miR-320-3p is involved in morphine pre-conditioning to protect rat cardiomyocytes from ischemia/reperfusion injury through targeting Akt3

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Abstract. Morphine pre-conditioning (MPC) can significantly reduce myocardial ischemic injury and inhibit cardiomyocyte apoptosis, but the underlying mechanism still remains unclear. The aim of the present study was to investigate the protective mechanism of MPC in myocardial hypoxia/reoxygenation (H/R) injury at the microRNA (miR) level. H9c2 cells were used as a model of H/R and subjected to morphine pre-treatment. The protective effects of MPC on H/R injury in cardiomyocytes were evaluated using MTT and colorimetric assay, as well as flow cytometry. In addition, reverse transcription-quantitative PCR, western blotting and dual-luciferase reporter assay experiments were performed to determine the relationship between MPC, miR-320-3p and Akt3, and their effects on H/R injury. The present study demonstrated that MPC enhanced cell activity, decreased LDH content, and reduced apoptosis in rat cardiomyocytes, suggesting that MPC could protect these cells from H/R injury. Moreover, MPC partially reversed the increase in miR-320-3p expression and the decrease in Akt3 levels caused by H/R injury. Inhibition of miR-320-3p expression also attenuated the effects of H/R on cardiomyocyte activity, LDH content and apoptosis. Furthermore, Akt3 was predicted to be a target gene of miR-320-3p, and overexpression of miR-320-3p inhibited the expression of Akt3, blocking the protective effects of MPC on the cells. The current findings revealed that MPC could protect cardiomyocytes from H/R damage through targeting miR-320-3p to regulate the PI3K/Akt3 signaling pathway.

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Introduction

Myocardial ischemia/reperfusion injury (MI/RI) refers to rapid tissue damage caused by the recovery of blood flow from myocardial cells following ischemia (1). MI/RI is a common complication during the perioperative period of cardiac and macrovascular surgeries that can lead to the aggravation of myocardial damage and seriously affect the recovery of postoperative cardiac function (2). Cardiomyocyte hypoxia/reoxygenation (H/R) injury can be used to mimic myocardial ischemia/reperfusion *in vitro* (3,4). Morphine is a widely used analgesic in cardiac and macrovascular anesthesia (5,6). Compared with ischemic pre-conditioning, morphine could be conveniently and easily administered and cause less trauma to patients (7). Previous studies demonstrated that morphine pre-conditioning (MPC) significantly reduced myocardial ischemic injury and inhibited cardiomyocyte apoptosis, although the mechanism remains unclear (8,9).

MicroRNAs (miRNAs/miRs) are a class of endogenous, single-stranded, non-coding RNAs that regulate multiple biological processes such as cell proliferation, differentiation and apoptosis (10). Cardiomyocyte apoptosis is an important characteristic of MI/RI (11). Accumulating evidence indicates that miRNAs can regulate apoptosis of cardiomyocytes through their target genes and downstream signaling pathways (12). For example, Dong *et al* (13) suggested that miR-21 could inhibit ischemia-induced apoptosis by studying acute myocardial infarction in rats. Cheng *et al* (14) demonstrated that miR-21 also protected cardiomyocytes from hydrogen peroxide-induced damage by regulating programmed cell death 4. Moreover, miR-320 was also implicated in the regulation of myocardial ischemia/reperfusion injury, and miR-320 upregulation could promote cardiomyocyte apoptosis (15). Additionally, miR-320 has multiple functions in different environments (16), and its expression level is closely related to tumor migration and invasion. Indeed, overexpression of miR-320 is associated with high risk of metastasis and poor prognosis (17).

Previous studies demonstrated that 5'-adenosine monophosphate-activated protein kinase (AMPK) could ameliorate myocardial ischemia through the regulation of oxidative stress (18,19), autophagy (20,21) and apoptosis (22)

in cardiomyocytes. Moreover, AMPK exerts its protective effects with other molecules that may have crosstalk with each other against myocardial ischemia, for example, mesenchymal stem cell (MSC)-derived exosomes could reduce MI/RI by inducing cardiomyocyte autophagy via AMPK/mTOR and Akt/mTOR pathways (21,23,24). Sun *et al* (25) demonstrated that dexmedetomidine protected mice against MI/RI by activating the AMPK/phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt/endothelial nitric oxide synthase pathway. PI3K/Akt signaling is an important signaling pathway associated with many diseases, including cancer and neurological diseases (26). Akt kinase consists of three subtypes, Aktl, Akt2 and Akt3, which are the key factors downstream of the PI3K signaling pathway that regulate cell proliferation, apoptosis and metastasis (27). Previous studies indicated that activation of the PI3K-Akt signaling pathway protected the heart from reperfusion injury (28). However, whether morphine pre-conditioning participates in myocardial H/R injury through the regulation of miR-320-3p and the PI3K/Akt signaling pathway is not fully understood. Therefore, the current study examined the myocardial protection mechanism of MPC following at the miRNA level, and aimed to elucidate the relationship between MPC, miR-320-3p and the PI3K/Akt signaling pathway in the regulation of H/R injury of cardiomyocytes.

