



Research Paper

Sufentanil protects rat myocardium against ischemia-reperfusion injury through miR-1 and Bcl-2 pathway

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ABSTRACT

Sufentanil, a lipophilic opioid, has been clinically used to treat patients with ischemic heart disease. The effects of sufentanil on the miR-1 and Bcl-2 pathways were explored in the context of ischemic heart disease. The effects of sufentanil were assessed in rats using an ischemic reperfusion (IR)-induced myocardium injury rat model. Sixty rats (body weight of $200 \text{ g} \pm 30 \text{ g}$) of either sex were randomly assigned to three groups (a sham operation group (SO group), an ischemia/reperfusion group (IR group), and an IR group treated with sufentanil (SUF group). Experiments were conducted to assess CK, LDH, MDA, SOD, Ca^{2+} - Mg^{2+} -ATPase, and Na^{+} - K^{+} -ATPase. qPCR was used to detect miR-1 expression. Protein levels of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9 were measured by western blot analysis and the protein expression of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9 were measured. The effect of sufentanil on the growth of H9C2 myocardium cells was determined *in vitro*. Serum CK, LDH and MDA increased in the IR group, while the SOD, Ca^{2+} - Mg^{2+} -ATPase, and Na^{+} - K^{+} -ATPase levels of the IR group reduced. Expression levels of miR-1, Bax, Caspase 3, Caspase 8 and Caspase 9 increased in the IR group, but the expression level of Bcl-2 did not increase. In H2C9 cells, the abundance of Bcl-2 was increased at 0, 4 and 8 h, but the abundance of miR-1, Bax, Caspase 3, Caspase 8, and Caspase 9 between 8 and 12 h time points decreased. Over-expression of miR-1 did not alter expression levels of Bax, Caspase 3, Caspase 8, or Caspase 9. Sufentanil ($10 \text{ } \mu\text{M}$) accelerated H9C2 cell growth. When Bcl-2 was silenced, miR-1 expression was reduced in H9C2 cells after 0, 4 and 8 h of growth; however, expression of Bax, Caspase 3, Caspase 8 and Caspase 9 did not change. The growth rate of H9C2 cells exposed to sufentanil was significantly more rapid than that of untreated H9C2 cells. The therapeutic effects of sufentanil in patients with ischemic heart disease are at least partially mediated by changes in signaling through the miR-1 and Bcl-2 pathways.

Key words: Sufentanil, Ischemia, miR-1, Bcl-2.

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INTRODUCTION

The clinical profile of myocardial ischemia/reperfusion (I/R) injury has changed profoundly over the last ten years, as the prevalence of systemic metabolic disorders has grown (Zhang et al., 2011). Early reperfusion is an absolute pre-requisite for the survival of the ischemic myocardium. However, reperfusion has been referred to as a “double-edged sword” because reperfusion itself may lead to accelerated and additional myocardial injury beyond that

generated by ischemia, which results in a spectrum of reperfusion-associated pathologies, collectively called reperfusion injury.

Sufentanil, a lipophilic opioid, is used in the clinic to treat patients with I/R injury and ischemic heart disease, in which it seems to produce therapeutic effects by regulating PI3k signaling (Wu et al., 2012).

MicroRNAs regulate many genes, including Bcl-2 (Zhang

and Cohen, 2013; Tay et al., 2014). The functions of several microRNAs are not completely understood, but several miRNAs are known to be regulated during apoptosis (Bisio et al., 2013). Recently, apoptosis-related gene expression and miRNA expression during cancer therapy were shown to be related (Beckman, 2007; He et al., 2007; Bisio et al., 2013). Several studies suggested that many miRNAs play important roles in I/R injury, including miR-1, miR-122, miR-124, miR-146a, miR-223, and miR-370 (Zhu et al., 2016). Bcl-2, insulin growth factor 1 (IGF-1), and heat shock protein 60 (Hsp60) are target genes of miR-1 that mediate the effect of miR-1 in aggravating myocardial injury by I/R or infarction (Zhu et al., 2016).

In this study, the effect of sufentanil on miR-1 and Bcl-2 was explored in the context of I/R injury and the function of sufentanil on miR-1 and Bcl-2 in cardiac cell H9C2 explored. The mechanism of sufentanil protective function of miR-1 and Bcl-2 was also shown.

MATERIALS AND METHODS

Unless otherwise specified, all chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA). Antibodies against IgG, GAPDH, Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9 and ABT-737 (Bcl-2 inhibitor) were purchased from Abcam (USA).

