fig. 2. Downregulated miR-200a-3p mediated cell viabilities, inflammation and apoptosis in HCM after hypoxia/reoxygenation treatment
A: MiR-200a-3p RNA expression in normal HCM cells and H/R HCM cells were validated using RT-qPCR, **P<0.05 while ^^P<0.01 versus HCM group
B: RT-qPCR was applied to check miR-200a-3p RNA levels after inhibition, **P<0.05 versus NC inhibitor group
C: Cell viabilities of H/R HCM cells with NC inhibitor and miR-200a-3p inhibitor were evaluated by CCK-8, **P<0.05 versus NC inhibitor group
D: Apoptosis rate of H/R HCM cells with NC inhibitor and miR-200a-3p inhibitor were measured with flow cytometry implement, **P<0.05 versus NC inhibitor group
E: Bax, caspase-3, Bcl-2 and c-IAP1 protein levels in H/R HCM cells with NC inhibitor and miR-200a-3p inhibitor were validated by western blot assay, **P<0.05 in comparison with NC inhibitor group.
F: IL-1β, IL-6, TNF-α and NLRP3 protein expressions were validated through western blot in H/R HCM cells with NC inhibitor and miR-200a-3p inhibitor, **P<0.05 versus NC inhibitor group

MiR-200a-3p Enhanced Apoptosis as well as Inflammation in HCMs Treated by H/R through Increasing Expressions of IGF2R

The expression of IFG2R in H/R HCMs was measured, showing that the level of IGF2R was distinctly upregulated time-dependently after H/R (Fig. 3A, $^{**}P<0.05$). The level of IGF2R was decreased by miR-200a-3p suppression and added siIGF2R resulted in a lower expression of IGF2R in HCMs (Fig. 3B, C, $^{**}P<0.05$ while $^{\#\#}P<0.05$). Moreover, added silenced IGF2R enhanced the suppres-

