Ligustrazine enhances the protective effect of remifentanil on myocardial ischemia-reperfusion injury by regulating miR-211/USP47 pathway

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Received: 22 August 2022; Accepted: 22 September 2022; Published: 18 October 2022

ORIGINAL ARTICLE

Abstract

Purpose: To assess the effects of ligustrazine on the progression of hypoxia-reoxygenation (H/R), and explore the possible role of miR-211 in myocardial ischemia-reperfusion (I/R) injury and identify its potential targeting gene. Methods: The level of miR-211 in H/R-induced cells was detected by quantitative polymerase chain reaction. CCK-8, flow cytometry (FCM), and Western blot assays were performed to examine the effect of miR-211 and USP47 on the role of remifentanil against H/R injury. Bioinformatic analysis and luciferase and Western blot assays were performed to identify and verify the potential target of miR-211. CCK-8 and FCM assays were performed to detect the mechanism of ligustrazine and miR-211 in the protection of remifentanil against H/R-induced cells. Results: Ligustrazine enhanced the effect of remifentanil on H/R-induced cardiomyocytes. MiR-211 enhanced the protective effect of remifentanil against H/R injury. Down-regulation of USP47 enhanced the protective effect of remifentanil against H/R injury. Ligustrazine enhanced the protective effect of remifentanil against H/R injury through miR-211/USP47 pathway. Conclusion: Ligustrazine enhanced the protective effect of remifentanil on myocardial I/R injury by regulating miR-211/USP47 pathway.

Keywords: ligustrazine; myocardial ischemia-reperfusion; remifentanil; miR-211; USP47

Introduction

Myocardial infarction (MI) is a major public health problem and one of the leading causes of death worldwide (Keshavaraz *et al.*, 2020). There is increased serum cardiac enzyme activity and progressive electrocardiographic changes that can be complicated by arrhythmias, shock, or heart failure, which can often be life-threatening. Cerebral ischemia-reperfusion (I/R) usually refers to patients who have suffered from MI, where the use of reperfusion is equivalent to ischamic preconditioning because of multiple occlusions occurring within minutes after reperfusion. Thrombolysis, primary angioplasty, and cardiac surgery are effective treatments to restore blood flow to ischemic myocardium (Jin *et al.*, 2020). As a disease with high mortality and morbidity, MI still lacks more effective treatments, and hence an in-depth study of its pathogenesis is urgently required (Singh *et al.*, 2021).

Ligustrazine (Lig) is one of the main active components of *Ligustrum chuanxiong*, which has a variety of biological activities in the cardiovascular system (Hao *et al.*, 2018). The possible mechanisms of ligustrazine in MI are antioxidant, anti-inflammatory, antiapoptotic, and improving coronary blood flow and myocardial metabolism.

ISSN 1757-837X online, DOI 10.15586/qas.v14i4.1179

Salvia miltiorrhiza and ligustrazine injection decreased the apoptotic rate of H9C2 cells by inhibiting the activation of caspase-3 and increasing the ratio of Bcl-2/Bax. Studies have shown that ligustrazine can increase the expression of Mir-211 (Hao *et al.*, 2018). In addition, the binding site between Mir-211 and USP47 was predicted on TargetScan website.

Remifentanil, an ultra short-acting, high-potency opioid pain reliever, is widely used in surgery. Preconditioning with remifentanil (RPC) normally reduces myocardial I/R damage. In the human body, it rapidly reaches the blood-brain barrier in about 1 min, and is hydrolyzed rapidly in tissues and blood (Yin *et al.*, 2021). Therefore, it has a rapid onset and a short maintenance time, in marked contrast to other fentanyl analogs (Aoki *et al.*, 2021). The analgesic effect of remifentanil and its adverse effects are dose-dependent and synergistic when combined with hypnotics, inhaled anesthetics, and benzodiazepines. Remifentanil has shown good therapeutic effects in a variety of cardiovascular diseases.

MicroRNAs (miRNAs) are distinguished as key regulators in the occurrence and progression of various cardiovascular diseases, such as cardiac hypertrophy, coronary atherosclerosis, and acute MI (Liu *et al.*, 2021). Therefore, many studies have suggested that miRNAs play a prominent role in myocardial functioning (e.g., contraction and morphogenesis) and are involved in the progression of myocardial I/R injury (Wang *et al.*, 2017). Studies have shown that expression of miR-211 is decreased during cerebral I/R (Ma *et al.*, 2020). Low expression and up-regulation of miR-211 during renal I/R inhibited hypoxia-reoxygenation (H/R)-induced apoptosis and increased cell viability (Liu *et al.*, 2020). However, its possible role in myocardial I/R injury is still unclear.