Cardiac ischemic reperfusion models building

Wistar rats were purchased from Weitong Lihua (Beijing, China). Sixty rats (body weight of 200 ± 30 g) of either sex were randomly assigned to three groups (a sham operation group treated with 300 mg/kg chloral hydrate (SO group), an ischemia/reperfusion group treated with 300 mg/kg chloral hydrate (IR group), and an IR group treated with sufentanil 1.1 mg/kg (SUF group). Before the experiment, the rats were prevented from eating for 12 h, but water was provided *ad libitum*. In summary, rats were anesthetized, after which the chest was opened through a left thoracic incision. A 6-0 silk suture slipknot was placed at the distal 1/3 of the left anterior descending artery. After 30 min of ischemia, the slipknot was released, after which the myocardium was reperused for 3 h. Sham-operated control rats underwent the same surgical procedures, but the suture placed under the left coronary artery was not tied. Cardiac function was continuously determined by electrocardiogram (Carlberg and Mannervik, 1975). The animal protocol was approved by The Chongqing Medical University Experimental Animal Management Committee.

Biochemical analysis

The serum levels of creatine kinase (CK, Abcam, ab155901)

and lactate dehydrogenase (LDH, Abcam, ab102526) were measured using commercially available kits. According to the methods described by Buege and Aust (1978), the lipid peroxidation level in the heart homogenate was determined by measuring MDA, the end product of lipid peroxidation, which reacts with thiobarbituric acid (TBA) as the TBA reactive substance (TBARS) to produce a red-colored complex with a peak absorbance at 532 nm. To precipitate proteins, the supernatant (125 ml) was homogenized by sonication with TBS (50 ml) and butylhydroxytoluene (TCA-BHT, 125 ml), followed by centrifugation ($1,000 \times g$, 10 min, 4°C). The supernatant (200 ml) was mixed with 0.6 M HCl (40 ml), while TBA was dissolved in 0.7 M Tris (160 ml), and the mixture was heated at 80°C for 10 min. The absorbance of the resulting supernatant was measured at 530 nm. The amount of TBARS was calculated by using an extinction coefficient and SOD activity measured according to the method described by McCord and Fridowich (1968), based on the production of superoxide radicals during the conversion of xanthine to uric acid by xanthine oxidase and the inhibition of cytochrome C reduction. One unit of SOD activity was defined as the amount of SOD that produces 50% inhibition of cytochrome C reduction. $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$ and $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ activities were assayed by spectrophotometrically measuring the amount of inorganic phosphate liberated following incubation of the tissue extract with disodium ATP (Sigma, Welwyn Garden City, UK) {Ko, 2011 #1060}{Li, 2003 #1693} (Greenwald, 1985).

Western blotting

Protein samples obtained from cells were separated by SDS-PAGE in 12% gels and transferred to nitrocellulose membranes. The membranes were blocked using milk. Thereafter, the membranes were incubated overnight at 4°C with primary antibodies (GAPDH, Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9). The membranes were washed thrice, incubated with secondary antibodies and developed using ECL reagents (Pierce, Rockford, IL, USA).

Cell culture

H9C2 cells (Chinese Academy of Sciences, China) were cultured in an incubator at 37°C with 5% CO_2 . The culture media was Dulbecco's Modified Eagle's Medium (DMEM) containing 15% fetal bovine serum (Gibco, USA).

Cell growth rate assay

The Cell Counting Kit 8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability and proliferation. Briefly, cells were seeded in 96-well plates (3000 cells/well, four replicates) and incubated with

