Tropical Journal of Pharmaceutical Research June 2020; 19 (6): 1173-1178
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i6.8

Original Research Article

Morphine pretreatment reduces myocardial ischemia-reperfusion injury in heart failure rats via GSK-3β/Cx43 signaling proteins and apoptosis-related gene, Bcl-2/Bax

Xuelian Zhu[1], Zhihai Geng[1], Xi Han[2], Xianfeng Xin[1]*
[1]Department of Anesthesiology, First Affiliated Hospital of Jiamusi University, [2]Department of Anatomy, School of Basic Medical Sciences, Jiamusi University, Jiamusi, PR China

For correspondence:* ***Email:* *g84613@163.com*

Sent for review: 16 April 2020 *Revised accepted: 28 May 2020*

Abstract

Purpose: *To investigate the effect of morphine preconditioning on myocardial ischemia reperfusion injury in heart failure rats, and the mechanism(s) of action involved*
Methods: *Seventy-two healthy male Sprague-Dawley rats were assigned to 4 groups: sham, model, morphine-preconditioning and SB203580 inhibitor groups, each with 18 rats. The expressions of P-p38, p-glycogen synthetase kinase-3, and p-gap junction protein 43 in rat myocardial cells were assayed by Western blotting. The mRNA expression levels of Bcl-2 and Bax, and Bcl-2/Bax were determined using real-time fluorescence quantitative PCR.*
Results: *The expression levels of P-p38, p-glycogen synthetase kinase-3, p-gap junction protein 43, Bcl-2 mRNA and Bcl-2/Bax were significantly higher in the pretreatment group than in the model group, while Bax mRNA was significantly lower ($p < 0.05$). Moreover, the mRNA expression levels of P-p38, p-glycogen synthetase kinase-3, p-gap junction protein, Bcl-2, and Bcl-2/Bax in inhibitor-treated rats decreased significantly, when compared to the values for pretreatment rats; furthermore, Bax mRNA was markedly upregulated ($p < 0.05$).*
Conclusion: *Morphine preconditioning significantly inhibits the expressions of GSK-3β and Cx43 signaling proteins, as well as apoptosis-related gene, Bcl-2 and Bax. In addition, it inhibits the apoptosis of rat cardiomyocytes, and reduces myocardial injury, after ischemia reperfusion, via activation of the p38 MARK signaling pathway. This provides a new strategy for clinical reduction of myocardial injury after ischemia-reperfusion.*

Keywords: *Morphine, Pretreatment, GSK-3β/Cx43 signaling protein, Bcl-2/Bax, Heart failure, Ischemia-reperfusion injury*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Coronary artery disease is considered the main cause of morbidity and mortality globally. According to statistics, the incidence of coronary heart disease in China is gradually increasing and showing a trend towards younger people [1]. Heart failure is a serious stage in various heart diseases. With increase in the aging population, heart failure patients are prone to perioperative

myocardial ischemia and reperfusion injury during cardiac or non-cardiac surgery, with serious effect on the quality of life and safety of patients [2].

Studies have found that post-myocardial ischemia reperfusion increases myocardial infarction area and myocardial cell apoptosis, thereby seriously affecting cardiac function recovery and endangering the lives and safety of patients [3]. Therefore, there is need to evolve effective methods for protecting the myocardium. Ischemic preconditioning, a traumatic stimulation, is the most classical and effective method for protecting the myocardium from ischemic perfusion injury [4]. Morphine is an opioid which significantly reduces myocardial suppression and the incidence of heart failure [5].

In the present study, the effect of morphine preconditioning on myocardial ischemia reperfusion injury in cardiac failure rats, and the mechanism involved, were investigated.

EXPERIMENTAL

Animals

A total of 72 healthy male SD rats [provided by Animal Experimental Center of Anhui Medical University; production license SCXK (Anhui) 2015-0001] weighing 211 ± 37 g, were used for the study. They were housed in a laboratory at a temperature of 26 ± 3℃, humidity of 47 ± 11%, and photoperiod of 12-h day/12-h night.

This research received approval from the Animal Ethical Committee of First Affiliated Hospital of Jiamusi University (approval no. 20195631, and was conducted according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [6].

