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Oxycodone protects cardiomyocytes from ischemia-reperfusion-induced apoptosis via PI3K/Akt pathway

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Ischemia/reperfusion (I/R) cause secondary myocardial damage following a blood reflow after myocardial infarction. This study aimed to explore oxycodone as a myocardial protector after an I/R injury in rats. Oxycodone reduced myocardial infarction volume, an I/R-induced apoptosis of the cardiomyocytes, the serum levels of CK-MB and LDH. The ejection fraction and fraction shortening in the I/R rats also increased. From the molecular mechanism, it was evident that oxycodone not only decreased the expression levels of Bax, active-caspase 3 protein but also increased the expression levels of Bcl2, p-PI3K, and p-Akt protein in heart tissue of the I/R rats. *In vitro*, oxycodone induced anti-H9c2 cell apoptosis after hypoxia/reoxygenation (H/R). However, its ability to act as a myocardial protector deteriorated in the presence of a PI3K/Akt pathway inhibitor.

1. Introduction

Myocardial infarction is the most common of all coronary artery diseases, which constitutes the highest morbidity and mortality rates worldwide. Coronary artery recanalization, such as coronary artery bypass grafting, percutaneous coronary intervention, and thrombolytic therapy is necessary to treat myocardial infarction, but ischemia-reperfusion (I/R) injury pose a significant obstacle in the treatment of blood reflow (Levine et al. 2011a,b). I/R injury could be due to several factors such as inflammation, oxidative stress, and mitochondrial damage (Xiao et al. 2011; Yu et al. 2017). However, cardiomyocyte apoptosis is the direct physiological manifestation of I/R injury that causes arrhythmia (Bozdoğan 2016; Park et al. 2017), myocardial contractile dysfunction (Ao et al. 2016; Rappaport 2000) and irreversible damage of cardiomyocytes (Hausenloy and Yellon 2013).

Previous studies have shown the presence of opioid receptors on the surface of cardiomyocytes. As such, the pre-administration of opioid receptor agonists could reduce an I/R injury (Irwin and Wong 2015; Tong et al. 2016). Oxycodone, a dual agonist of μ and κ opioid receptors, is a semi-synthetic opioid extracted from thebaine and has been used as a potent analgesic for more than 80 years (Kalso 2005; Lalovic et al. 2006). Yang et al. (2016) reported that oxycodone could reduce lipopolysaccharide-induced acute lung injury in rats. Typically, the U50, 488H, also a κ opioid receptor-like oxycodone, were reported to show myocardial protection in I/R myocardium (Irwin and Wong 2015; Tong et al. 2016). However, the effect of oxycodone on myocardial protection during an I/R injury is still not fully elucidated. In general, the PI3K/Akt pathway does not cause mammalian apoptosis, but the activated pathway could reduce the chances of an I/R-induced cardiomyocyte apoptosis (Li et al. 2018; Zhang et al. 2017).

2. Investigations and results

2.1. Oxycodone attenuated an I/R-induced heart injury

Twenty rats were used to establish an I/R model. Ten rats were intravenously injected with oxycodone (Fig. 1A) before reperfusion and were sacrificed two hours after reperfusion to recover their heart tissue. As shown in Fig. 1B, the myocardial infarction volume was significantly smaller in the oxycodone-pretreated I/R rats. Before the sacrifice, the echocardiograph data revealed that the ejection fraction and fraction shortening in rats of the I/R group was less than those of the sham group (Fig. 1C-D). Furthermore, oxycodone-pretreated I/R rats showed reduced levels of CK-MB and LDH in the rats (Fig. 1E).

2.2. Oxycodone attenuated an I/R-induced cardiomyocyte apoptosis

Cardiomyocytes are the prime functional cells in the heart tissues, and hence, we detected the apoptosis of cardiomyocytes. The results indicated that an I/R injury could induce cardiomyocyte apoptosis; however, the pretreatment of the I/R rats with oxycodone could significantly reduce cardiomyocyte apoptosis (Fig. 2A, 30.40±4.10% vs 20.20±4.87%). The expression levels of apoptosis-related proteins in the heart tissue of rats were measured. After an I/R injury as shown in Fig. 2B, the expression levels of Bax, Bcl2, active-caspase 3 protein, the ratios of active-caspase 3/caspase 3, and Bax/Bcl2 significantly increased in the heart tissue. In contrary, oxycodone-pretreated I/R rats showed an increase in the expression of Bcl2 protein and a decrease in the expression of Bax, active-caspase 3 protein, active-caspase 3/caspase 3, and Bax/ Bcl2 ratios in the heart tissue.