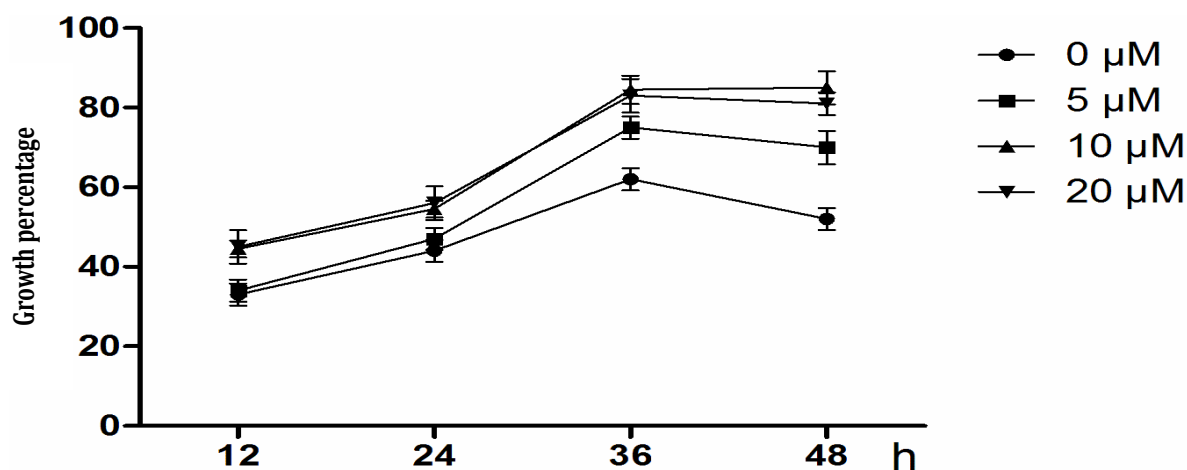


Figure 1: Expression levels of CK, LDH, MDA, and SOD, and the activity levels of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase. A: Expression level of CK in blood. B: Expression level of LDH in blood. C: Expression level of MDA in myocardium. D: Expression level of SOD in myocardium. E: Activity level of Na⁺-K⁺-ATPase in myocardium. F: Activity level of Ca²⁺-Mg²⁺-ATPase in myocardium. *Significantly different from the IR group (P < 0.05).

different final concentrations of sufentanil (0, 5, 10 and 20 μM) for different periods of time (12, 24, 36 and 48 h), respectively. The cells were replenished with medium containing CCK-8 solution (10 μl CCK-8 in 100 μl medium) and incubated for 2 h, after which the absorbance at 450 nm was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The growth rate of the cells was calculated using the formula:

$$\% \text{ Growth rate} = \frac{\text{Mean experimental absorbance}}{\text{Mean control absorbance}} \times 100$$

Quantitative reverse transcriptase polymerase chain reaction analysis (qRT-PCR)

Total cellular RNA was obtained by AccuZol reagent (Bioneer, Daedeok-gu, Daejeon, Korea). Complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed using a Rotor-Gene 6000 system (Corbett Life Science, Mortlake, NSW, Australia) with SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan) (Mansoori et al., 2016). The cDNA was diluted with 1:10 and the following primers: *miR-1-F*, 5'-GAGGAGGCTGGAGAA-3'; *miR-1-R*, 5'-GGTGAATGTAAAGAAGTC-3'; *GAPDH-F*, 5'-AGAAGGCTGGGGCTCATTG-3'; *GAPDH-R* and 5'-CTCGCTCCTGGAAGATGGTG-3' were used. GAPDH was used as an internal control.

Transient transfection

Logarithmically growing H2C9 cells were seeded in a 10-cm dish (6×10⁶ cells per flask), and then transfected with

GV268-miR-1 (hsa-miR-1-1) for miR-1 over-expression with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The culture medium was replaced after 6 h and sufentanil added; thereafter, protein or RNA was extracted from subconfluent cells after transfection for 0, 4, 8 and 12 h, respectively.

Bcl-2 silenced through ABT-737

ABT-737 (60 nM) with 10 μM sufentanil was added into the H2C9 cells. The cells were obtained after 0, 4, 8 12 h, respectively and the miR-1, Bax, Caspase 3, Caspase 8, and Caspase 9 detected.

Statistical analysis

GraphPad Prism version 5 software (GraphPad Software, La Jolla, CA, USA, www.graphpad.com/company/) was used to conduct one-way analysis of variance (ANOVA) followed by post-hoc tests. The threshold for significance was p < 0.05. Data are presented as mean ± S.D (n=5).

RESULTS

Serum parameters and activity levels of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase

Serum CK activity and serum LDH activity levels increased in the IR group in comparison with the same measurements in the SO group and SUF group (P < 0.05, Figure 1A and B). The myocardial MDA activity of the IR group also increased in comparison with the SO and SUF groups (P < 0.05, Figure