Major equipment and reagents

The major instruments and reagents used, and their makers (in brackets) were: Animal Ventilator (Shanghai Yuyan Scientific Instrument Co. Ltd, model: SAR-1000); Low-temperature High-speed Centrifuge (Shanghai Hetian Scientific Instrument Co. Ltd, model: TG18G); Optical microscope (Shanghai Optical Instrument Factory, model: SG-51); CytoFLEX flow cytometer (Shanghai Murui Biotechnology Co. Ltd); phosphate buffer (Shanghai Thermo Fisher Technology Co. Ltd); fetal bovine serum (Shanghai Huiying Biotechnology Co. Ltd); Rabbit anti-rat Bax polyclonal antibody (Wuhan Aimejie Technology Co. Ltd); Rabbit anti-rat p38 MARK polyclonal antibody (Shanghai Xuanling Biotechnology Co. Ltd); Rabbit anti-mouse GSK-3β antibody polyclonal antibody (Aimekit Technology Co. Ltd); Mouse anti-Cx43 monoclonal antibody (Shanghai Huzhen Industrial Co. Ltd), and morphine (Shenyang First Pharmaceutical Co. Ltd, production batch no. 20173351, specification: 10 mg/mL).

Animal groups

A rat model of myocardial reperfusion injury was established, and the rats were randomly divided into sham group, model ischemic group, morphine pretreatment group (pretreatment group) and SB203580 inhibitor group i.e. inhibitor of p38 mitogen activated protein kinase (p38 MARK), with 18 rats in each group. Sham operation rats received 1mL physiological saline 10 min before perfusion without ligation.
Rats in the model group were ligated at the coronary artery anterior descending arm for 35 minutes, and then released and ligated for 2 h after reperfusion. Normal saline (1 mL) was injected into the tail vein 10 min before perfusion. The rats in the pretreatment group were ligated at the coronary artery anterior descending arm for 35 minutes, and then released and ligated for 2 h for reperfusion. The rats were injected with 1 mol/L morphine 10 min before perfusion, while rats in the inhibitor group were given p38MARK inhibitor 5 min before morphine pretreatment.

Treatment indicators

The TCC staining was used to determine changes in myocardial infarction area in each group. After 2 h of myocardial reperfusion, the rat hearts were removed and injected with 0.5 mL of 0.25 % evanlan to expose the ischemic and non-ischemic areas: the ischemic areas were uncolored, while the non-ischemic areas were colored blue. The areas of myocardial infarction in the various groups were compared.

Cell viability

Changes in viability of cardiomyocytes were determined using CCK-8 method. The cardiomyocytes were seeded in 96-well plates at a density of 400000 cells/mL, followed by addition of 10 μL of CCK-8 and incubation for 2 h. The absorbance of each well was read at 450 nm.

Evaluation of apoptosis

Apoptosis of rat cardiomyocytes was measured with flow cytometry, while the expressions of P-p38, p-glycogen synthetase kinase-3 (p-GSK-3) and p-gap junction protein 43 (p-Cx43) were

determined with Western blotting assay. The mRNA expression levels of Bcl-2 and Bax, and Bcl-2/Bax ratio were determined using real-time fluorescence quantitative PCR.

Statistical evaluation

Measurement data are presented as mean ± standard deviation (SD), and were statistically compared with one-way ANOVA and LSD *t*-test using SPSS 22.0 software package. Values of $p < 0.05$ were taken as indicative of significant differences.

RESULTS

Myocardial infarction area

Myocardial infarction area was significantly higher in model rats than in sham rats. Myocardial infarction area in pretreatment rats was markedly decreased, relative to model rats, but it was markedly increased in inhibitor-treated rats. These results are shown in Figure 1 and Table 1.

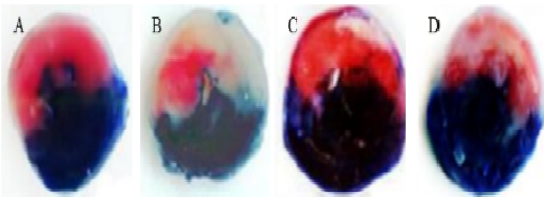

Figure 1: Myocardial infarctions area of the groups: A (sham surgery), B (model), C (pretreatment) and D (inhibitor).