In this study, the functioning of ligustrazine and miR-211 was investigated in myocardial I/R injury. Ligustrazine could serve as a promising drug for the treatment of MI.

Materials and methods

Cell culture and treatment

Embryonic rat myocardium cells H9C2 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) mixed with 10% fetal bovine serum (FBS; Invitrogen; Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin, in a culture hood supplied with 5% CO_2 at 37°C.

For the construction of I/R model, cells were cultured in serum and glucose-deficient DMEM, and maintained in an anaerobic chamber with no oxygen supply (95% N_2 and 5% CO_2) at 37°C for 10 h after maintaining in normal medium for 24 h. Subsequently, cell culture condition was changed to normal condition with complete medium and oxygen access for another 24 h. Cells were divided into the following groups: control, H/R, H/R+RPC, and H/R+ligustrazine+RPC. Ligustrazine was administrated at a dose of 10 μg/mL (Baomanbio, D0188). As a pre-treatment, Remifentanil hydrochloride (Ultiva, U22B, dissolved in sterile 0.9% NaCl) was administrated at a dose of 8 ng/mL.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNAs from indicated cells were isolated with Trizol reagent (Invitrogen, Shanghai, China). Then, total RNAs were used to produce cDNA with PrimeScript reverse transcription reagent kit (Takara Biotechnology Co. Ltd., Dalian, China). Quantitative PCR was performed with SYBR mixture (Takara Biotechnology). The primer sequences of miR-211 and U6 were listed as follows: miR-211: 5'-CCGGAATTCCGGTTTTACAACA CCCCATTTCACC-3', 5'-CGCGGATCCGCGCGAGC AACAGAGTAGAACAGG-3' and U6: 5'-GTCGTATCC AGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACAAAATATGGAAC-3'. The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. U6 was used as an internal control.

Cell transfection

The sequences were listed as follows: miR-211 mimic: 5'-UAACGACGAAUAACGCAAAAUGU-3'; miR-211 inhibitors: 5'-AGGCAAAGGATGACAAAGGGAA-3'. MiR-211 mimics, miR-211 inhibitors, and corresponding controls were synthesized by GenePharma (Shanghai, China). Cells were transfected using lipofectamine 3000 (Thermo Fisher Scientific, San Jose, CA, USA) for 24 h, according to the manufacturer's protocol.

Cell counting kit 8 (CCK-8) assay

To measure cell viability, quantitative colorimetric assay was performed with CCK-8. Briefly, cells were placed into 96-well plate at a density of 6×10^3 cells per well and cultured for 24 h. Thereafter, cells were supplied with CCK-8 solution and incubated for 1.5 h at 37°C. Subsequently, the values of each well were determined at 450 nm by an enzyme-linked immunosorbent serologic assay (ELISA) microplate reader.

Cell apoptosis

To detect cell apoptosis, cells were treated with Annexin V-FITC apoptosis detection kit (Clontech Laboratories Inc., Palo Alto, CA, USA). Cells were seeded in 6-well plates at a density of about 4×10^6 cells. Cells were typsinized, washed with cold phosphate-buffered saline (PBS), and harvested at 1,000 rpm. Cells were stained with Annexin V-FITC in binding buffer, cultured for 30 min, and protected from light. Then, cells were stained with propidium iodide in dark and measured by a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Western blotting analysis

Proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Jiangsu, China). Then the samples were collected and subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes, followed by blocking with 5% fat-free milk in tris-buffered saline with Tween 20 (TBST) buffer. Subsequently, membranes were conjugated with primary antibodies targeting Bax (1:1,000 dilution, Abcam, Cambridge, MA, USA), Bcl-2 (1:1,000, Cell Signaling Technology Inc., Danvers, MA, USA), cleaved-caspase3 (1:1,000 dilution; Abcam), USP47 (1:1,000 dilution, Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:10,000, ab8226; Abcam) for 2 h at room temperature. Subsequently the membranes were incubated with specific secondary antibodies at room temperature for 1 h. The blots were analyzed with ECL kit. The blots were quantified by the ImageJ 9.0 software.