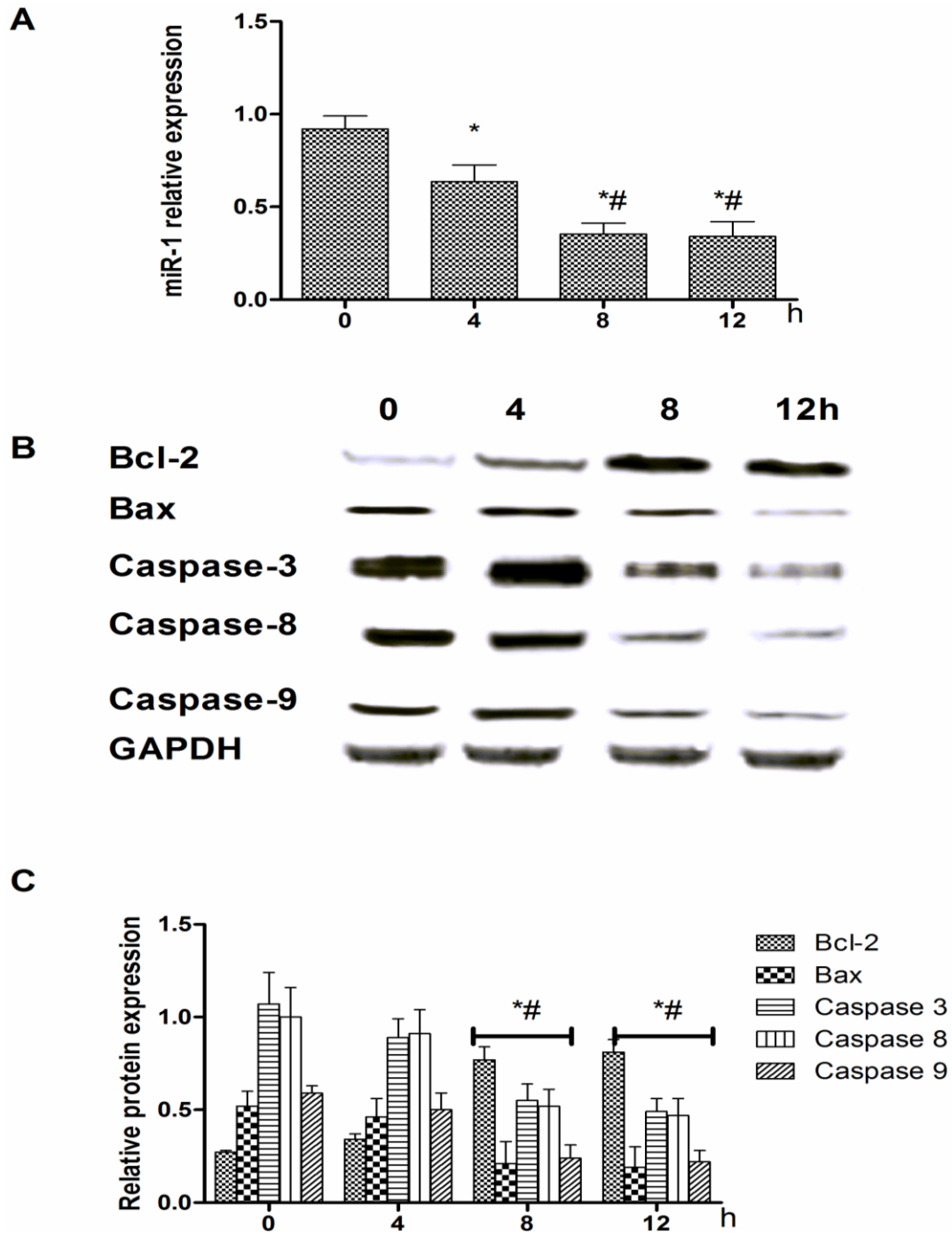


Figure 2: Effects of sufentanil on the miR-1 and Bcl-2 pathways in the IR group, SO group and SUF group. (A) Measurement of miR-1 expression by qPCR. (B) Representative western blots showing levels of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9. (C) Contrast gray value of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9 proteins based on the western blot. Results are expressed as mean $\pm$ S.D. (n = 5). *, significantly different from the IR data point (P < 0.05).

1C). The myocardial SOD activity of the IR group reduced in comparison with the SO group and SUF group (P < 0.05, Figure 1D). The Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase activity levels of the IR group reduced in comparison with those of the SO and SUF groups (P < 0.05, Figure 1E and F).