Materials and methods

Cell culture. H9c2 cells (cat. no. CRL-1446) were obtained from the American Type Culture Collection and divided into four groups: i) Control, ii) MPC, iii) H/R, and iv) MPC+H/R. Cells from the control group were cultured in DMEM/F-12 containing 10% FBS, and 1% penicillin/streptomycin at 37˚C with 5% CO_2 (all from Thermo Fisher Scientific, Inc.). After one day of culture, the original medium was discarded, and the cells were washed once or twice with PBS, then resuspended in 5 ml fresh DMEM/F-12 medium and returned to the CO_2 incubator. The cells were passaged when grown to 80 to 90% confluence. In the MPC group, cells were cultured normally following a 10-min treatment with 1 μM morphine (cat. no. 121206-2; Northeast Pharmaceutical Group Co., Ltd.). In the H/R group, DMEM/F12 medium containing 10% serum was replaced by anoxic solution (i.e., glucose-free and serum-free medium containing 2.3 mM $CaCl_2$, 5.6 mM KCl, 154 mM NaCl, 5 mM Hepes, and 3.6 mM $NaHCO_3$; pH 7.4). The culture plate was maintained in anoxic chamber for 5 h at 37˚C, and then reoxygenation for 1 h in DMEM/F12 medium containing 10% serum under a humidified atmosphere (95% air and 5% CO_2) at 37˚C. For the MPC + H/R group, the cells were treated with 1 μM morphine in serum-free DMEM/F12 for 10 min. Following the treatment, serum-free DMEM/F12 was replaced by DMEM/F12 medium containing 10% serum and the MPC pre-treatment was completed after 30 min of normal culture. Cells then received the same treatment as the H/R group.

Cell transfection. Cells were cultured for 12-16 h to 60-70% confluence, and then transfected with Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. miR-320-3p mimic (miR10000903-1-5; 5'-AAA AGCUGGGUUGAGAGGGCGA-3'), mimic negative control (miRB160401025525-2-1; 5'-UUUGUACUACACAAAAGU ACUG-3'), and miR-320-3p inhibitor (miR20000903-1-5; 5'-UCGCCCUCUCAACCCGCUUUU-3'), and inhibitor negative control (miR2N0000001-1-5; 5'-CAGUACUUUUGUGUA GUACAAA-3') were purchased from Guangzhou RiboBio Co., Ltd. Briefly, when cells were at 60 to 70% confluence, the transfection was performed using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.). miR-320-3p mimic, inhibitor, corresponding control and Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Inc.) were diluted in Opti-MEM medium (Thermo Fisher Scientific, Inc.). Then, the diluted RNAs were added into the diluted Lipofectamine® reagent and incubated for 10 min at room temperature. The mixture containing the RNA was added to the cells. Following an 8-h incubation in the presence of transfection reagent, the medium was replaced, and the cells incubated for 2-4 days at 37˚C prior to subsequent experimentation.

Bioinformatics prediction. TargetScan7.2. (http://www.targetscan.org) was used for the biological prediction of miR-320-3p target genes.

Dual luciferase activity assay. The sequence of Akt3-3'-UTR was cloned into the pmirGLO luciferase vector (cat. no. E1330; Promega Corp.). H9c2 cells were transfected with miR-320-3p mimic to detect the binding of Akt3 and miR-320-3p. Cells transfected with miR-320-3p mimic negative control were used as the blank group. The cell transfection was performed by transfected with Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Luciferase activity was determined using the Double-Luciferase Reporter Assay kit (cat. no. FR201-01; TransGen Biotech) according to the manufacturer's protocol. Briefly, the cell culture medium was removed, and the cells were carefully washed twice in PBS, then added to 20 μl 1X Cell Lysis Buffer from the kit. The cells were lysed for 10 min at room temperature and centrifuged at 12,000 x g for 10 min at 4˚C to collect supernatant for later use. After centrifugation, 100 μl Luciferase Reaction Reagent was added to the tube at room temperature, and 20 μl cell lysate was carefully pipetted into the tube and mixed gently to measure the activity of the firefly luciferase reporter gene in a luminometer (SpectraMaxL, Molecular Devices, LLC). Finally, 100 μl Luciferase Reaction Reagent II was added to the above reaction tube at room temperature, vortexed, and the activity of the *Renilla* luciferase reporter gene was detected using a SpectraMaxL luminometer (Molecular Devices, LLC).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA extraction from lysed H9c2 cells was carried out at 4˚C using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), and all the consumables and reagents used in the extraction process were subjected to DEPC treatment. The RT of miRNA and mRNA was conducted using TaqMan MicroRNA Reverse Transcription kit (cat. no. 4366597; Thermo Fisher Scientific, Inc.) and TaqMan Reverse Transcription Reagents (cat. no. N8080234; Thermo Fisher Scientific, Inc.). GAPDH served as internal control for mRNA, while U6 served as internal control for miRNA quantification. The primer sequences

were as follows: miR-320-3p-forward (F), 5'-TAAGTGCTT CCATGTTTTGGTGA-3'; miR-320-3p-reverse (R), 5'-GAA CATGTCTGCGTATCTCAGACTTC-3'; Akt3-F, 5'-TCCCCC GAACACTCTCTTCA-3'; Akt3-R, 5'-CCCTCCACCAAG GCGTTTAT-3'; GAPDH-F, 5'-ATGACTCTACCCACGGCA AG-3'; GAPDH-R, 5'-GGAAGATGGTGATGGGTTTC-3'; U6-F, 5'-CTCGCTTCGGCAGCACA-3'; U6-R, 5'-AACGCT TCACGAATTTGCGT-3'. RT-qPCR was performed using the ABI7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The thermocycling conditions were set as follows: Initial denaturation at 95˚C for 10 min, followed by 45 cycles at 95˚C for 30 sec, 59˚C for 1 min, and finally at 60˚C for 1 min, and preservation at 4˚C. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (29).

MTT assay. Cell viability was detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (cat. no. M6494; Thermo Fisher Scientific, Inc.). H9c2 cells were seeded in 96-well plates at a density of $5x10^4$/ml and incubated for 24, 48 and 72 h at 37˚C. A volume of 10 μl MTT reagent was added into each well and cells were then cultured for an additional 4 h at 37˚C. After removing the MTT, 100 μl DMSO was added. Finally, the optical density was measured at 490 nm using a microplate reader (cat. no. 24072800; Thermo Fisher Scientific, Inc.). Cells were seeded in triplicate wells and the experiment was repeated three times.