2.3. Oxycodone activated PI3K/Akt pathway in the I/R rats

The PI3K/Akt pathway is not related to mammalian apoptosis but plays a vital role in I/R injury. Therefore, we measured the expression of a key molecule in the PI3K/Akt pathway in heart tissue of the I/R rats. The results revealed that the expression of p-PI3K, p-Akt protein, p-PI3K/PI3K, and p-Akt/Akt ratios increased after I/R injury of the heart tissue in the rats (Fig. 3). Oxycodone enhanced the expression of p-PI3K, p-Akt protein, p-PI3K/PI3K, and p-Akt/Akt ratios in heart tissue of the I/R rats. Moreover, the proportion of p-PI3K/PI3K or p-Akt/Akt were negatively correlated with myocardial infarct volume or cardiomyocyte apoptosis in the I/R rats (Fig. 4).

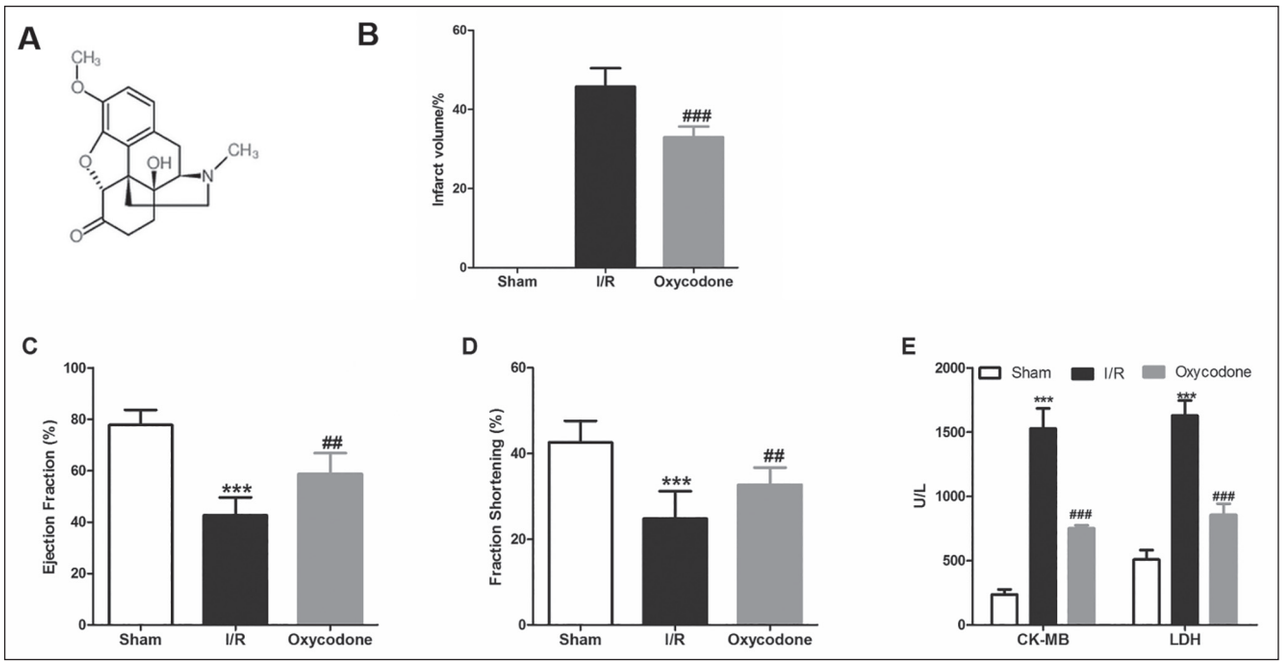

Fig. 1: Oxycodone reduced I/R-induced myocardial infarction volume and cardiac dysfunction. A: Chemical structure of oxycodone; B: Statistics of myocardial infarct volume; C-D: Ejection fraction (C) and fraction shortening (D) which were measured by echocardiography in rats of different group; E: Serum levels of LDH and CK-MB in of different group; 10 rats per group; *** was P<0.001 versus (vs) sham group, and ### was P<0.001 vs I/R group.