Luciferase Assay

For luciferase assay, cells were transfected with miR-211 inhibitors or mimics and USP47 wide-type (WT) or mutant plasmids using lipofectamine 3000 (Invitrogen, China). USP47 with 3'-UTR was amplified by PCR. The forward primer was USP47: 5'-GGCAGGACGCTCATTAGGT-3', 5'-GCACAACATGATTCCAA-3'. To produce mutant USP47, the mutations (wild type 3'-UTR, 5'-AAAGGGA-3'; mutated, 3'-UTR: 5'-AAATTC-3') was incorporated with QuikChange II XL site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). Each well was transfected with 0.2 μg of luciferase reporter plasmids and 2.5 ng of SV-Renilla luciferase plasmids. Cells were harvested and lysed and subjected to luciferase activity detection with Dual Luciferase Reporter assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Firefly luciferase activities were normalized to Renilla luciferase activity.

Statistical analyses

Data were shown as mean ± standard deviation (SD). For statistical analysis, one-way ANOVA followed by Turkey's *post hoc* was performed; $P < 0.05$ was considered statistically significant.

Results

Ligustrazine enhances the effect of remifentanil on H/R-induced cardiomyocytes

H9C2 cells were subjected to ischemia and H/R treatment. To investigate the effect of ligustrazine on remifentanil-pretreated H/R-injured cells, a CCK-8 assay was performed in H9C2 cells. H/R injury significantly impaired cell viability. Remifentanil treatment alone had little effect on cell viability. However, the combination of remifentanil and ligustrazine dramatically improved H/R-induced cell viability (Figure 1A). In addition, H/R injury induced severe apoptosis, as evidenced by increased proportion of apoptotic cells, and elevated expression levels of cleaved-caspase 3 and Bax (Figures 1B–D). Similarly, the combined usage of remifentanil and ligustrazine reduced cell apoptosis, as evidenced by decreased apoptotic cell proportion and reduced expression levels of cleaved-caspase 3 and Bax (Figures 1B–D). These results suggest that ligustrazine has a protective effect on remifentanil-pretreated H/R injured cells.

MiR-211 enhances the effect of remifentanil on H/R-induced cardiomyocytes

Level of miR-211 was detected by RT-qPCR. MiR-211 was down-regulated in H/R cells compared to control cells (Figure 2A). Level of miR-211 was monitored by transfection of miR-211 mimic. Overexpression efficiency was analyzed by RT-qPCR. As expected, transfection of miRNA mimics significantly increased the level of miR-211 (Figure 2B). To investigate the effect of miR-211 on remifentanil-pretreated H/R-injured cells, miR-211 was overexpressed in H9C2 cells. H/R injury then significantly impaired cell viability. Treatment of miR-211 or remifentanil alone had little effect on cell viability. However, the combination of remifentanil and miR-211 dramatically improved H/R-induced cell viability (Figure 2C). Furthermore, H/R injury induced severe apoptosis, as evidenced by an increased proportion of apoptotic cells, and elevated expression levels of cleaved-caspase 3 and Bax (Figures 2D–G). Similarly, the combined usage of remifentanil and miR-211 reduced apoptosis, as evidenced by a decrease in the proportion of apoptotic cell as well as a decrease in the expression levels of cleaved-caspase 3 and Bax (Figures 1D–G). These results suggest a protective role for miR-211 in remifentanil-pretreated H/R injured cells.