Lactate dehydrogenase (LDH) content determination. LDH content was measured using the Cytotoxicity LDH Assay Kit-WST (cat. no. CK12; Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. Briefly, the cell suspension was diluted in 50 μl medium, added to the 96-well plate with 50 μl medium and incubated in a CO_2 incubator at 37˚C for 24 h. A volume of 10 μl Lysis Buffer was added into the control well and the reaction was incubated at 37˚C with 5% CO_2 for 30 min. After adding 100 μl Working Solution into each well, the cells were incubated in the dark at room temperature for 30 min. Next, 50 μl Stop Solution was added to terminate the reaction, and the absorbance at 490 nm was determined immediately using a microplate reader (cat. no. 24072800; Thermo Fisher Scientific, Inc.).

Flow cytometry. A total of $1x10^6$ H9c2 cells were harvested then centrifuged at 1,200 x g for 1 min at 4˚C, then resuspended in 300 μl pre-chilled PBS containing 10% calf serum. Cells were then fixed in 700 μl absolute ethanol at -20˚C for >24 h, and centrifuged at 140 x g for 30 sec at 4˚C. Fixed cells were resuspended in 500 μl cold PBS, and centrifuged at 1,200 x g for 1 min at 4˚C. After discarding the supernatant, the pelleted cells were suspended in 480 μl cold PBS and 10 μl of 0.1 mg/ml RNase A, and allowed to stand at 37˚C for 30 min. Annexin V-FITC and propidium iodide (PI) staining solution (5 μl each; cat. no. APOAF; Sigma-Aldrich; Merck KGaA) were added to the cell suspension for 15 min at room temperature in the dark. After the incubation, cells were filtered once through a 400-mesh sieve, and apoptosis was detected using a BD FACS Canto™ flow cytometer. Analysis was carried out using FlowJo version 10.0 (FlowJo, LLC). The lower left quadrant (FITC-H^-/PI^-) represents healthy living cells, while the lower right quadrant (FITC-H^+/PI^-) represents early apoptotic cells. Late apoptotic cells are gated in the upper right quadrant (FITC-H^+/PI^+) and necrotic cells are found in the upper left quadrant (FITC-H^-/PI^+).

Western blot analysis. Total protein was extracted from H9c2 cells using RIPA buffer (cat. no. 89901; Thermo Fisher Scientific, Inc.) containing protease inhibitor and phosphatase inhibitor. The protein concentrations were measured using Pierce Rapid Gold BCA Protein Assay kit (cat. no. A53227; Thermo Fisher Scientific, Inc.). 30 μg protein was separated by SDS-PAGE on 10% gels, then transferred to PVDF membranes (cat. no. HVLP04700; EMD Millipore). The films were fully rinsed in TBS, and then transferred to a dish containing 5% skim milk solution and blocked at 37˚C for 1 h, then shaken for 2 h at room temperature on a decolorizing shaker. The membranes were then incubated with rabbit primary antibody against Akt3 (72 kDa; 1:2,000; cat. no. ab152157; Abcam) or mouse anti-GAPDH (36 kDa; 1:20,000; cat. no. ab8245; Abcam) overnight at 4˚C. The membrane was then incubated with an HRP-conjugated secondary antibody (1:5,000; cat. nos. ab205718 and ab205719; Abcam) for 2 h at room temperature. Finally, SignalFire electrochemiluminescence reagent (cat. no. 6883; Cell Signaling Technology, Inc.) was used for visualization. The ImageJ version 5.0 software (Bio-Rad Laboratories, Inc.) for quantification. GAPDH was used as the reference protein.

Statistical analysis. Data are presented as the mean ± SD. Multi-group comparisons were conducted using one-way ANOVA, followed by Tukey's post hoc test. Statistical analysis was carried out using GraphPad Prism 7.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

MPC protects rat cardiomyocytes from H/R damage. To investigate the effects of MPC on reperfusion injury in rat cardiomyocytes, H9c2 cells were divided into Control, MPC, H/R, MPC+H/R groups. An MTT assay demonstrated that the viability of cells in the H/R group was reduced, compared with the control. However, cell viability in the MPC+H/R group was significantly higher than that of the H/R group ($P<0.05$; Fig. 1A).

LDH levels indicate the number of dead and damaged cells. In the H/R group, the LDH content was increased, compared with the control. However, the LDH content in the MPC+H/R group was significantly lower than that in the H/R group ($P<0.05$; Fig. 1B). In addition, the levels of apoptosis were also examined in each of the four groups of cells. The frequency of apoptotic cells in the H/R group was increased, compared with control, but significantly decreased in the MPC+H/R group, compared with the H/R group ($P<0.05$; Fig. 1C).

MPC regulates the expression of miR-320-3p and Akt3. To further investigate the molecular mechanism of MPC in rat cardiomyocytes, RT-qPCR was performed to detect the expression of miR-320-3p. MPC treatment alone did not affect the expression of miR-320-3p. The expression of miR-320-3p in the MPC+H/R group was significantly lower than that in the H/R group ($P<0.05$; Fig. 2A). Moreover, the mRNA