Table 1: Size of myocardial infarction area (mean ± SD, n = 18)

Group	Myocardial infarction (%)
Sham	0.02 ± 0.01
Model	0.15 ± 0.03[a]
Pretreatment	0.07 ± 0.03[ab]
Inhibitor	0.12 ± 0.02[ac]
F	102.26
P-value	< 0.001

[a,b,c]$P < 0.05$, where [a, b,] and [c] refer to comparison with sham, model and pretreatment groups, respectively

Myocardial cell viability

Table 2 shows that the viability of myocardial cells was markedly lower in model rats than in sham rats. Myocardial cell viability was significantly higher in pretreatment rats than in model rats, but was markedly less in inhibitor-treated rats than in pretreatment rats ($p < 0.05$).

Table 2: Myocardial cell viability amongst the groups (mean ± SD, n = 18)

Group	Cell viability (%)
Sham	1.01 ± 0.12
Model	0.62 ± 0.09[a]
Pretreatment	0.83 ± 0.03[ab]
Inhibitor	0.71 ± 0.05[ac]
F	79.02
P-value	< 0.001

[a, b, c]$P < 0.05$, where [a, b,] and [c] refer to comparison with sham, model and pretreatment rats, respectively

Apoptosis of rat cardiomyocytes

Apoptosis of myocardial cells was significantly higher in the model group than in sham group, but was markedly higher in pretreatment group than in the model group ($p < 0.05$). Compared with the pretreatment group, apoptosis of myocardial cells in the inhibitor group was significantly decreased ($p < 0.05$). These results are shown in Table 3.

Table 3: Apoptosis of rat myocardial cells (mean ± SD, n = 18)

Group	Apoptosis of myocardial
Sham	1.01 ± 0.07
Model	2.14 ± 0.73[a]
Pretreatment	1.37 ± 0.11[ab]
Inhibitor	1.79 ± 0.75[ac]
F	15.68
P-value	< 0.001

[a, b, c]$P < 0.05$, where [a, b,] and [c] refer to comparison with sham, model and pretreatment rats, respectively

Expression levels of P-p38, p-glycogen synthetase kinase-3 and p-gap junction protein 43 in rat myocardial cells

The expression levels of P-p38, p-glycogen synthetase kinase-3 and p-gap junction protein 43 were markedly downregulated in model rats, relative to sham operation rats, but they were markedly higher in pretreatment rats than in model rats ($p < 0.05$). However, the expression levels of p-p38, p-GSK-3 and p-Cx43 in the inhibitor group were significantly lower than corresponding levels in pretreatment group ($p < 0.05$). These data are presented in Figure 2 and Table 4.

Expression levels of Bcl-2 mRNA and Bax mRNA, and Bcl-2 /Bax ratio in rat muscle cells

Table 5 shows that mRNA level of Bcl-2, and Bcl-2/Bax in rat myocardial cells in the model group were significantly lower than those in the sham group, while Bax mRNA was significantly higher ($p < 0.05$). In contrast, mRNA level of Bcl-2, and

Bcl-2/Bax ratio were markedly higher in pretreatment rats than in model rats, while Bax mRNA was significantly lower ($p < 0.05$). However, mRNA level of Bcl-2, and Bcl-2/Bax ratio were markedly downregulated, relative to pretreatment group, while Bax mRNA was significantly higher ($p < 0.05$).

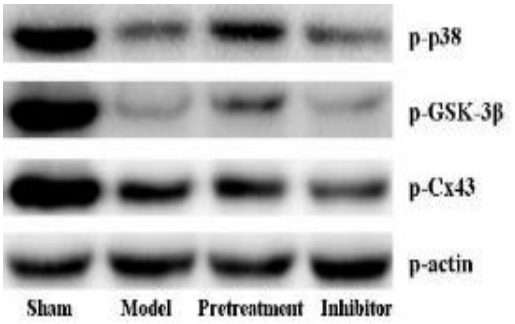

Figure 2: Expression levels of p-p38, p-GSK-3, and p-Cx43 in rat myocardial cells in each group

Table 4: Expression levels of P-p38, p-glycogen synthetase kinase-3 and p-gap junction protein 43 in rat myocardial cells (mean ± SD, n = 18)

Group	p-p38	p-glycoprotein synthetase kinase-3β	p-Gap junction protein-43
Sham	1.01 ± 0.35	0.99 ± 0.47	1.01 ± 0.36
Model	0.17 ± 0.23[a]	0.24 ± 0.12[a]	0.26 ± 0.22[a]
Pre-treatment	0.53 ± 0.19[ab]	0.65 ± 0.09[ab]	0.71 ± 0.23[ab]
Inhibitor	0.24 ± 0.12[ac]	0.45 ± 0.24[ac]	0.47 ± 0.24[ac]
F	46.41	24.36	25.96
P-value	< 0.001	< 0.001	< 0.001