2.4. Oxycodone protected H9c2 cells by activating the PI3K/Akt pathway in vitro

Hypoxia-reoxygenation (H/R) treatment was used to simulate an I/R injury in the H9c2 cells *in vitro*, and LY294002 was used as a specific inhibitor of the PI3K/Akt pathway. As shown in Fig. 5A, oxycodone reduced the apoptosis of the H9c2 cells (induced by H/R), but the percentage of apoptosis of the H9c2 cells in H/R+Ox+LY group was significantly higher than those in the H/R+O/X group. For protein expressions, we found out that the H/R increased the expression levels of p-PI3K, p-AKT, Bax, active-caspase 3 protein, and decreased the expression levels of Bcl2 protein. The expression levels of PI3K, Akt, caspase 3, and GAPDH protein in each group were not significantly different; however, as compared with the H/R group, oxycodone decreased the expression levels of p-PI3K, p-AKT, Bax, active-caspase 3 protein, and increased the expression levels of Bcl2 protein. Interestingly, the expression levels of p-PI3K, p-AKT, Bax and active-caspase 3 protein in the H/R+Ox+LY group was significantly higher than those in H/R+Ox group, whereas, the expression levels of Bcl2 protein was significantly lower.

3. Discussion

With improvements in the social economy and the development of urbanized spaces, the risk of major cardiovascular-related disease, such as myocardial infarction among the Chinese population has increased (Bundy and Jiang 2016; Wu et al. 2016). Treatments for myocardial infarction include thrombolysis, coronary angioplasty, and coronary artery bypass grafting, which restores the ischemic myocardium to blood perfusion. Besides protection of the heart muscle, reperfusion facilitates additional myocardial damage, also known as the myocardial ischemia/reperfusion (I/R) damage (Levine et al. 2011a, b).

In this study, we found that oxycodone could reduce myocardial infarct volume that improved the cardiac function of I/R rats. Oxycodone is a μ (analgesic role) and κ opioid receptor (analgesic, and cardioprotective role) agonist, which is used for surgical analgesia (Chang et al. 2010; Olesen et al. 2010). Shi et al. (2013) reported that agonists could activate κ receptors before reperfusion, which stabilized CX43 and reduced the incidence of reperfusion arrhythmias. It was also reported that the intravenous administration of a selective κ receptor agonist (0.1 mg/kg of U50488) before reperfusion could significantly reduce myocardial infarct size in rats (Hajime et al. 2004). Likewise, the intravenous or oral administration of atorvastatin, nicorandil, scopolamine, morphine, and other drugs before reperfusion also seems to protect the myocardium from I/R injury (Ibáñez et al. 2015). Typically, endogenous or exogenous opioids bind at receptors (μ, κ, and δ) that are linked to the inhibitory G protein *via* seven-transmembrane structures. During myocardial ischemia and reperfusion, the body produces endogenous opioid peptides such as endorphin, enkephalin, and dynorphin, which bind with μ-, κ-, and δ-opioid receptors, respectively (Tanaka et al. 2014). Enkephalin, and dynorphin bind with δ-, κ-opioid receptors on the heart, and activates the protein kinase C in the cardiomyocytes *via* the PI3K pathway. Such an association inhibits the opening of ATP-dependent potassium channels in the mitochondrial membranes and defends ischemic cardiomyocytes (Tanaka et al. 2014). In this respect, scientists have explored the delivery of morphine and remifentanil tablets before reperfusion to simulate the binding of endogenous opioid peptides with the corresponding receptors; this not only promoted the recovery of myocardial infarction but also reduced the incidence of myocardial infarction volume and reperfusion arrhythmia (Headrick et al. 2015).