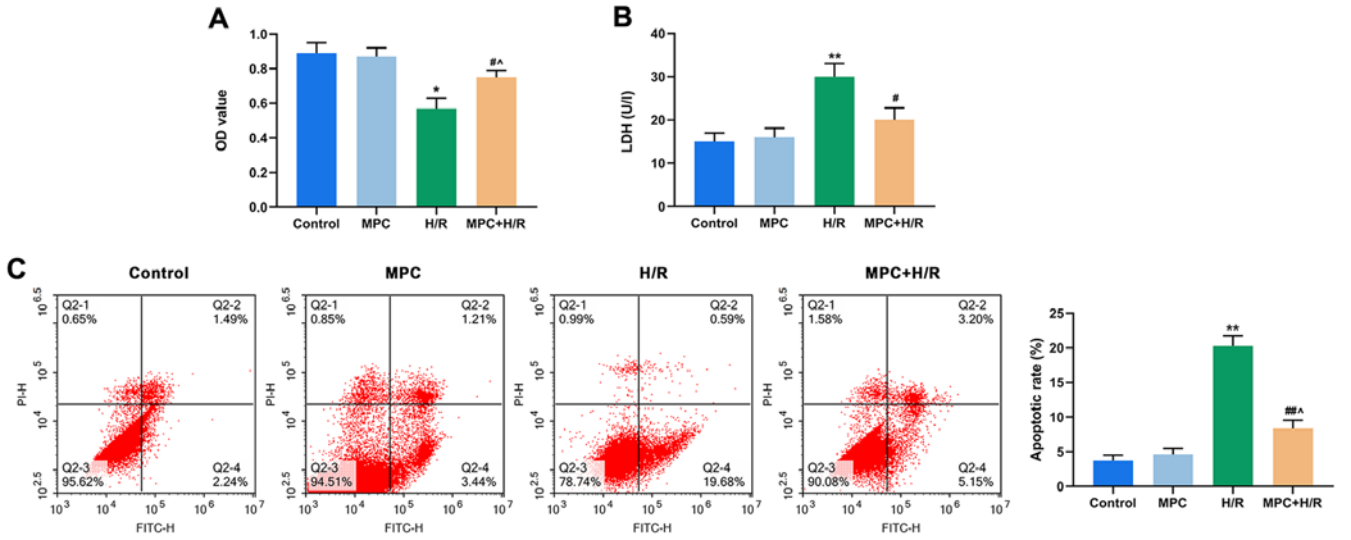

Figure 1. MPC protects rat cardiomyocytes from H/R damage. (A) MTT assay was used to assess the viability of H9c2 cells in the control, MPC, H/R and MPC+H/R groups. (B) Colorimetric assay was used to measure LDH levels in the control, MPC, H/R and MPC+H/R groups. (C) Flow cytometry was used to detect apoptosis in the control, MPC, H/R and MPC+H/R groups. *P<0.05 and **P<0.001 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.001 vs. H/R; $^{\wedge}$P<0.05 vs. MPC group. MPC, morphine pre-conditioning; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; OD, optical density; PI, propidium iodide; FITC, fluorescein isothiocyanate.