[a, b, c]$P < 0.05$, where [a, b,] and [c] refer to comparison with sham, model and pretreatment rats, respectively

Table 5: m-RNA Bcl-2 and Bax, and Bcl-2/Bax in rat myocardial cells (mean ± SD, n = 18)

Group	Bcl-2mRNA	Bcl-2/Bax	Bax mRNA
Sham	0.99 ± 0.03	1.00 ± 0.03	1.00 ± 0.01
Model	0.76 ± 0.13[a]	0.59 ± 0.22[a]	1.39 ± 0.11[a]
Pretreatment	1.03 ± 0.17[b]	1.15 ± 0.24[ab]	1.11 ± 0.06[ab]
Inhibitor	0.87 ± 0.02[ac]	0.76 ± 0.02[ac]	1.28 ± 0.05[ac]
F	22.87	41.54	118.69
P-value	< 0.001	< 0.001	< 0.001

[a]$P < 0.05$, vs sham; [b]$p < 0.05$, vs model rats; [c]$p < 0.05$, vs pretreatment rats

DISCUSSION

Heart failure is a clinical syndrome associated with progressive dysfunction and circulatory failure. The stimulation of hypertension and coronary heart disease leads to hypertrophy, fibrosis and apoptosis of cardiomyocytes. At the same time, the expressions of marker genes also change, resulting in decreased contractility and enlargement of the myocardium, which eventually lead to heart failure [7]. The prevention and treatment of ischemia reperfusion injury (one of the postoperative complications of coronary artery treatment in clinical practice) has received extensive attention, with continuous development of various clinically-difficult cardiac techniques [8]. It has been reported that ischemic preconditioning of myocardial tissue before perfusion significantly reduces myocardial ischemia reperfusion injury for a long time thereafter, thereby playing a protective role [9]. Morphine is an important opioid. It is known that opioids are frequently used as analgesics during surgery. Studies have confirmed that opioid receptors are involved in the myocardial protective effect of opioid preconditioning against heart ischemia [10]. Morphine activates G-protein through cardiac opioid receptors, and then activates protein kinase C, thus playing a role in alleviating myocardial ischemia [11].

Myocardial infarction refers to the occlusion of coronary arteries, interruption of blood flow, and partial myocardial necrosis due to severe and persistent ischemia. Some studies have found that the myocardial infarction area is an important index for evaluating myocardial injury [12]. Apoptosis refers to the orderly death of cells, which is also the most significant feature in ischemia-reperfusion injury. Studies have revealed that the more severe the myocardial cell apoptosis, the more severe the myocardial infarction [13]. The results of this study showed that morphine preconditioning significantly improved the viability of myocardial cells, inhibited the apoptosis of myocardial cells, and reduced the area of myocardial infarction.

Studies on rabbit myocardial ischemia reperfusion injury have shown that p38 MAPK expression significantly changed due to the mitigating influence of morphine preconditioning on ischemia reperfusion-induced injury on rabbit myocardium [14]. The p38 MARK signaling pathway may be involved in morphine preconditioning-induced protection against myocardial ischemia-reperfusion injury. A member of the MAPK family, P38 phosphorylates transcription factors and effector proteins, a process which is important for initiation of

apoptosis and quiescence of cell cycle. The GSK-3β is a serine/threonine kinase found mainly in mammalian eukaryotic cells. Studies have revealed that GSK-3β beta affects blood glucose concentration and regulates Bax/hexokinase (HK II) ratio, the proportion of which is involved in regulating cell apoptosis [15]. Increased levels of p-gsk-3 morphine constitute an important link in the myocardial protective effect of morphine.

An important connexin, Cx43 is widely found in human tissues and cells, and is considered a unique component of cardiac gap junction [16]. Tania et al [17] found that the expression level of Cx43 plays an important role in maintaining normal heart function. It is known that Bcl-2 is a tumor suppressor gene which significantly inhibits apoptosis, while Bax is an apoptotic gene which inhibits Bcl-2. It has been reported that the Bcl-2/Bax ratio is a key factor that determines the degree of apoptosis [18]. The results of this study showed that morphine pretreatment significantly raised mRNA levels of p-p38, p-GSK-3β, p-Cx43, Bcl-2 and Bcl-2/Bax, while Bax mRNA levels were decreased.