Effect of sufentanil on miR-1 and Bcl-2 signaling in rats

To assess miR-1 and Bcl-2 signaling activity in the IR group, SO group and SUF group, miR-1 abundance was measured by qPCR (Figure 2A), and protein levels of Bcl-2, Bax,

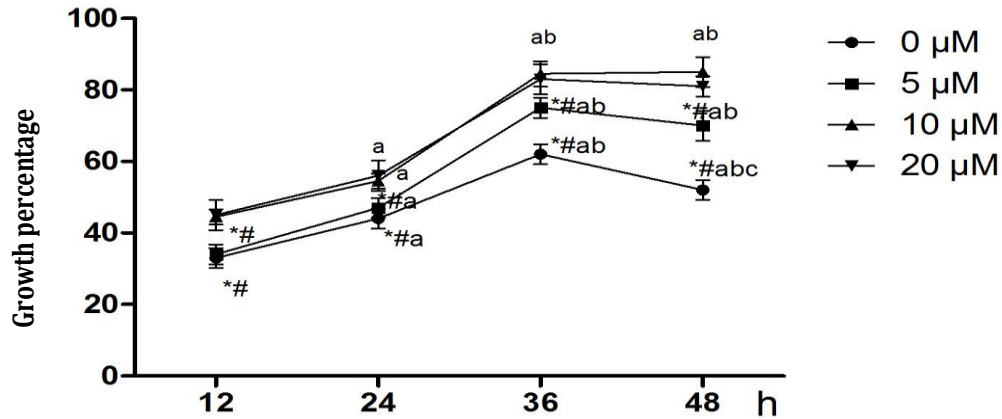


Figure 3: H9C2 cell growth percentage following exposure to sufentanil (0 μ M, 5 μ M, 10 μ M, 20 μ M) for 12 h, 24 h, 36 h, and 48 h. * significantly different from the 0 μ M data point ($P < 0.05$). #, significantly different from the 5 μ M data point ($P < 0.05$). a: significantly different from the 12 h data point ($P < 0.05$). b: significantly different from the 24 h data point ($P < 0.05$) and c: significantly different from the 36 h data point ($P < 0.05$).

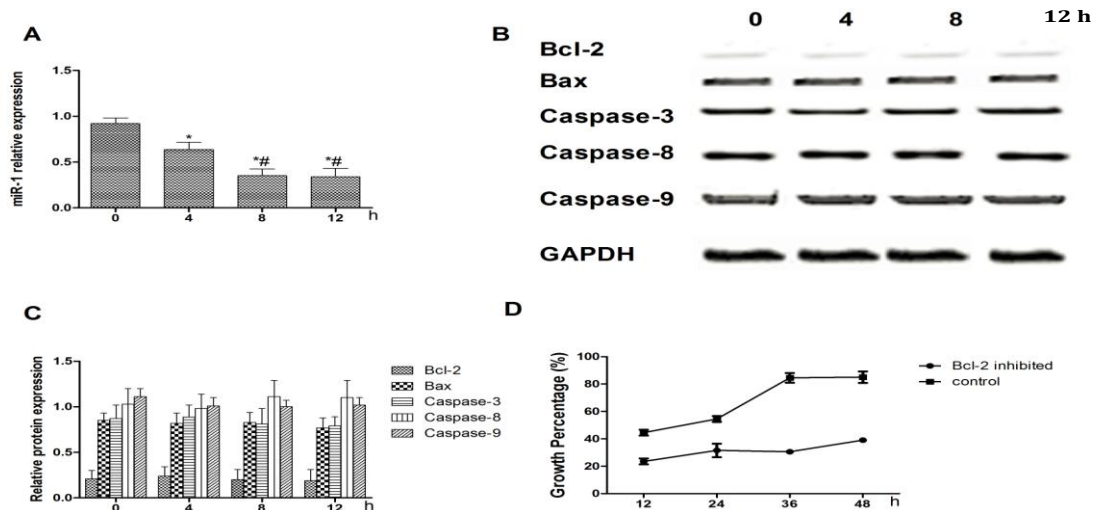


Figure 4: Effects of sufentanil on the miR-1 and Bcl-2 pathways in H9C2 cells after 0, 4, 8 and 12 h. (A) Measurement of miR-1 expression by qPCR. (B) Representative western blots showing levels of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9. (C) Contrast gray value of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9 proteins based on the western blot. Results are expressed as mean $\pm$ S.D. ($n = 5$). *, significantly different from the 0 h data point ($P < 0.05$), and #, significantly different from the 4 h data point ($P < 0.05$).

Caspase 3, Caspase 8 and Caspase 9 measured by western blot analysis (Figure 2B and C). In comparison with the SO group and SUF group, the IR group showed increased abundance of miR-1, Bax, Caspase 3, Caspase 8, and Caspase 9 ($P < 0.05$). However, the IR group showed reduced expression of Bcl-2 in comparison with that of the SO group and SUF group ($P < 0.05$).