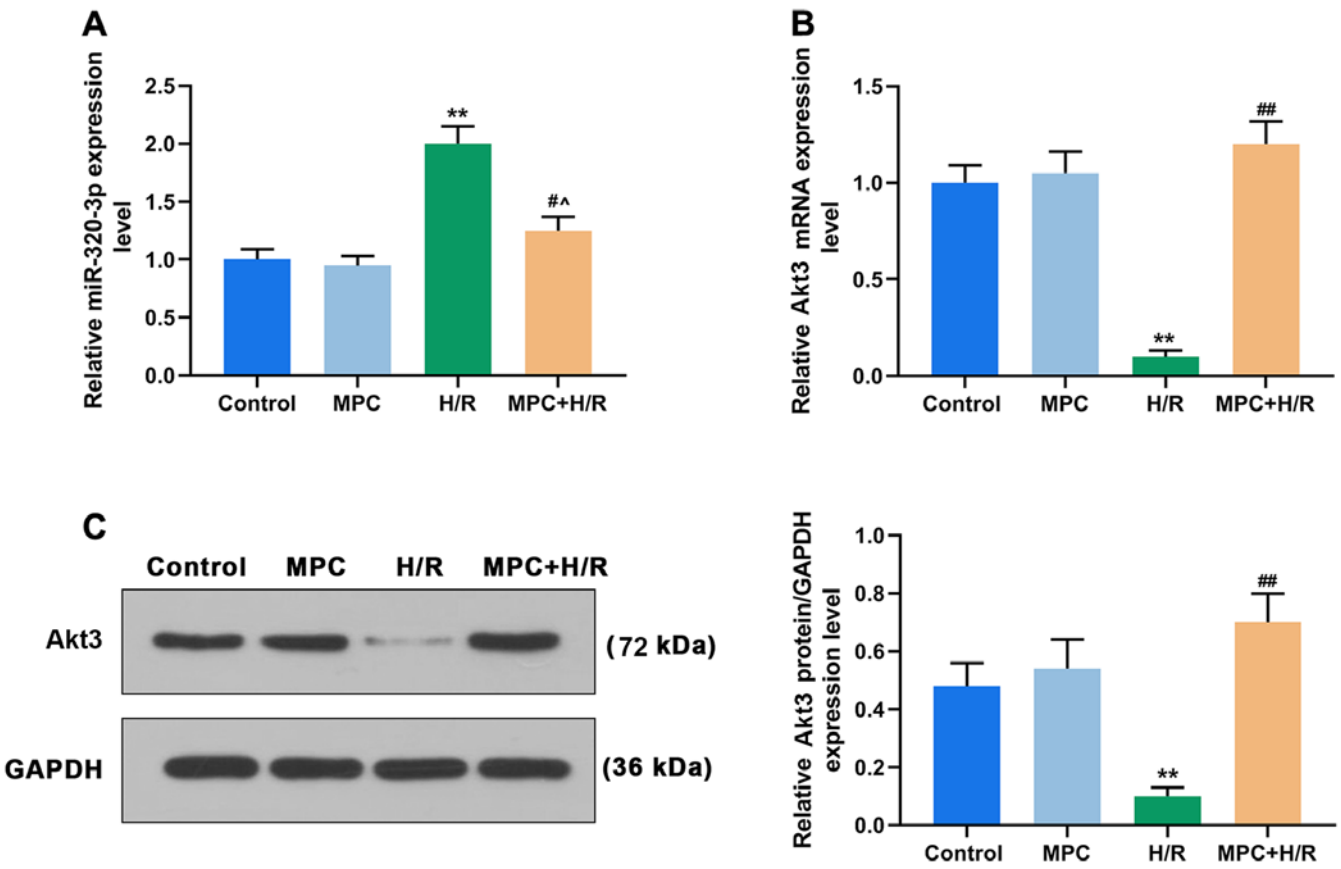

Figure 2. MPC regulates the expression of miR-320-3p and Akt3. (A) RT-qPCR was used to detect the miR-320-3p expression in the control, MPC, H/R and MPC+H/R groups. (B and C) RT-qPCR and western blot analysis were used to quantify Akt3 expression in the control, MPC, H/R and MPC+H/R groups. **P<0.001 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.001 vs. H/R; $^{\wedge}$P<0.05 vs. MPC. MPC, morphine pre-conditioning; H/R, hypoxia/reoxygenation; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

and protein expression levels of Akt3 were also examined. MPC treatment alone did not affect the expression of Akt3. However, the expression of Akt3 in the H/R group was reduced, compared with the control. Additionally, expression of Akt3 in the MPC+H/R group was significantly higher than that in the H/R group (P<0.05; Fig. 2B and C).