In our study, oxycodone reduced the apoptosis ratio of cardiomyocytes in heart tissue of I/R rats. It also decreased the Bax/Bcl2, and active-caspase 3/caspase 3 ratios in the heart tissue of the I/R rats. Gottlieb et al. (1994) reported that reperfusion injury promoted cardiomyocyte apoptosis in a rabbit model of myocardial I/R. Subsequent studies found out that apoptotic cardiomyocytes mainly occurred in the marginal zone of infarcted myocardium, while no cell apoptosis occurred in the non-infarcted zone. Typically, the apoptotic cardiomyocytes are involved in myocardial reperfusion injury, and reperfusion accelerates the ischemic zone. Cardiomyocyte apoptosis also significantly increases with ischemia and reperfusion time (Hoffman et al. 2004; Sabbah et al. 1998). Previous studies reported that the expression levels of Bax and active-caspase 3 protein increased in the heart tissue after reperfusion. In contrast, the ratio of Bcl-2 family apoptosis inhibitory gene to apoptosis-promoting gene decreased, whereas, the myocardial cell apoptosis increased significantly (Rajtik et al. 2016; Zhu et al. 2016). These observations were consistent with our results.

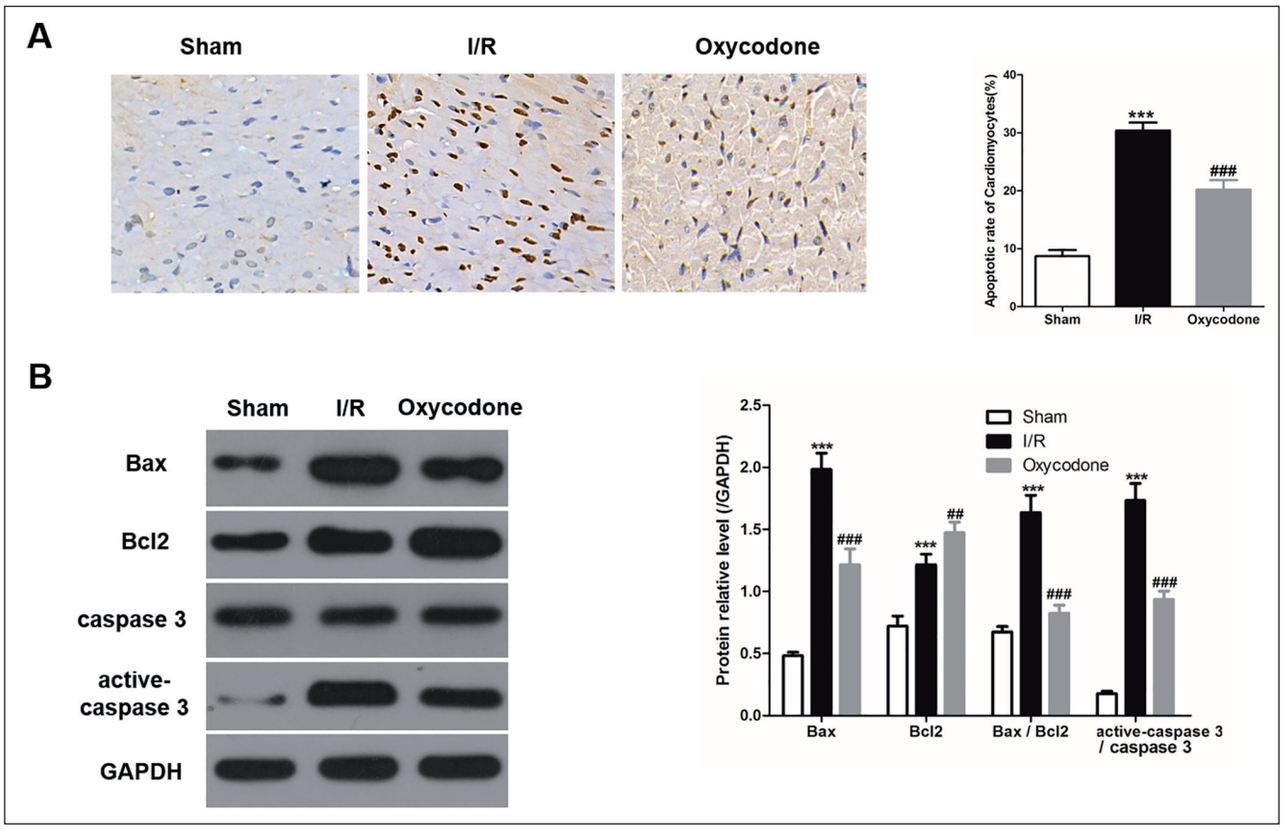

Fig. 2: Oxycodone reduced I/R-induced cardiomyocyte apoptosis. A, TUNEL staining was used to detect apoptosis of cardiomyocytes in cardiac tissue and statistical comparison of the proportion of cardiomyocyte apoptosis in different groups; B, Western blot was used to measure the expression of Bax, Bcl2, caspase 3 and active-caspase 3 protein, and representative strip temporary and gray value comparison. Three independent repetitions per experiment. *** was P<0.001 versus (vs) sham group, ## was P<0.01 and ### was P<0.001 vs I/R group.