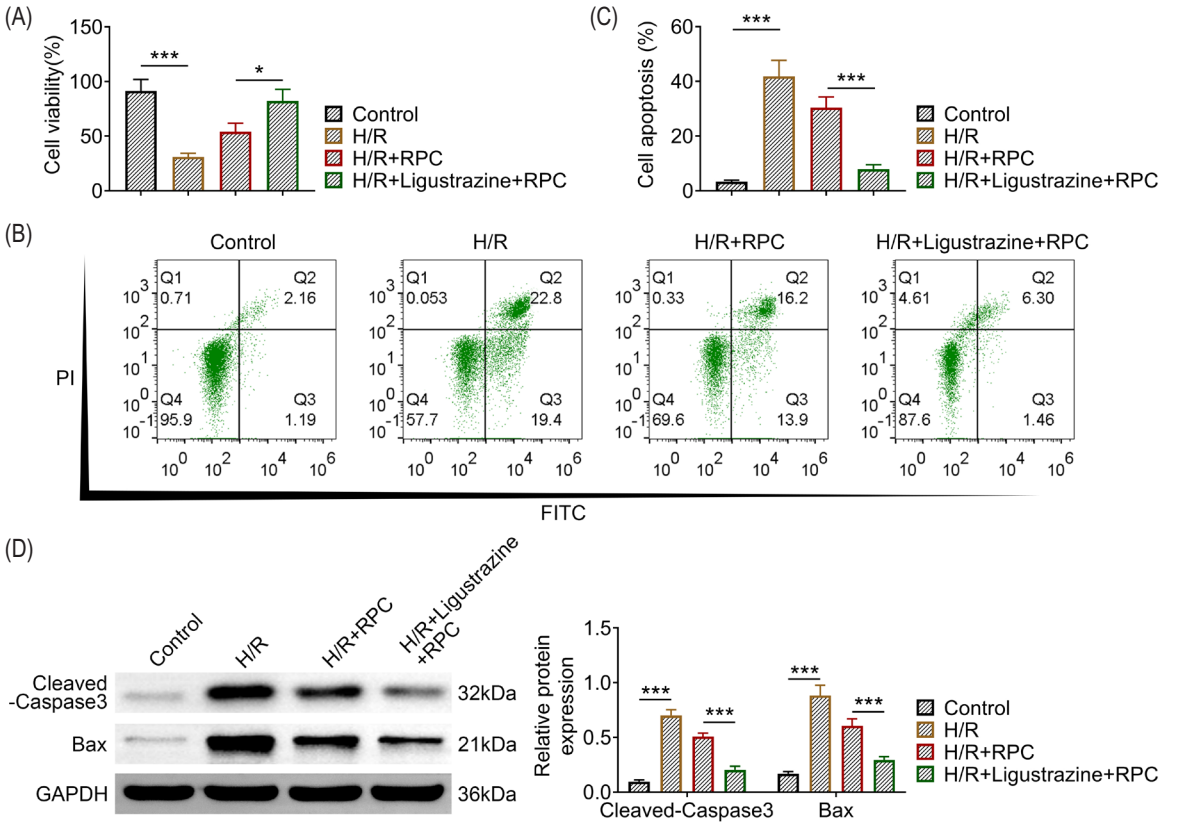

Figure 1. Ligustrazine enhances the effect of remifentanil on H/R-induced cardiomyocytes. (A) Cell viability in indicated groups as detected by CCK-8 assay. (B and C) Cell apoptosis in indicated groups as detected by flow cytometry. (D and E) Cell apoptosis in indicated groups as detected by Western blot analysis. Data are shown as SD. $^{*}p < 0.05$, $^{***}p < 0.001$.