Effect of sufentanil on the growth of H9C2 myocardial cells

The growth percentage of H9C2 cells increased as the

concentration of sufentanil increased. Moreover, the growth percentage of H9C2 cells increased as the duration of sufentanil exposure was lengthened. Optimal growth of H9C2 cells was achieved through exposure to 10 μ M sufentanil for 36 h (Figure 3).

Effect of sufentanil on miR-1 and Bcl-2 signaling in H9C2 myocardial cells

To assess miR-1 and Bcl-2 signaling activity in H9C2 cells, miR-1 abundance was measured by qPCR (Figure 4A), whereas Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9

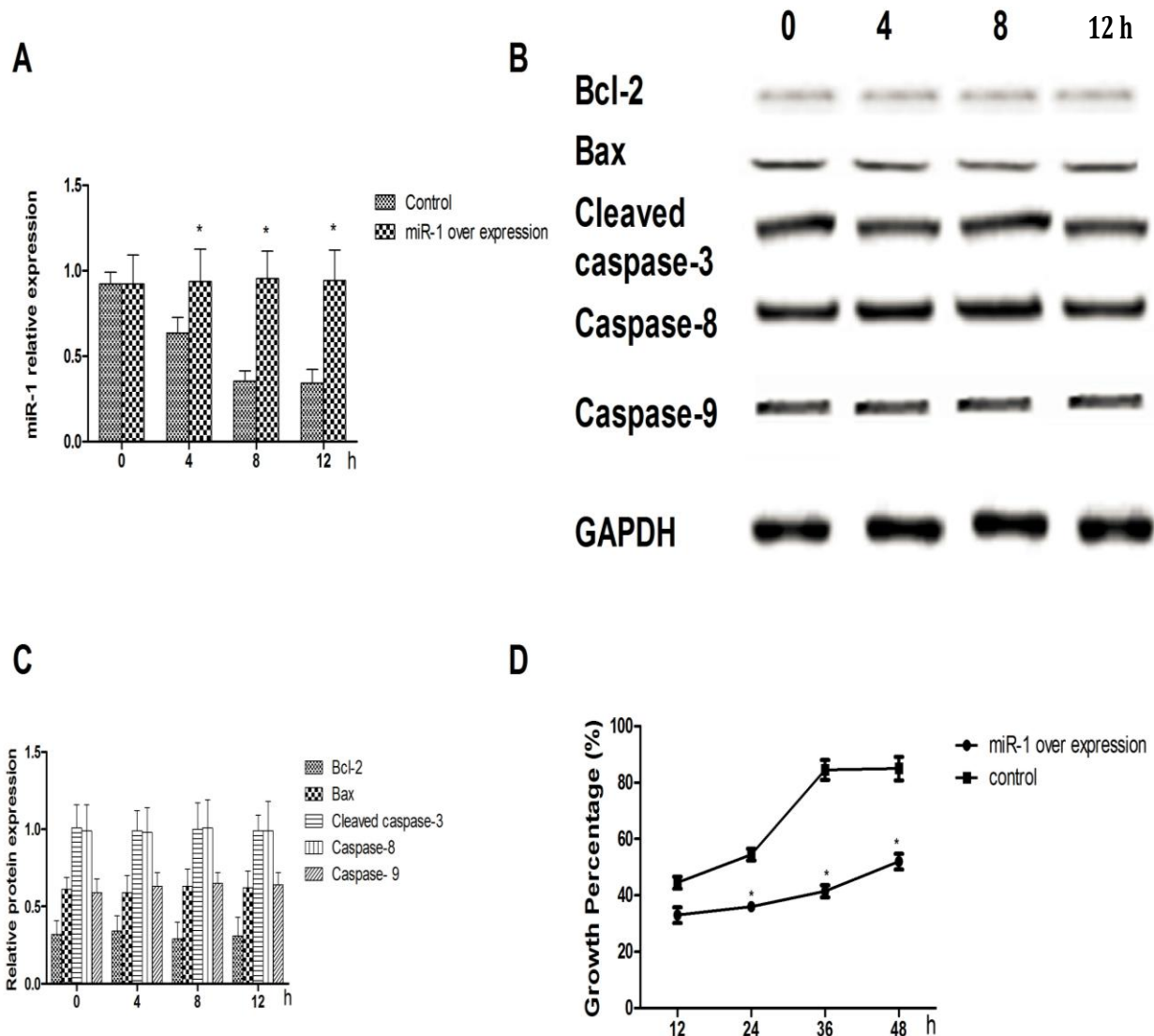


Figure 5: Effects of sufenanil on the miR-1 and Bcl-2 pathways after miR-1 over-expression after 0, 4, 8 and 12 h. (A) Measurement of miR-1 expression by qPCR. (B) Representative western blots showing levels of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9. (C) Contrast gray value of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9 proteins based on the western blot. Results are expressed as mean $\pm$ S.D. (n = 5). (D) H9C2 cell growth percentage following miR-1 over-expression. *, significantly different from the control group ($P < 0.05$).