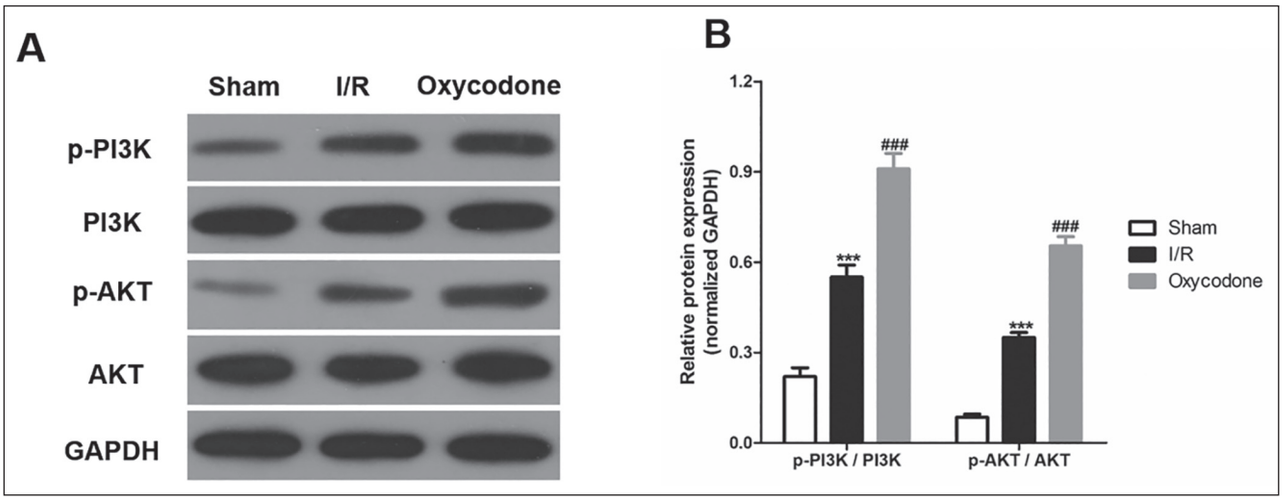

Fig. 3: Oxycodone activated PI3K/AKT signaling pathway in myocardial tissue of I/R rats. A-B, Western blot was used to measure the expression of p-PI3K, PI3K, p-AKT and AKT protein, and representative strip temporary (A) and gray value comparison (B). 3 independent repetitions per experiment. *** was P<0.001 versus (vs) sham group, and ### was P<0.001 vs I/R group.

The PI3K family is considered to be an essential molecule in the signal transduction process of the growth factor superfamily. Upon activation of the PI3Kfamily, it phosphorylates the third hydroxyl group of phosphatidylinositol, which produces an inositol lipid substance having a second messenger action. The primary function of the AKT pathway is to process vital information initiated by PI3K and Akt (Franke 2008). After PI3K activation, a large amount of second messenger, i.e. PIP3 is produced on the plasma membrane, which combines with the intracellular signal proteins Akt and phosphoinositidedependentkinase-1 to activate Akt (Franke 2008). Activated Akt either activates or inhibits downstream target proteins Bad, NF-kappaB, FKHR, p21Cip1, GSK-3, p27 Kip1 and caspase9 by phosphorylation, and thereby takes part in various biological processes such as cell proliferation, differentiation, apoptosis and migration (Franke 2008).