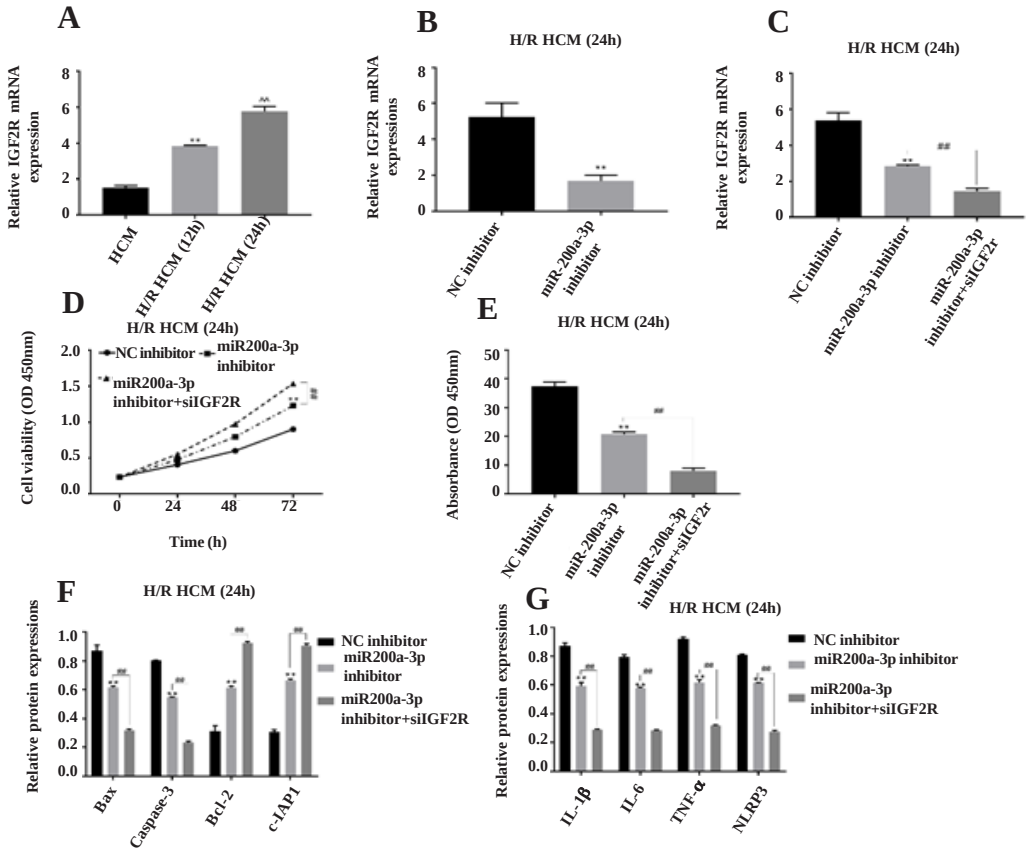

Fig. 3. MiR-200a-3p enhanced apoptosis and inflammation in hypoxia/reoxygenation-treated HCM cells through increasing expressions of IGF2R

A: Expressions of IGF2R in HCM cells and H/R HCM cells were evaluated by RT-qPCR, $^{**}P<0.05$ while $^{\wedge\wedge}P<0.05$ in comparison with HCM group

B: RNA expressions of IGF2R were evaluated through RT-qPCR with transfected NC inhibitor and suppressed miR-200a-3p, $^{**}P<0.05$ versus NC inhibitor group

C: RT-qPCR was carried out to evaluate IGF2R RNA expression with inhibitor of NC and miR-200a-3p and suppressed IGF2R with miR-200a-3p, $^{**}P<0.05$ while $^{\#\#}P<0.05$

D: CCK-8 was applied for evaluating cell viabilities with miR-200a-3p inhibitor and siIGF2R in H/R HCM cells, $^{**}P<0.05$ while $^{\#\#}P<0.05$

E: Apoptosis rate of H/R HCM cells transfected by inhibited miR-200a-3p and IGF2R were validated through flow cytometry, $^{**}P<0.05$ while $^{\#\#}P<0.05$

F: Western blot was applied for analyzing Bax, caspase-3, Bcl-2 and c-IAP1 protein levels in H/R HCMs transfected by inhibited miR-200a-3p and IGF2R, $^{**}P<0.05$ while $^{\#\#}P<0.05$

G: Western blot was applied to examine TNF-α, IL-1β, IL-6 and NLRP3 protein expressions in H/R HCMs with suppressed miR-200a-3p and IGF2R, $^{**}P<0.05$ while $^{\#\#}P<0.05$

sion of cell viabilities in HCMs caused by inhibited miR-200a-3p (Fig. 3D, $^{**}P<0.05$ while $^{\#\#}P<0.05$). Meanwhile, suppressed miR-200a-3p and IGF2R caused a higher apoptosis rate of HCMs than miR-200a-3p inhibition alone (Fig. 3E, $^{**}P<0.05$ while $^{\#\#}P<0.05$). Knockdown of IGF2R also significantly amplified downregulation of Bax and caspase-3 but elevated Bcl-2 and c-IAP1 after miR-200a-3p downregulation (Fig. 3F, $^{**}P<0.05$ and $^{\#\#}P<0.05$). Furthermore, miR-200a-3p inhibitor decreased expressions of IL-6, TNFα, IL-1β and NLRP3 and IGF2R suppression enhanced the inhibition (Fig. 3G, $^{**}P<0.05$ while $^{\#\#}P<0.05$). Based on these experiments, IGF2R was the target induced by miR-200a-3p in H/R-treated HCMs.

MiR-200a-3p Regulated Hypoxia/Reoxygenation-induced Inflammation and Apoptosis of HCMs through Wnt/β-catenin Signalling Pathway and Activating HIF-1α

After miR-200-3p and IGF2R were measured respectively, HIF-1α related to H/R, Wnt3a and β-catenin were examined, showing that H/R treatment in HCMs greatly enhanced protein levels of HIF-1α, Wnt3a and β-catenin (Fig. 4A, $^{**}P<0.05$