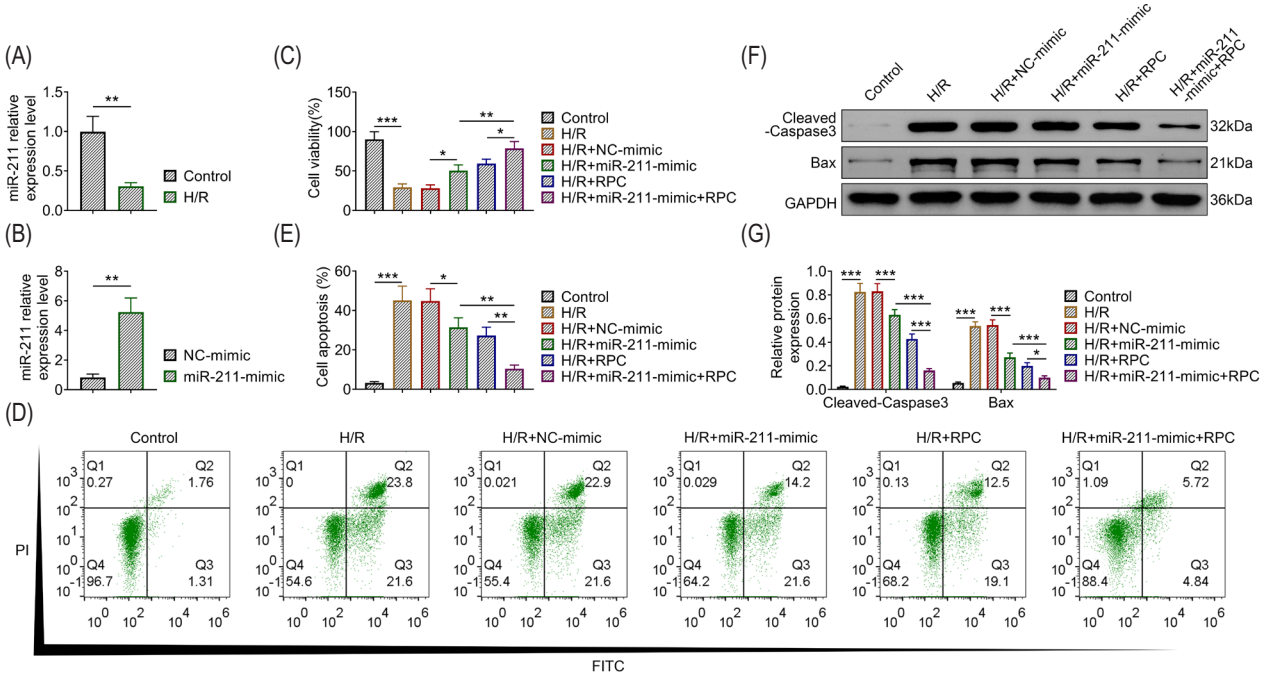

Figure 2. MiR-211 strengthens the effect of remifentanil on H/R-induced cardiomyocytes. (A) Expression of miR211 in H/R and control cells. (B) Transfection efficiency of miR211 overexpression. (C) Cell viability in indicated groups as detected by CCK-8 assay. (D and E) Cell apoptosis in indicated groups as detected by flow cytometry. (F and G) Cell apoptosis in indicated groups as detected by Western blot analysis. Data are shown as SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

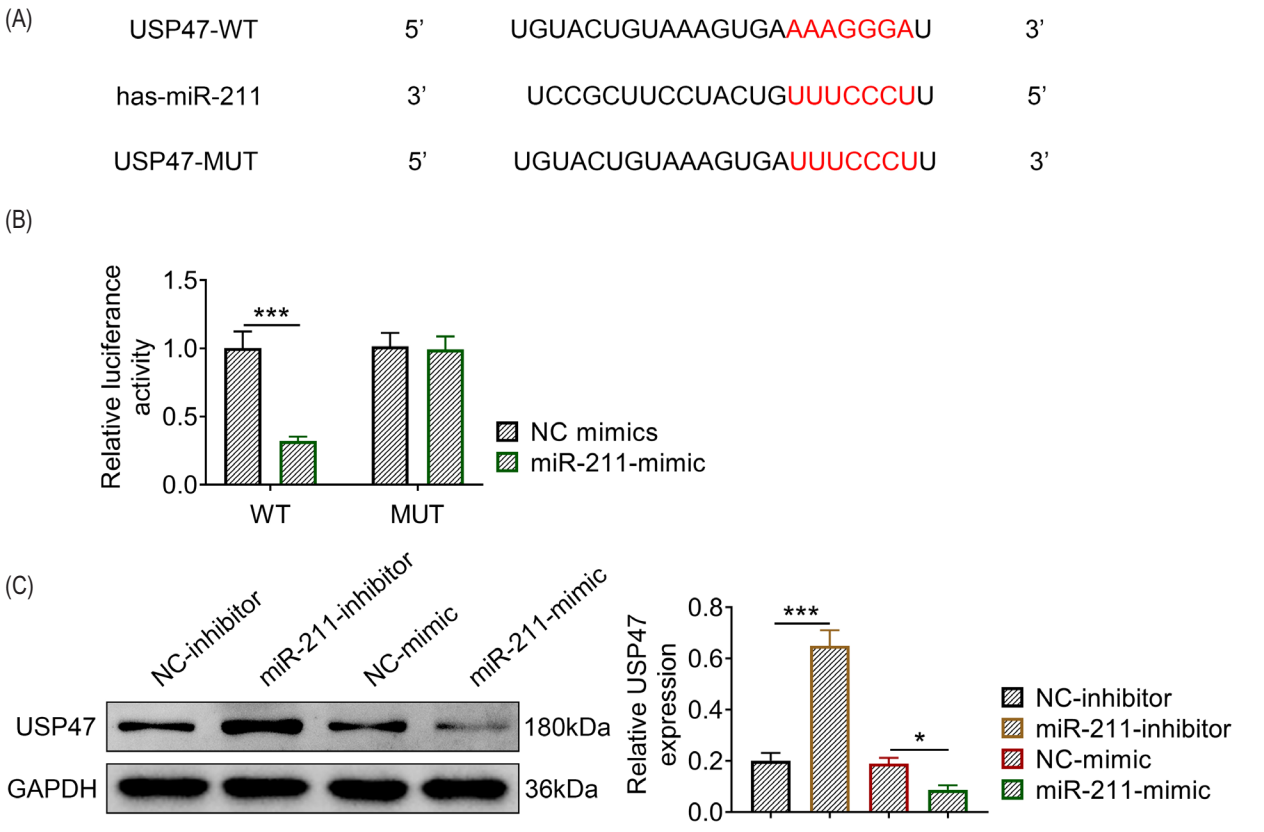

Figure 3. MiR-211 negatively regulates USP47. (A) Bioinformatic prediction of target sequence of miR-211. (B) Luciferase assay detected the regulatory role of miR-211 in USP47 expression. (C) Effect of miR-211 mimics/inhibitor on USP47 expression. Data are shown as SD. $^{*}p < 0.05$, $^{***}p < 0.001$.