In conclusion, we found that oxycodone could activate the PI3K/Akt signaling pathways both as *in vitro* and *in vivo*. Furthermore, an inhibitor of the PI3K/Akt pathway, i.e. LY294002 could reverse

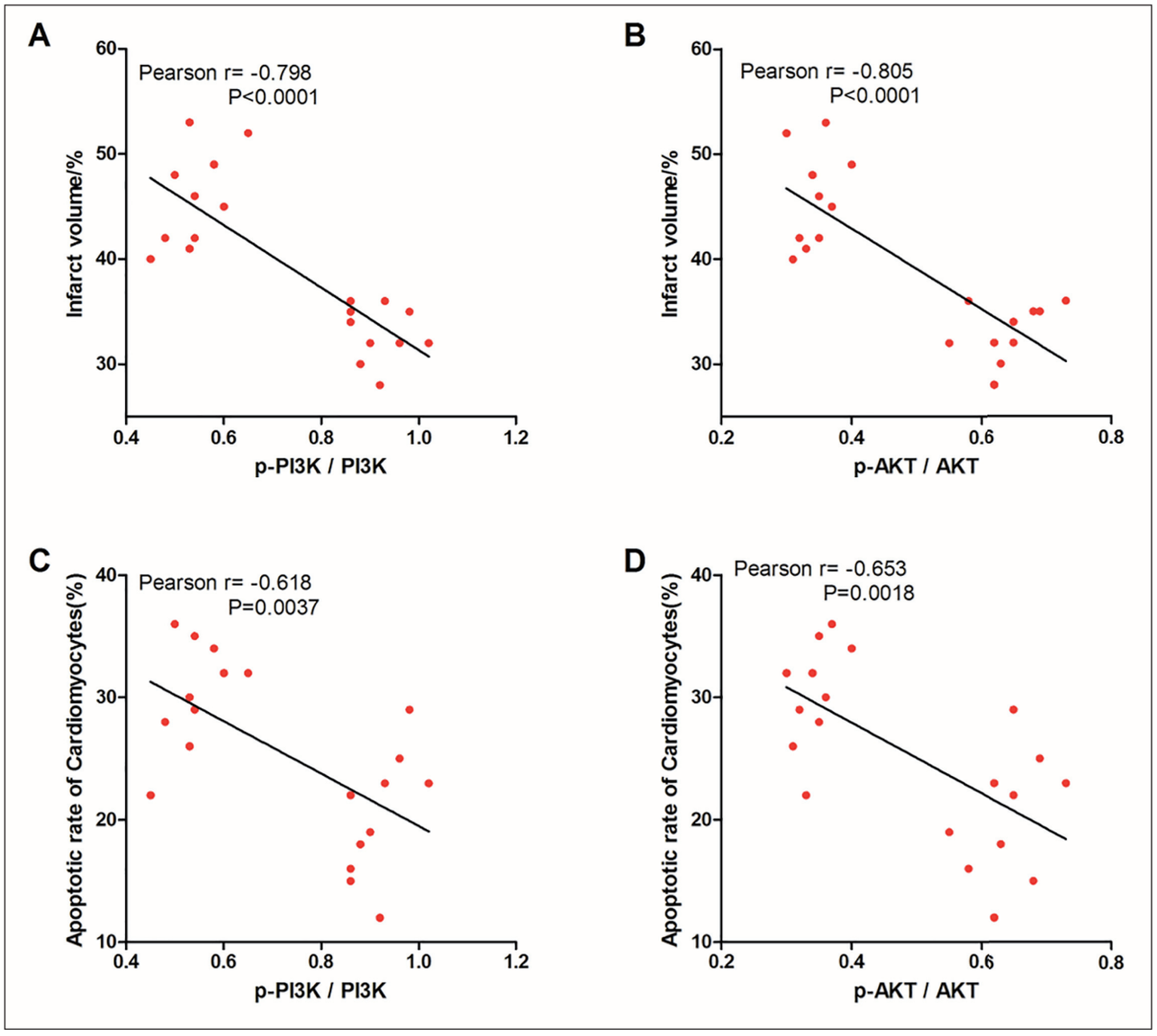

Fig. 4: PI3K/AKT pathway was negatively correlated with myocardial infarct volume and myocardial apoptosis in I/R rats. A-B, The expression of p-PI3K/PI3K or p-AKT/AKT were negatively correlated with myocardial infarct volume; C-D, The expression of p-PI3K/PI3K or p-AKT/AKT were negatively correlated with myocardial apoptosis.

the protective effect of oxycodone on the H9c2 cells after the H/R treatment. Based on these observations, it was confirmed that oxycodone could protect cardiomyocytes from I/R-induced apoptosis by activating the PI3K/Akt pathway.