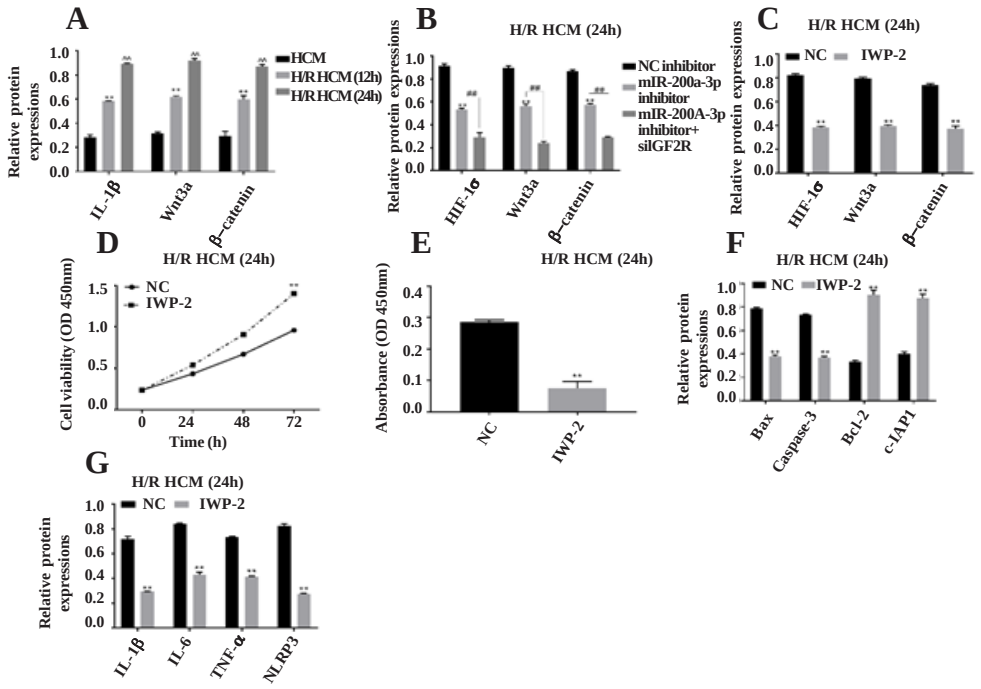

Fig. 4. MiR-200a-3p inhibited hypoxia/reoxygenation-induced inflammation and apoptosis of myocardial cells via Wnt/β-catenin signaling pathway and activation of HIF-1α.
A: Western blot was applied for examining protein changes of HIF-1α, Wnt3a and β-catenin in HCMs and H/R HCMs, $^{**}P<0.05$ while $^{\#\#}P<0.01$ versus HCM group
B: Expressions of HIF-1α, Wnt3a and β-catenin with inhibited NC, miR-200a-3p and IGF2R were validated through western blot, $^{**}P<0.05$ while $^{\#\#}P<0.05$
C: Levels of HIF-1α, Wnt3a and β-catenin after IWP-2 added were checked by western blot, $^{**}P<0.05$ versus NC group
D: CCK-8 was used to validate cell viabilities of H/R HCMs after IWP-2 used, $^{**}P<0.05$ versus NC group.
E: Apoptosis rate of H/R HCM cells with IWP-2 treatment were examined using flow cytometry, $^{**}P<0.05$ versus NC group
F: Bax, caspase-3, Bcl-2 and c-IAP1 protein levels in H/R HCM cells were validated through western blot assay after treated by IWP-2, $^{**}P<0.05$ versus NC group
G: Expressions of IL-6, TNF-α, IL-1β and NLRP3 were validated with western blot after IWP-2 added into H/R HCM cells, $^{**}P<0.05$ versus NC group

while $^{\wedge\wedge}P<0.01$). Moreover, knockdown of IGF2R enhanced the role of miR-200a-3p suppression by causing much lower levels of HIF-1β, Wnt3a and α-catenin in HCMs (Fig. 4B, $^{**}P<0.05$ and $^{\#\#}P<0.05$). To figure out the participation of Wnt/β-catenin signalling pathway with H/R treatment, IWP-2, a Wnt/β-catenin signalling pathway inhibitor, was used and it significantly downregulated protein expressions of HIF-1α, Wnt3a and β-catenin in comparison with the NC group (Fig. 4C, $^{**}P<0.05$). Meanwhile, results of CCK-8 revealed that IWP-2 addition greatly increased cell viabilities of H/R-treated HCMs (Fig.4D, $^{**}P<0.05$) while the apoptosis was markedly decreased (Fig. 4E, $^{**}P<0.05$). Besides that, IWP-2 treatment resulted in lower levels of Bax and caspase-3 but higher expressions of Bcl-2 and c-IAP1 (Fig. 4F, $^{**}P<0.05$). Moreover, in H/R-treated HCMs, IWP-2 addition distinctly downregulated protein levels of IL-6, TNF-α, IL-1β and NLRP3 (Fig. 4G, $^{**}P<0.05$). From these detections, the Wnt/b-catenin signalling pathway was activated by IGF2R during H/R treatment and suppression of this pathway blocked expressions of pro-inflammatory cytokines and apoptosis in HCMs.