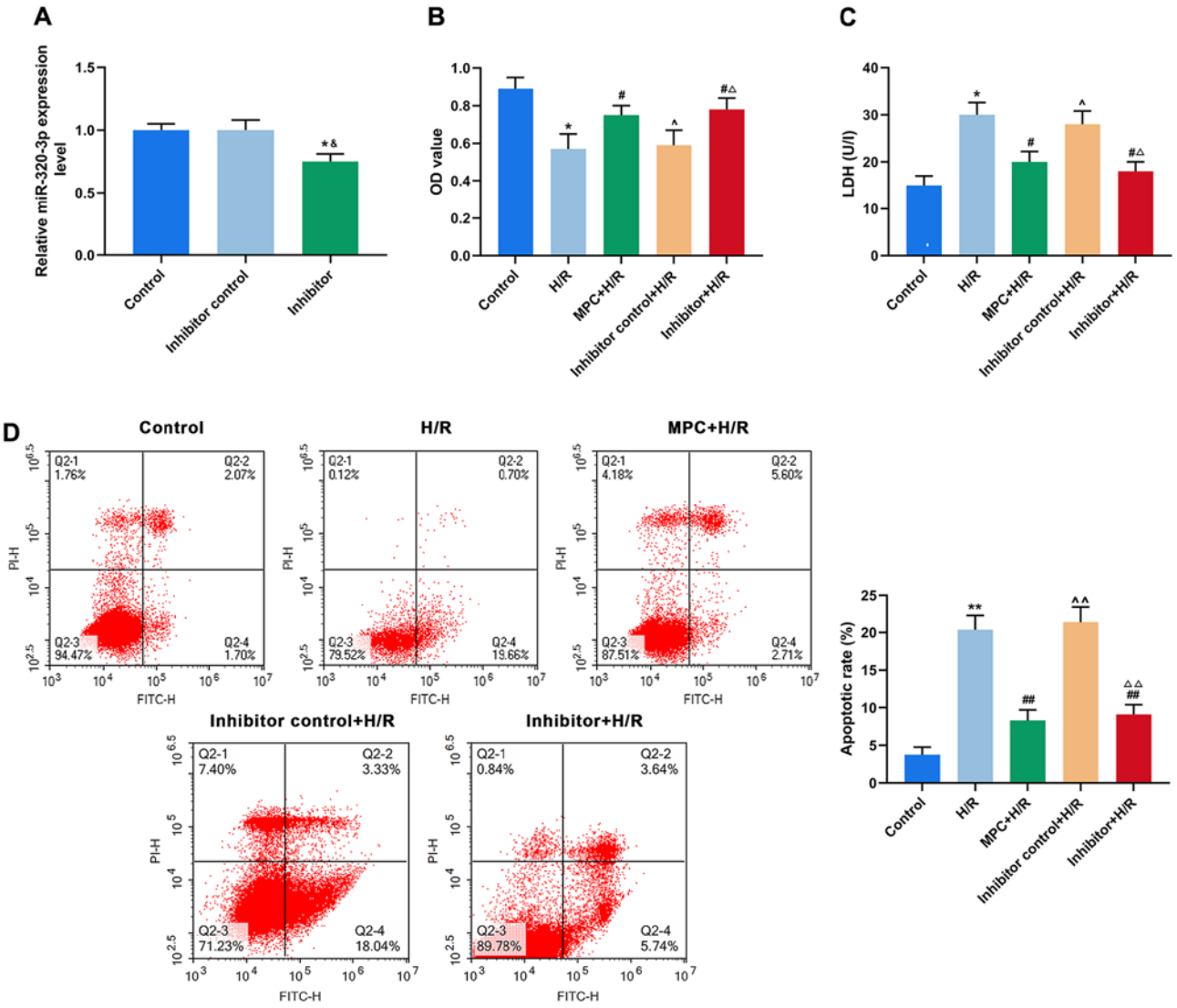

Figure 3. Downregulation of miR-320-3p attenuates H/R damage to cells. (A) Reverse transcription-quantitative PCR was used to determine the effect of miR-320-3p inhibitor transfection. (B) H9c2 cell viability in the control, H/R, MPC+H/R, inhibitor control+H/R and inhibitor+H/R groups using an MTT assay. (C) LDH content was measured in H9c2 cells in the control, H/R, MPC+H/R, inhibitor control+H/R and inhibitor+H/R groups using a colorimetric assay. (D) Apoptosis of H9c2 cells in the control, H/R, MPC+H/R, inhibitor control+H/R and inhibitor+H/R groups was detected using flow cytometry. $^{\&}$P<0.05 vs. Inhibitor control; *P<0.05 and **P<0.001 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.001 vs. H/R; $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.001 vs. MPC+H/R; $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.001 vs. Inhibitor control+H/R. MPC, morphine pre-conditioning; H/R, hypoxia/reoxygenation; miR, microRNA; LDH, lactate dehydrogenase; OD, optical density; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Downregulation of miR-320-3p reduces H/R damage to H9c2 cells. To further understand the biological role of miR-320-3p in reperfusion injury of rat cardiomyocytes, H9c2 cells were transfected with an miR-320-3p inhibitor (Fig. 3A). In the H/R group, an MTT assay demonstrated that the viability of cells transfected with the inhibitor (Inhibitor+H/R) was significantly higher than untransfected cells and was similar to the MPC+H/R group (P<0.05; Fig. 3B). Furthermore, the LDH content in the Inhibitor+H/R group was lower than that in the unstranfected H/R group and was similar to the MPC+H/R group (P<0.05; Fig. 3C). As indicated by flow cytometry, the rates of apoptosis of in the Inhibitor+H/R group was similar to that of the MPC+H/R group. However, this was significantly lower than that of the H/R group (P<0.001; Fig. 3D).

Upregulation of miR-320-3p targeting Akt3 inhibits the protective effect of MPC on H9c2 cells. Using TargetScan v7.2 bioinformatics analysis and luciferase assay, Akt3 was identified as the direct target of miR-320-3p (Fig. 4A and B). Both at the mRNA and protein levels, the expression of Akt3 was significantly reduced in the untransfected cells of the H/R group, compared with control. However, Akt3 expression in the H/R-treated cells transfected with the inhibitor was significantly higher, compared to the untransfected cells (P<0.001; Fig. 4C and D).

To better understand the effects of MPC, miR-320-3p and Akt3 on reperfusion injury of rat cardiomyocytes, H9c2 cells were transfected with an miR-320-3p mimic (Fig. 5A). The expression of Akt3 in transfected cells of the MPC+H/R group (Mimic+MPC+H/R group) was significantly lower than that in the untransfected MPC+H/R group (P<0.05; Fig. 5B and C). Moreover, an MTT assay suggested that cell viability in the Mimic+MPC+H/R group was significantly lower than that in the MPC+H/R group (P<0.05; Fig. 5D). To further assess the

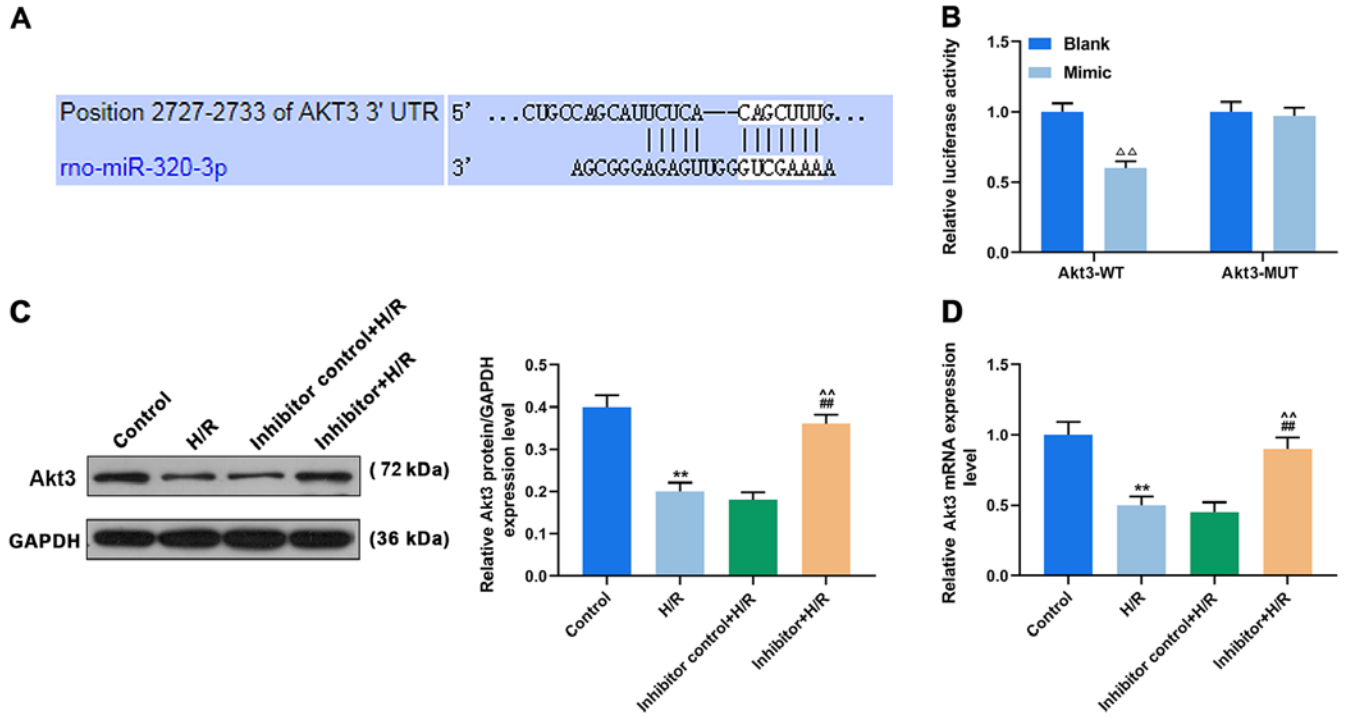

Figure 4. miR-320-3p directly regulates Akt3. (A) TargetScan v7.2 and (B) dual luciferase assay were used to identify the binding sites of miR-320-3p. (C) Reverse transcription-quantitative PCR and (D) western blot analysis were used to quantify Akt3 expression in the control, H/R, inhibitor control+H/R and inhibitor+H/R groups. $^{\Delta\Delta}$P<0.001 vs. blank group, **P<0.001 vs. control; $^{\#\#}$P<0.001 vs. H/R; $^{\wedge\wedge}$P<0.001 vs. Inhibitor control+H/R. H/R, hypoxia/reoxygenation; miR, microRNA; WT, wild-type; MUT, mutant.