4. Experimental

4.1. Experimental animals and I/R model

SD rats (male, 240-260 g) were fed for a week (room temperature 20-24 °C, half-day and night, air humidity 60%). The left anterior descending coronary artery (LAD) was used to build a myocardial I/R model, as described previously (Liu et al. 2017). Before reperfusion (5 min), the rats in the oxycodone group were intravenously injected with 0.3 mg/kg oxycodone (O-002, SIGMA, USA). The surgical procedure in the sham group was the same as before, except for the ligating LAD. After the operation, the animals were allowed to stay at room temperature (25- 28 °C) and fed. The animal protocol was approved by the Animal Care and Use Committee in the Third Hospital of Xingtai. The guidelines of the National Institution of Health were followed for all the procedures.

4.2. Cell culture and H/R stress

H9c2 (crl-1446, ATCC, VA, USA) cells were cultured in DMEM medium (12491-15, ThermoFisher, CA, USA) containing 10% of fetal bovine serum (10100-147, ThermoFisher, CA, USA) and 1% penicillin-streptomycin (15640055, ThermoFisher, CA, USA) in this paper. Hypoxia-reoxygenation (H/R) treatment was used to establish I/R injury in the H9c2 cells, which were sequentially exposed to hypoxia for 8h (CO_2/N_2 at a 95:5 ratio) and reoxygenated (O_2/CO_2 at a 95:5 ratio) for 2 h.

For the H/R+Ox group, 1 ng/mL oxycodone was added into the medium to culture H9c2 cells for 1 h. For the H/R+Ox+LY group, 50 μmol/L LY294002 (L9908, SIGMA, USA) and 1 ng/mL oxycodone was added into the medium to culture H9c2 cells for 1 h.

4.3. TTC staining and infarct size measurement

Rats were sacrificed immediately after 30 min of reperfusion, and hearts were taken directly. The preparation of heart tissue sections and TTC staining were done as described previously (Liu et al. 2017). Image J software (National Institutes of Health, Maryland, USA) was used to study the area of myocardial infarction per layer. The myocardial infarction volume was calculated after multiplying the infarct size of each heart slice by the thickness (2 mm).

4.4. Elisa assay

Rat creatine kinase isoenzyme MB (CK-MB) Elisa kit (XF2852B, xinfan.biomart.cn, China) was used to detect the serum levels of CM-MB. Rat Lactate dehydrogenase Elisa kit (www.goybio.com, China) was used to detect the serum levels of LDH.

4.5. Annexin V/PI double staining

The apoptosis of H9c2 cardiomyocytes was determined by annexin V-FITC and PI double staining assay using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer's protocols. The fluorescence was measured by a FACSCalibur flow cytometry (Becton-Dickinson). The annexin V-positive cells are defined as apoptotic cells.