DISCUSSION

Pathological basis of myocardial fibrosis is increased proportion of myocardial matrix in cardiac muscle tissues, which is fibrous connective tissue compounded by fibroblast, myofibroblast, pericytes, valvular interstitial cells and other cells and extracellular matrix (ECM) (Segura et al. 2014). Clinically, myocardial fibrosis can be found in common cardiovascular diseases such as hypertensive heart disease, ischemic heart disease and diabetic cardiomyopathy and so on. In myocardial fibrosis, ECM is a tangible component synthesised and secreted by cardiac fibroblasts, which can maintain normal cardiac structure and regulate repair after myocardial injury (Fan et al. 2012). Changes in the content and composition of extracellular matrix of myocardium are the pathological basis of myocardial fibrosis caused by various etiologies (Li et al. 2018). Myocardial fibroblasts can directionally move to injured parts influenced by endothelial injury and ischemic hypoxic injury and so on. Moreover, it can be mediated to produce plenty of ECMs by inflammatory factors, cytokines and neurohumoral factors. In hypoxic myocardial tissues, myofibroblast existed in scar tissues of myocardium is the key effector causing myocardial fibrosis (Lee and Chintalgattu 2019). Therefore, in this study, the researchers selected HCMs to verify effects and the molecular mechanism of hypoxia/reoxygenation. HCMSs were first divided into normal group and H/R groups, indicating that cell viabilities of HCMs were significantly decreased after H/R treatment in a time dependent manner. Besides that, apoptosis rate was dramatically increased and expressions of Bax and caspase-3 were upregulated while levels of Bcl-2 and c-IAP1 were decreased. As for inflammatory cytokines, IL-6, TNF-α, IL-1β and NLRP3 expressions were also significantly upregulated by hypoxia/reoxygenation treatment. Same as previous studies, researchers found that increased inflammatory cytokines and apoptosis were induced by H/R in HCMs.

According to previous research, miR-200a-3p expression was distinctly higher in patients with myocardial fibrosis (Fang et al. 2015). Moreover, Li et al. have demonstrated that miR-200a-3p was notably promoted in cardiac progenitor cells (CPC) after hypoxia stimulus, which repressed proliferation and migration through targeting Sirt1 and long non-coding RNA H19 (Li et al. 2018). Moreover, miR-200a-3p was remarkably increased in hypertrophic H9c2 cells, and accelerated development of myocardial hypertrophy through declining WDR1 via PTEN/PI3K/AKT/CREB signalling pathway (Yang et al. 2019). Insulin-like growth factor type 2 receptor (IGF2R) has participated in progressions of heart diseases, which was upregulated in H9c2 cells in diabetic rat models and caused upregulation of atrial natriuretic peptide and brain natriuretic peptide, cardiac hypertrophy related proteins, leading to apoptosis of myocardial cells (Feng et al. 2018). In hypoxia-induced H9c2 cells, IGF2R expression induced by hypoxia was suppressed by CREB overexpression and reduced apoptosis rate of H9c2 cells (Chen et al. 2015). Lin et al. have also shown that IGF2R overexpression caused cardiac hypertrophy and myocardial fibrosis, which was inhibited by NFIL3 (Lin et al. 2015). Moreover, miR-200a-3p overexpressions were demonstrated to target and downregulate endogenous expression of IGF2 in mouse trophoblast stem cells (Saha et al. 2015). Therefore, potential connections might exist in miR-200a-3p and IGF2R in HCMs. In this study, miR-200a-3p was markedly promoted in