were measured after 0, 4, 8 and 12 h of sufenanil exposure by western blot analysis. The abundance of Bcl-2 was increased after 0, 4 and 8 h of sufenanil exposure ($P < 0.05$), but the abundance of miR-1, Bax, Caspase 3, Caspase 8, and Caspase 9 decreased at the 8 and 12 h time points, respectively (Figure 4).

Effect of sufenanil on miR-1 and Bcl-2 signaling in H9C2 myocardial cells after miR-1 over-expression and Bcl-2 silenced

For further study, miR-1 was over-expressed in H9C2 cells.

Over-expression of miR-1 (Figure 5A) did not change the expression levels of the target proteins (Figure 5B and C). When miR-1 was over-expressed, sufenanil (10 μ M) accelerated H9C2 cell growth (Figure 5D); the growth rate of sufenanil-treated cells was significantly greater than that of untreated H9C2 cells. These findings show that miR-1 can influence Bcl-2 expression. Therefore, to test this hypothesis Bcl-2 was silenced by ABT-737 (60 nM). H9C2 cells lacking Bcl-2 expression showed reduced miR-1 abundance at 0, 4 and 8 h (Figure 6A); however, the abundance of Bax, Caspase 3, Caspase 8 and Caspase 9 remained unchanged (Figure 6B and C). The growth rate of cells lacking Bcl-2 expression was significantly greater than