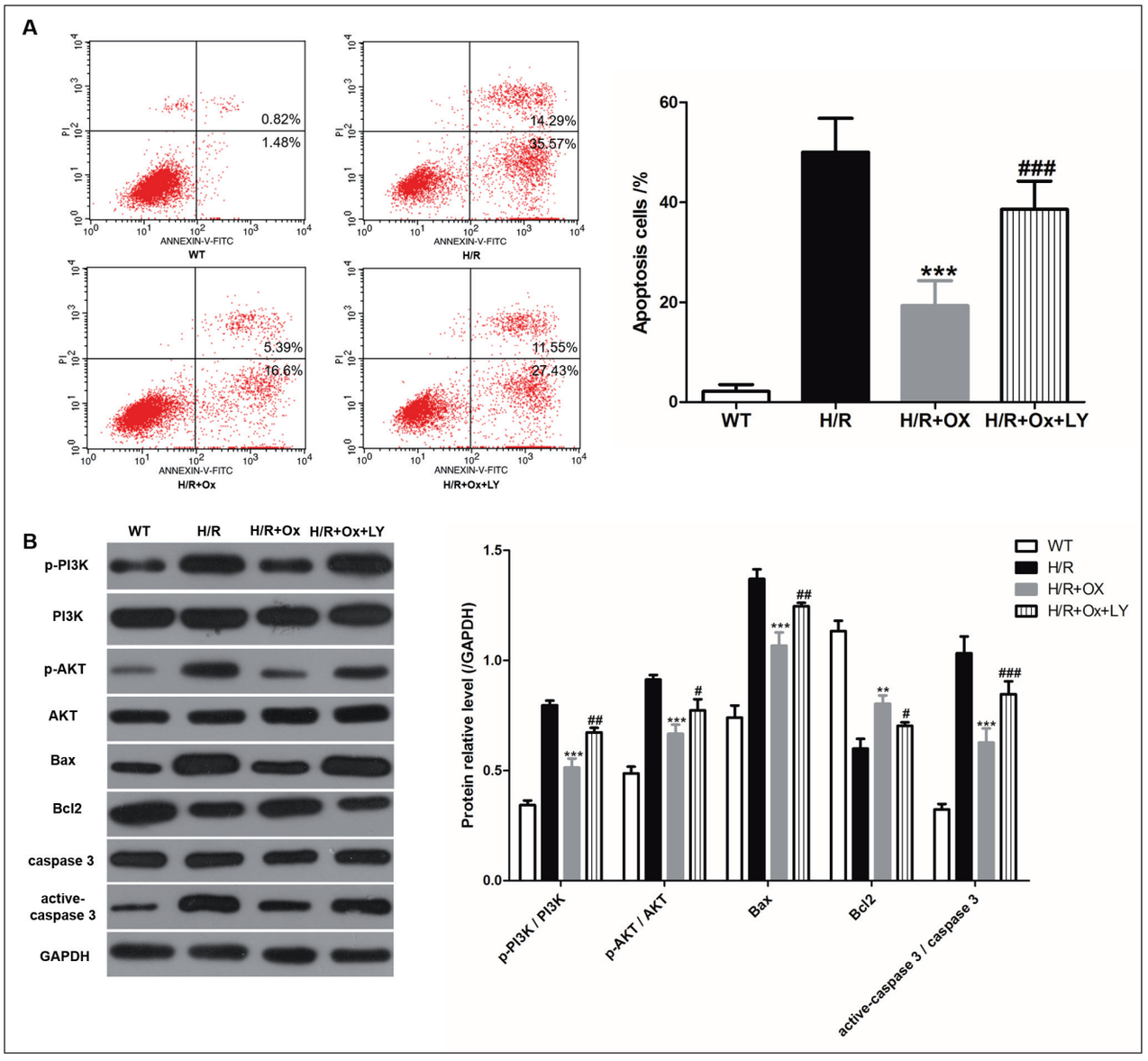

Fig. 5: Oxycodone reduces H/R-induced apoptosis in H9c2 cells by activating the PI3K/AKT pathway. A, detecting apoptosis of H9c2 cells in different treatments and statistical comparison; B, Western blot was used to measure the expression of protein and representative strip temporary and gray value comparison. Three independent repetitions per experiment. * was P<0.05, ** was P<0.01 and *** was P<0.001 versus (vs) sham group; # was P<0.05, ## was P<0.01 and ### was P<0.001 vs I/R group.

4.6. TUNEL staining

The rats' heart was fixed by paraformaldehyde and cut into paraffin section. TUNEL staining was used to detect the apoptosis of cardiomyocytes in each group. All reagents and procedures for TUNEL assay were from TUNEL Cell Apoptosis Detection Kit (TA201-02, TRANSGEN, ShangHai, China).

4.7. Western blot

Protein levels were analyzed by western blot technique, and GAPDH protein transcription was used as the internal loading control (Tao et al. 2018). Primary antibody to Bax (1:1000), Bcl2 (1:1000), active-caspase 3 (1:200), caspase 3 (1:500), p-PI3K (1:1000), PI3K (1:1000), p-AKT (1:1000) and AKT (1:1000) antibodies were purchased from Abcam.

4.8. Statistical analysis

The results were analyzed by SPSS 20.0. and the data were presented as mean±standard deviation. Student's t-test was used to compare the differences between the two groups, and one-way ANOVA with Duncan's posthoc test was used to compare the multiple groups. The correlation between groups was analyzed by Pearson's correlation coefficient (P<<0.05), which indicated statistically significant results.

Conflicts of interest: None declared.

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