biological function of miR-320-3p in H9c2 cells, LDH content was measured by colorimetry in each group. The LDH content of the Mimic+MPC+H/R group was significantly higher than that of the MPC+H/R group (P<0.05; Fig. 5E). In addition, the rates of apoptosis in the Mimic+MPC+H/R group was significantly increased, compared with the MPC+H/R group (P<0.05; Fig. 5F).

Discussion

Myocardial ischemia/reperfusion injury (MI/RI) is a pathophysiological process that occurs after revascularization in patients with ischemic heart disease, such as myocardial infarction (30,31). Morphine is a non-selective opioid receptor agonist widely used as an anesthetic and in the treatment and myocardial infarction (32). Previous research has demonstrated that morphine has an early pre-conditioning effect on cardiomyocytes that is triggered by activation of opioid receptors (33). In the present study, the H9c2 cardiomyocyte hypoxia/reoxygenation injury model was established as previously described (34) but modified to incorporate 1 μM morphine as pre-treatment (35). In the present study, MPC significantly enhanced the viability of cardiomyocytes, reduced the levels of LDH resulting from H/R injury, and inhibited the apoptosis of cardiomyocytes, indicating that MPC alleviates H/R injury and plays a role in myocardial protection.

miRNAs are abundantly expressed in cardiomyocytes and participate in pathophysiological processes, such as cardiomyocyte apoptosis, myocardial remodeling and heart failure (10). Several previous studies suggest that MI/RI could be alleviated through the upregulation or downregulation of the expression of target miRNAs, pointing to a new therapeutic target for clinical myocardial protection (36,37). Yang *et al* (38) demonstrated that miRNA-22 can reduce apoptosis in MI/RI in a rat model by inhibiting CREB-binding protein expression and its downstream signaling pathway. Li *et al* (39) suggested that miRNA-145 expression was downregulated in an MI/RI model, whereas upregulation of miRNA-145 inhibited hydrogen peroxide-induced apoptosis, reactive oxygen species production, and activation of key signaling proteins in the mitochondrial apoptotic pathway. In addition, it has been reported that morphine can upregulate the expression of miR-133b-5p and protect cardiomyocytes from H/R injury, indicating that the biological function of morphine may be achieved by regulating miRNA expression (9).

Previous studies indicate that the regulation of multiple signaling pathways could improve myocardial injury. For example, melatonin protects the diabetic heart against ischemia-reperfusion injury through membrane receptor-dependent activation of cyclic GMP-dependent guanosine monophosphate protein kinase (40). The inhibition of miR-101a-3p alleviates H/R injury in H9c2 cells by regulating the JAK2/STAT3 pathway (41). Downregulation of miR-134 protects against myocardial H/R injury through targeting nitric oxide synthase 3 and regulating the PI3K/Akt pathway (42). However, whether myocardial protection by MPC involves the regulation of miR-320-3p and PI3K/Akt signaling pathways is still unclear.

The present findings demonstrated that miR-320-3p expression was upregulated and Akt3 expression was downregulated in cardiomyocytes after H/R injury, while MPC partially reversed the changes in miR-320-3p and Akt3