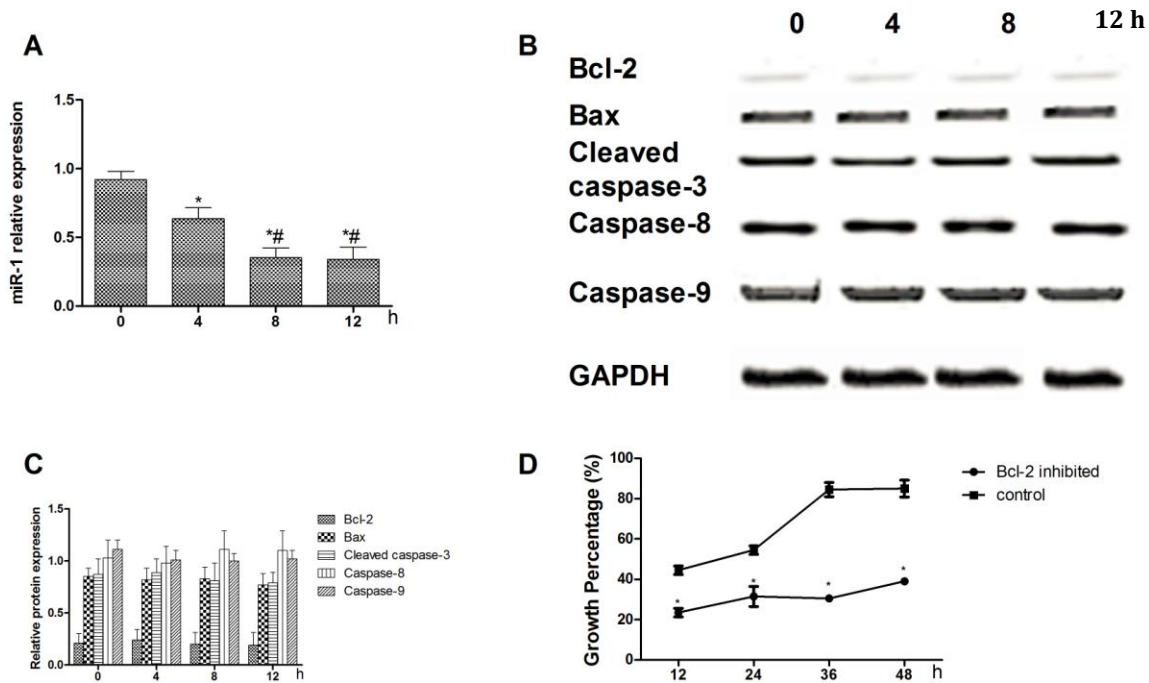


Figure 6: Effects of sufenanil on the miR-1 and Bcl-2 pathways after Bcl-2 silencing after 0, 4, 8 and 12 h. (A) Measurement of miR-1 expression by qPCR. (B) Representative western blots showing levels of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9. (C) Contrast gray value of Bcl-2, Bax, Caspase 3, Caspase 8 and Caspase 9 proteins based on the western blot. Results are expressed as mean $\pm$ S.D. (n = 5). (D) H9C2 cell growth percentage following miR-1 over expression. *, significantly different from the control group (P < 0.05).

that of untreated cells (Figure 6D).

DISCUSSION

In the present study, changes in hemodynamic parameters confirmed that ischemic-reperfusion injury was induced in the hearts of the untreated rats. Creatine kinase (CK) and lactate dehydrogenase (LDH) are routinely measured in clinical laboratories to diagnose and monitor myocardial infarction (Zhang et al., 2012; Sun et al., 2012; Yin et al., 2015; Ye et al., 2010). In this study, the serum LDH and CK levels of the IR group increased in comparison with the SO and SUF group. The amounts of intracellular enzymes, CK and LDH released from the ischemic hearts of the SUF group were significantly reduced, indicating protection against cell membrane damage.

In the present study, the activity levels of myocardial Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase decreased in IR rats. Our findings is in line with previous observations indicating that abnormalities in the activity levels of Na⁺-K⁺-ATPase, Na⁺-Ca²⁺ exchangers, and Ca²⁺-pumps led to intracellular calcium overload in experimental rat models of myocardial ischemia reperfusion. The SUF group showed significantly increased myocardial SOD activity, which indicates that sufenanil may alleviate oxidative injury associated with IR. miRNAs are non-coding single stranded RNAs that

contain approximately 21 to 24 nucleotides and regulate gene expression by base-pairing of nucleotides 2 to 8 with the 5'- or 3'-untranslated regions of target mRNAs, primarily in the cytoplasm (Abdel-Aleem et al., 2016; Ali et al., 2012). Some miRNAs are encapsulated into microvesicles, exosomes, or apoptotic bodies, whereas other miRNAs form complexes with RNA-binding proteins (Aras et al., 2016; Bostjancic et al., 2015; Bostjancic et al., 2010). MiRNAs were demonstrated to be involved in myocardial I/R injury (Zhang et al., 2011). miR-1 regulates apoptosis (Sygitowicz et al., 2015).

In this study, we observed that miR-1 regulated H9C2 cell growth in sufenanil-treated cells. In addition, the Bcl-2 pathway was also regulated by miR-1. These results suggest that miR-1 and Bcl-2 play key roles in mediating the accelerating effect of sufenanil on H9C2 cell growth. miR-1 over-expression did not change the abundance of Bcl-2, Bax, Caspase 3, Caspase 8 or Caspase 9. Inhibition of Bcl-2 did not significantly change the abundance of miR-1. Moreover, inhibition of Bcl-2 did not change the abundance of Bax, Caspase 3, Caspase 8 or Caspase 9. In a previous study, miR-1 suppressed HEI-193 cell proliferation, inhibited colony formation and enhanced apoptosis (Bostjancic et al., 2015). A recent clinical investigation of renal cancer samples revealed significant down-regulation of miR-1, an event significantly associated with the advanced clinical stage and poor overall survival

(Sygitowicz et al., 2015).

miR-1 is one of the most abundant miRNAs in the heart and plays a protective role against cardiac hypertrophy (Sygitowicz et al., 2015). Cardiomyocyte death is the direct and main cause of I/R-induced myocardial injury. Bcl-2 and Bax are two important regulators of the mitochondrial apoptotic signaling pathway, and Bcl-2 can prevent permeabilization of the outer mitochondrial membrane by inhibiting activation of Bax/Bak, leading to attenuation of the activation of caspase-9 and caspase-3 (Zhu et al., 2016). This study reveals that the Bcl-2 was regulated by miR-1. Therefore, inhibition of Bcl-2 did not significantly change the abundance of miR-1.

This study further reveals that Bcl-2 regulated by miR-1 plays an important role in sufentanil-treated H2C9 cells by affecting cell proliferation and apoptosis. Therefore, sufentanil should be considered as a potential treatment for patients with myocardial dysfunction associated with I/R injury.

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