MiR-211 negatively regulates USP47 expression

To search the potential targets of miR-211, screening was performed by TargetScan (http://www.targetscan.org/vert_71/). The 3'-UTR was complementary to miR-211 according to bioinformatic analysis (Figure 3A). To verify this, a luciferase assay was performed, and it was observed that the luciferase activity of USP47 was significantly inhibited by miR-211 mimic (Figure 3B). However, mutant sequences of USP47 luciferase activity exhibited no significant difference in luciferase activity, indicating that USP47 was a target of miR-211. Consistently, transfection of miR-211 mimics reduced the expression of USP47 (Figure 3C). Taken together, these data suggested that miR-211 regulated USP47 expression.

MiR-211 enhanced the protective effect of remifentanil against H/R injury by negatively regulating USP47

To illustrate further the mechanism of miR-211 against remifentanil-induced H/R injury, cell viability was detected in cells that co-transfected with miR-211 mimics and USP47 overexpressed USP47. As previously described previously, miR-211 improved the viability of remifentanil-pretreated H/R injury cells. Moreover, USP47 counteracted the effect of miR-211 on the improvement of cell viability (Figure 4A). Cell apoptosis was examined by flow cytometry (FCM) and Western blot assays. Consistently, miR-211 improved cell apoptosis in remifentanil-pretreated H/R injury cells. In contrast, USP47 counteracted the effect of miR-211 on the improvement of cell apoptosis (Figures 4B–D). Therefore, miR-211 enhances the protective effect of remifentanil against H/R injury by negatively regulating USP47.

Ligustrazine enhanced the protective effect of remifentanil against H/R injury through miR-211/USP47

To illustrate further the mechanism of ligustrazine against remifentanil-induced H/R injury, cell viability was detected in ligustrazine-treated cells that transfected with miR-211 inhibitor. As described previously, H/R treatment reduced miR-211 level and enhanced USP47 level. Ligustrazine ameliorated the effect of H/R on miR-211 and USP47. MiR-211 antagonized the effect of ligustrazine on USP47 (Figures 5A and B). Cell apoptosis was then examined by FCM and Western blot assays. Consistently, ligustrazine improved cell apoptosis