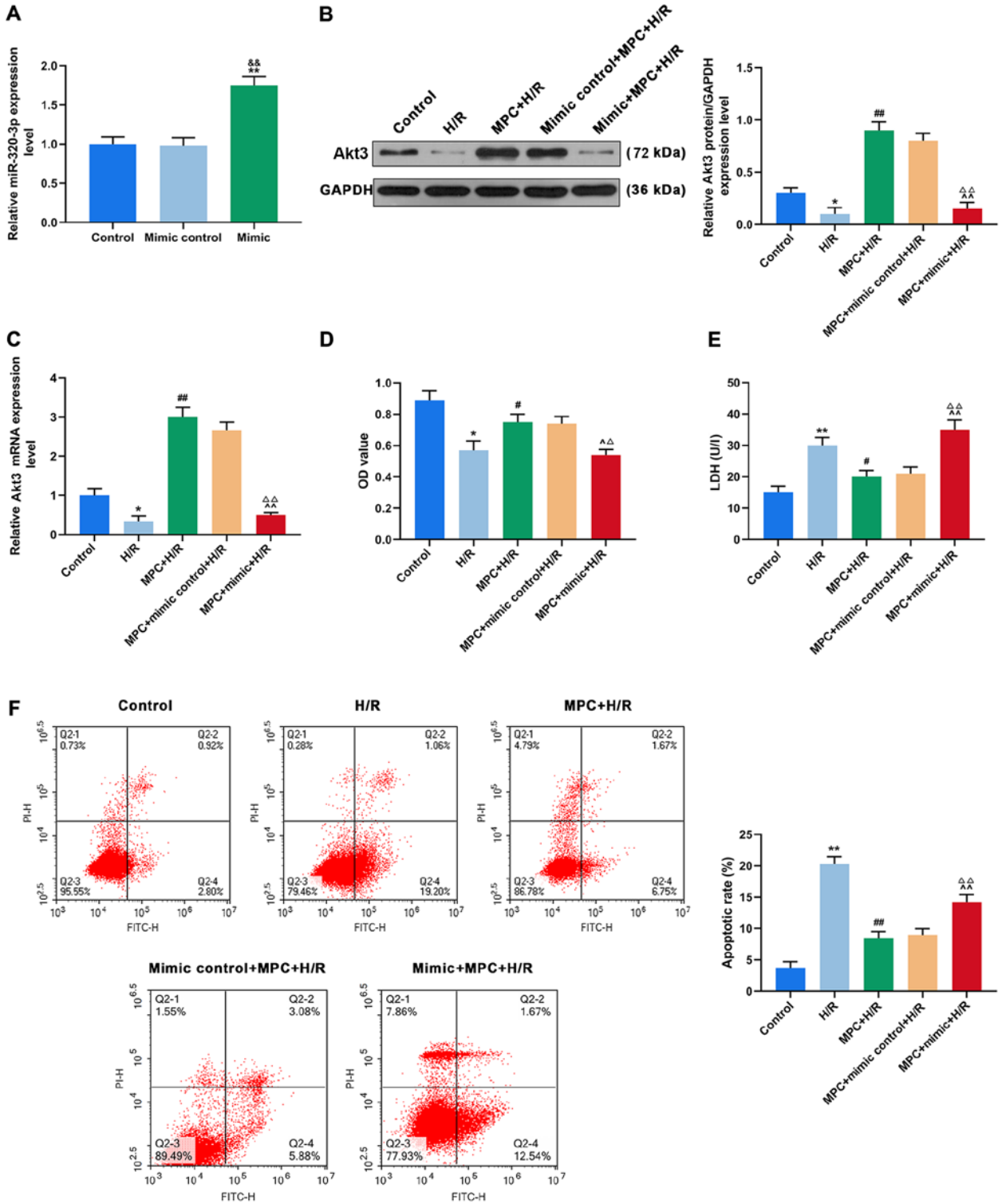

Figure 5. Upregulation of miR-320-3p targeting Akt3 inhibits the protective effect of MPC on H9c2 cells. (A) RT-qPCR was used to determine the effect of miR-320-3p mimic transfection. (B) RT-qPCR and (C) western blot analysis were used to detect Akt3 expression in the control, H/R, MPC+H/R, MPC+Mimic control+H/R and MPC+Mimic+H/R groups. (D) MTT assay was used to assess H9c2 cell viability in the control, H/R, MPC+H/R, MPC+Mimic control+H/R and MPC+Mimic+H/R groups. (E) LDH content was measured in the control, H/R, MPC+H/R, MPC+Mimic control+H/R and MPC+Mimic+H/R groups using a colorimetric assay. (F) Flow cytometry was used to detect the apoptosis of H9c2 cells in the control, H/R, MPC+H/R, MPC+Mimic control+H/R and MPC+Mimic+H/R groups. $^{\&\&}$P<0.001 vs. mimic control, *P<0.05 and **P<0.001 vs. control, $^{\#}$P<0.05 and $^{\#\#}$P<0.001 vs. H/R; $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.001 vs. MPC+H/R, $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.001 vs mimic control+MPC+H/R. MPC, morphine pre-conditioning; H/R, hypoxia/reoxygenation; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; LDH, lactate dehydrogenase; OD, optical density; PI, propidium iodide; FITC, fluorescein isothiocyanate.

expression induced by the injury. This indicated that MPC plays an important role in the protection of H9c2 cardiomyocytes by regulating miR-320-3p expression and the PI3K/Akt signaling pathway. Previous studies also demonstrated that miR-320 serves critical functions in many diseases such as glioma, ovarian cancer and osteosarcoma (16,43-46).

Another study reported that miR-320 targeted A-kinase interacting protein 1 to induce mitochondrial apoptosis, and contributed to MI/RI (47). In the present study, the role of miR-320-3p in myocardial ischemia/reperfusion was further investigated using transfection of an miR-320-3p inhibitor, which indicated that inhibition of miR-320-3p expression enhanced cell viability, reduced LDH content and inhibited apoptosis. Moreover, these results were consistent with a previous study that suggested that miR-320-3p is involved in MI/RI (47).

In line with previous studies, it was hypothesized that downregulation of miR-320-3p may be involved in mediating MPC to protect myocardial cells from H/R injury through the regulation of apoptosis. Through bioinformatics analysis and experimental validation, it was determined that miR-320-3p could directly interact with Akt3. The Akt3 gene encodes protein kinase B and plays an important role in the regulation of several physiological processes (48,49). Transgenic mice display significant cardiac hypertrophy, suggesting that one of the functions of Akt3 is to promote cell growth (28). The PI3K/Akt signaling pathway is also involved in cardiac hypertrophy, myocardial cell remodeling and the prevention of inflammation, thereby alleviating myocardial ischemia-reperfusion injury (50-52). The present study suggested that overexpression of miR-320-3p inhibited the expression Akt3 following MPC, and reduced the protective effect of MPC on H/R injury in cardiomyocytes. This indicated that MPC may protect cardiomyocytes from H/R injury through inhibiting miR-320-3p expression and the PI3K/Akt signaling pathway.

In conclusion, the present study demonstrated that MPC could significantly reduce H/R injury of H9c2 cardiomyocytes, and its mechanism of action may be related to miR-320-3p and the PI3K/Akt3 signaling pathway. The present findings provide a basis for further research on miRNA regulation mechanism of morphine pre-treatment.

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Availability of data and materials

The analyzed data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LC and SC made substantial contributions to conception and design. LC and SC acquired, analyzed and interpreted data. LC and SC drafted the article and critically revised the draft for important intellectual content. LC and SC agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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