H/R-treated HCMs while inhibitor of miR-200a-3p reduced its level. Then, results of cell viabilities indicated that miR-200a-3p inhibition increased survival of cells. Meanwhile, reduced apoptosis rate, suppressed Bax and caspase-3 but upregulated Bcl-2 and c-IAP1 showed that suppressed miR-200a-3p inhibited apoptosis in hypoxia/reoxygenation-treated HCMs. Moreover, lower expressions of IL-6, TNF-α, IL-1β and NLRP3 revealed that inhibited miR-200a-3p remarkably decreased expressions of pro-inflammatory cytokines. After that, IGF2R was measured, showing that its expression was significantly promoted in hypoxia/reoxygenation-treated HCMs, which was suppressed by downregulated miR-200a-3p and IGF2R knockdown. Results in CCK-8 also showed that silenced IGF2R enhanced roles of miR-200a-3p inhibitor by increasing viabilities of hypoxia/reoxygenation-treated HCMs and reducing apoptosis rates. Additionally, knockdown of IGF2R accelerated decreases of Bax and caspase-3 but promotions of Bcl-2 and c-IAP1 caused by miR-200a-3p inhibition. Beyond that, inflammatory factors, TNF-α, IL-1β, IL-6 and NLRP3 were also suppressed with downregulated IGF2R after miR-200a-3p inhibition. In this study, researchers have found that miR-200a-3p and IGF2R were promoted after H/R treatment while miR-200a-3p and IGF2R inhibition reduced apoptosis and inflammation but promoted cell viability in HCMs with hypoxia/reoxygenation treatment. Compared to former studies, researchers have analysed correlation between miR-200a-3p and IGF2R and functions of these two genes.

Suppression of Wnt and b-catenin expressions could retard development of fibrosis after inflammation according to research of Blyszczuk et al. (2017). In myocardial infarction (MI), CGX1321 was determined to suppress Wnt signalling pathway resulting in promoted proliferation of myocardial cells (Yang et al. 2017). Besides that, HIF-1α is a known factor in hypoxia of myocardial cells, which facilitates injuries of hypoxia/reoxygenation. Therefore, expressions of HIF-1α and proteins in Wnt/b-catenin signalling pathway were checked and results showed that hypoxia/reoxygeneration upregulated levels of HIF-1α, Wnt3a and α-catenin while they were then suppressed by inhibition of miR-200a-3p and IGF2R. IWP-2 has been well known as a Wnt/β-catenin signalling pathway inhibitor (Gao et al. 2020; Liu et al. 2015). Therefore, IWP-2 was added to figure out functions of Wnt/β-catenin signalling pathway. Expressions of HIF-1a, Wnt3a and b-catenin were suppressed after IWP-2 treatment with lower apoptosis and inflammation while cell viabilities were increased, revealing that Wnt/b-catenin signalling pathway could promote process of hypoxia/reoxygenation in HCM cells. In summary, this study has found that miR-200a-3p facilitated apoptosis and inflammatory cytokines' expressions in H/R-induced HCMs. Furthermore, it also promoted expressions of IGF2R regulate progression of H/R treatment via Wnt/²-catenin signalling pathway in vitro.

CONCLUSION

MiR-200a-3p promoted apoptosis and inflammation but inhibited cell viability in hypoxia/reoxygenation-treated HCMs through increasing levels of IGF2R and activating Wnt/β-catenin signalling pathway, suggesting miR-200a-3p and IGF2R might be factors to treat H/R damages in myocardial cells. Nevertheless, studies in vivo and clinical are necessary to verify this finding.

RECOMMENDATIONS

H/R treatment accelerated apoptosis and expressions of pro-inflammatory cytokines but repressed cell viability in HCMs. MiR-200a-3p and IGF2R were upregulated after H/R treatment and reduced cell viability but accelerated apoptosis and inflammation. HIF-1α, Wnt3a and β-catenin were also promoted by H/R treatment and activated after inhibition of miR-200a-3p and IGF2R while IWP-2 treatment inhibited apoptosis and proinflammatory cytokines' expressions but increased cell viability of H/R-induced HCMs through suppressing Wnt/β-catenin signalling pathway. Hence, the study recommended that miR-200a-3p and IGF2R might be potential biomarkers for H/R treatment.

ABBREVIATION LIST

- MiR-200a-3p: microRNA-200a-3p
- CCK-8: Cell Counting Kit-8.
- IGF2R: Insulin-like growth factor 2 receptor
- H/R: Hypoxia/Reoxygenation
- HCM: Human cardiac myocytes
- PI: Propidium Iodide

- RT-qPCR: Quantitative reverse transcription PCR
- ECM: Extracellular matrix
- CPC: Cardiac progenitor cell
- MI: Myocardial infarction.

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