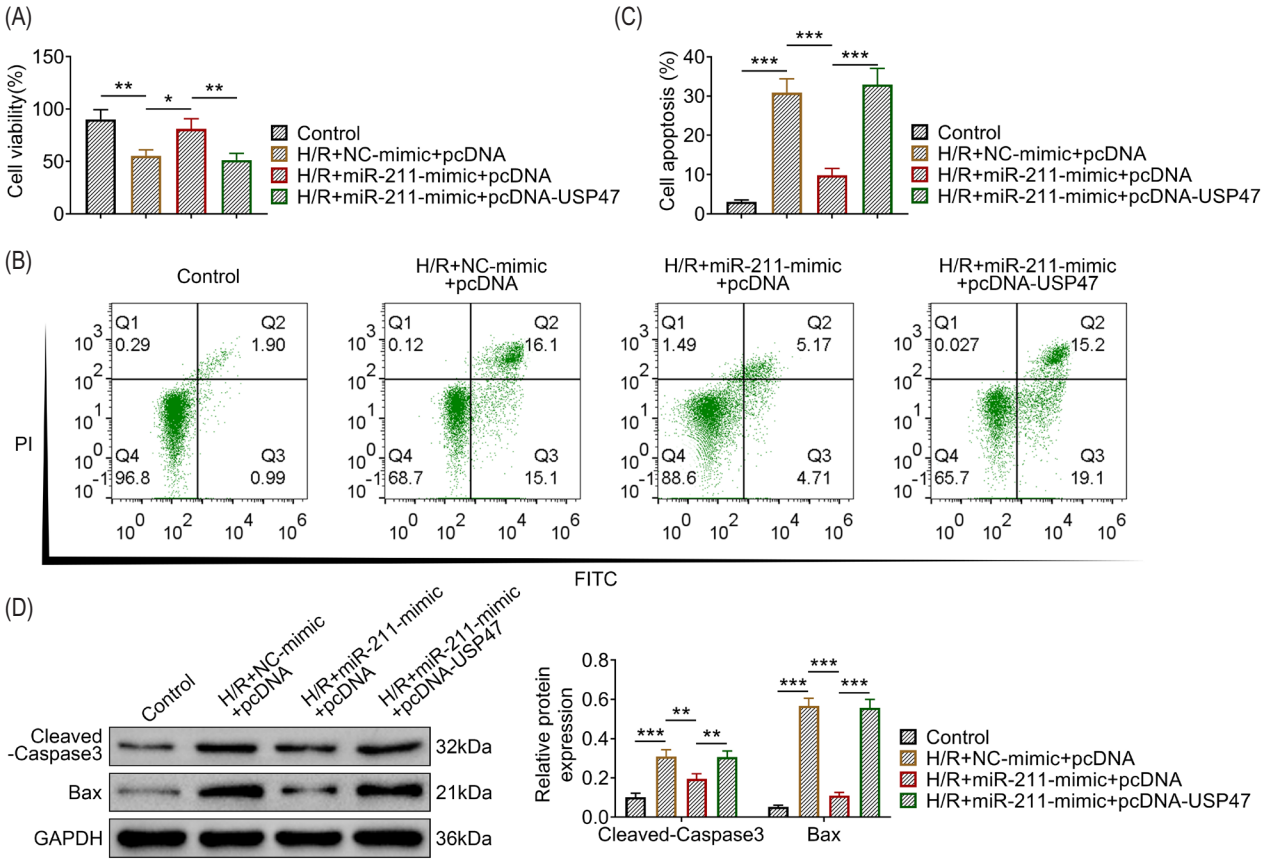

Figure 4. MiR-211 enhanced the protective effect of remifentanil on H/R injury by negatively regulating USP47. (A) Cell viability in indicated groups as detected by CCK-8 assay. (B and C) Cell apoptosis in indicated groups as detected by flow cytometry. (D) Cell apoptosis in indicated groups as detected by Western blot analysis. Data are shown as SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

in remifentanil-pretreated H/R injury cells. Inhibition of miR-211 counteracted the effect of ligustrazine on the improvement of cell apoptosis (Figure 5C). Therefore, ligustrazine enhanced the protective effect of remifentanil against H/R injury through miR-211/USP47.

Discussion

Myocardial infarction is the occlusion of a coronary artery and interruption in blood flow, resulting in localized necrosis of part of the myocardium because of severe and persistent ischemia. Its treatment mainly includes thrombolysis, surgery, etc. Reperfusion can cause damage to the myocardium, including inflammation, apoptosis, oxidative stress injury, and calcium overload (Kuramoto *et al.*, 2021). Therefore, it is necessary to further elucidate the mechanisms and develop effective drugs for the treatment of MI. During the rescue and treatment of ischemic disease, medical scientists have come to realize that the main factor causing tissue damage is not ischemia itself but excessive free radicals attacking the cells in this part of the tissue, which is I/R injury, where the blood supply has been restored. Treatment of cerebral I/R injury is mainly through cerebral protective drugs, such as edaravone, butylphthalide, etc., which reduce damage to the brain caused by calcium overload and excessive oxygen-free radicals. Remifentanil is a commonly used drug in the treatment of MI, but its therapeutic effects require further research (Karacsonyi *et al.*, 2021). Previous studies have confirmed that ligustrazine can improve cerebral circulation. Ligustrazine has a protective effect against cerebral I/R-induced brain injury, alleviates neuronal and microvascular endothelial cell damage and improves neurological signs, provides short-term improvement in complete cerebral ischemia, and to some extent promotes cerebral resuscitation.

MiRNAs are recognized as key regulators in the occurrence and progression of several cardiovascular diseases, such as cardiac hypertrophy, coronary atherosclerosis, and acute MI (Chen *et al.*, 2020). Therefore, many studies have suggested that miRNAs play a key role in myocardial functioning (e.g., contraction and morphogenesis) and are involved in the progression of myocardial I/R injury (Song *et al.*, 2021). The expression of miR-211 was decreased during cerebral I/R, and it could protect cerebral I/R injury by inhibiting apoptosis (Pan *et al.*, 2021).

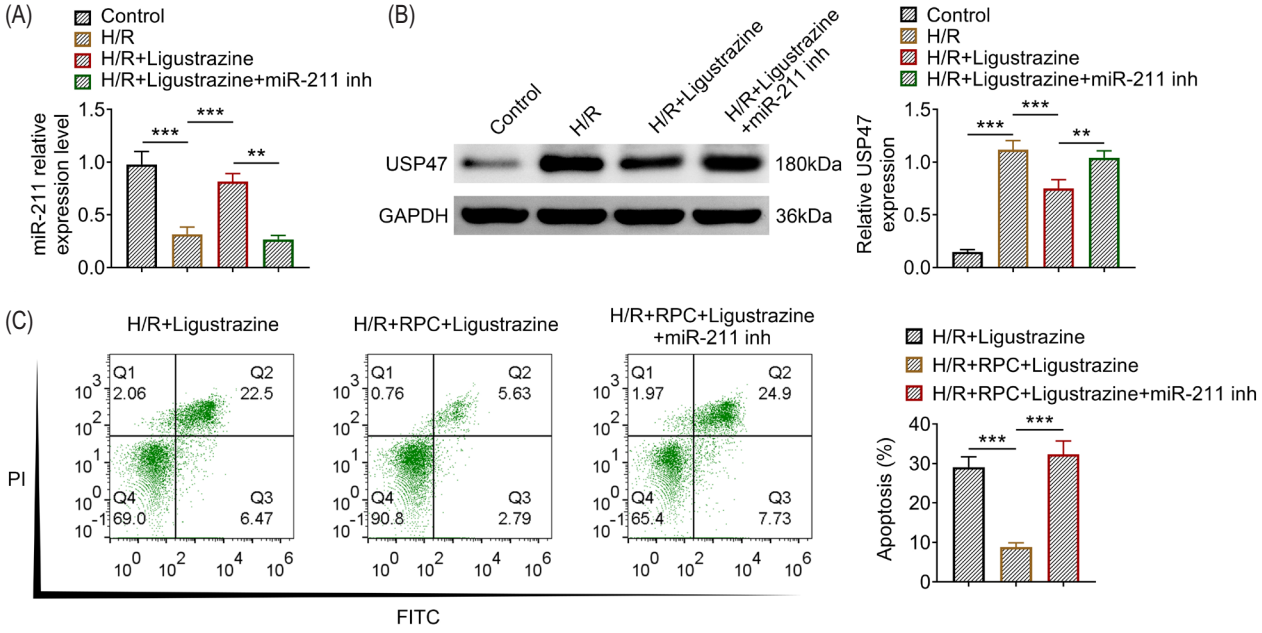

Figure 5. Ligustrazine enhanced the protective efffect of remifentanil against H/R injury through miR-211/USP47. (A) Expression of miR-211 in indicated groups as detected by RT-qPCR. (B) Expression of USP47 in indicated groups as detected by Western blot analysis. (C) Cell apoptosis in indicated groups as detected by flow cytometry. Data are shown as SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

Low expression and up-regulation of miR-211 during renal I/R inhibited H/R-induced apoptosis and increased cell viability. As shown in CCK-8 and FCM assays, miR-211 enhanced the protective effect of remifentanil against H/R injury through USP47.

In this study, a novel function of miR-211 was identified in the progression of MI. Indeed, the possible roles of miR-211 in different cellular processes are revealed broadly. Knockdown of nuclear-enriched abundant transcript 1 (NEAT1), a long non-coding RNA (lncRNA), could relieve inflammatory response to spinal cord injury through targeting miR-211. In addition, the neuroprotective effects of miRNA-211 on chronic stress-induced neuronal apoptosis have been reported. Hippocampal miR-211 could mediate neurogenesis and depression-like behaviors in mice (Li *et al.*, 2021). MiR-211 also regulates TAB1 expression and inhibits the nuclear factor *kappa B* (NF-κB) pathway in lipopolysaccharide-induced endometritis. These studies, together with these findings, confirmed that miR-211 could act as a promising target for the treatment of multiple diseases.

USP47, a member of the ubiquitin-specific protease (USP) family, was reported to be increased in rat H9C2 cardiomyocytes in an I/R injury model. USP47 knockdown reduced I/R injury-induced apoptosis (Thogersen *et al.*, 2021). Interestingly, in this study, miR-211 enhanced the protective effect of remifentanil on myocardial I/R injury by suppressing the expression of USP47. Targeting USP47 could overcome resistance to tyrosine kinase inhibitor and further eradicate stem cells in leukemia (Thogersen *et al.*, 2021). According to these data, USP47 could serve as a promising target for MI treatment.

To illustrate further the mechanism of ligustrazine on remifentanil-induced H/R injury, cell viability was assayed in ligustrazine-treated cells that transfected with miR-211 inhibitor. Interestingly, it is found that ligustrazine enhanced the protective effect of remifentanil against H/R injury through miR-211/USP47. Studies have demonstrated that ligustrazine could increase the expression of miR-211, although this mechanism requires further investigation. One of the limitations of this study was that molecular mechanism was not studied and verified; this requires to be confirmed in the future studies.

Conclusion

Ligustrazine enhances the protective effect of remifentanil on myocardial I/R injury by regulating miR-211/USP47 pathway. Therefore, ligustrazine could serve as a promising drug for the treatment of myocardial I/R injury.

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