CONCLUSION

The findings obtained in this study suggest that morphine preconditioning may significantly influence the expressions of p-glycogen synthetase kinase-3 and p-gap junction protein 43 signaling proteins, and the expressions of the apoptosis-related genes, Bcl-2 and Bax, by activating p38 MARK signaling pathway, inhibiting apoptosis of rat cardiomyocytes, and reducing myocardial injury after ischemia reperfusion. Thus, this research provides a new strategy for clinical reduction of myocardial injury after ischemia-reperfusion.

DECLARATIONS

Acknowledgement

This study was supported by scientific research project of Heilongjiang Health and Family Planning Commission (no. 2018-314).

Conflict of interest

No conflict of interest is associated with this work.

Authors' contributions

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Xuelian Zhu; Xuelian Zhu, Zhihai Geng, Xi Han, Xianfeng Xin collected and analyzed the data; Xianfeng Xin wrote the text. All authors read and approved the manuscript for publication.

REFERENCES

1. *Ostuzzi G, Turrini G, Gastaldon C, Papola D, Rayner L, Caruso R, Grassi L, Hotopf M, Barbui C. Efficacy and acceptability of antidepressants in patients with ischemic heart disease: systematic review and meta-analysis. Int Clin Psychopharmacol 2018; 34(2): 1.*
2. *Chen C, Lu W, Wu G, Lv L, Chen W, Huang L, Wu X, Xu N, Wu Y. Cardioprotective effects of combined therapy with diltiazem and superoxide dismutase on myocardial ischemia-reperfusion injury in rats. Life Sci 2017; 183(5): 50-59.*
3. *Zeng G, Liu H, Wang H. Amelioration of myocardial ischemia-reperfusion injury by SIRT4 involves mitochondrial protection and reduced apoptosis. Biochem Biophys Res Commun 2018; 502(1): 113-118.*
4. *Yuan Y, Cao W, Hong Y, Guo X, Wang Y, Wang Y, Wang X, Hu P. Tilianin pretreatment prevents myocardial ischemia-reperfusion injury via preservation of mitochondrial function in rat heart. Phytomedicine 2017; 34(2): 106-114.*
5. *Wang XH, Zeng JF, Lin C, Chen SB. Effects of morphine and sufentanil preconditioning against myocardial ischemic-reperfusion injury in rabbits. Int J Clin Exp Med 2015; 8(9): 15692-15699.*
6. *World Health Organization. Principles of laboratory animal care. WHO Chron 1985; 39: 51-56.*
7. *Bradic J, Jeremic N, Petkovic A, Jeremic J, Zivkovic V, Srejovic I, Sretenovic J, Matic S, Jakovljevic V, Tomovic M. Cardioprotective effects of Galium verum L. extract against myocardial ischemia-reperfusion injury. Arch Physiol Biochem 2019; 53(1): 1-8.*
8. *Jiang B, Liu Y, Liang P, Li Y, Liu Z, Tong Z, Lv Q, Liu M, Xiao X. MicroRNA-126a-5p enhances myocardial ischemia-reperfusion injury through suppressing Hspb8 expression. Oncotarget 2017; 8(55): 94172-94187.*

9. Li Y, Xiang Y, Zhang S, Wang Y, Yang J, Liu W, Xue F. Intramyocardial injection of thioredoxin 2-expressing lentivirus alleviates myocardial ischemia-reperfusion injury in rats. Am J Transl Res 2017; 9(10): 4428-4439.
10. Dorsch M, Behmenburg F, Raible M, Blase D, Grievink H, Hollmann MW, Heinen A, Huhn R. Morphine-Induced Preconditioning: Involvement of Protein Kinase A and Mitochondrial Permeability Transition Pore. PLoS ONE 2016; 11(3): 151025.
11. Lu Y, Hu J, Zhang Y, Dong CS, Wong GT. Remote intrathecal morphine preconditioning confers cardioprotection via spinal cord nitric oxide/cyclic guanosine monophosphate/protein kinase G pathway. J Surg Res 2015; 193(1): 43-51.
12. Hashmi S, Al-Salam S. Acute myocardial infarction and myocardial ischemia-reperfusion injury: A comparison. Int J Clin Exp Pathol 2015; 8(8): 8786-8796.
13. Yu L, Di W, Dong X, Li Z, Xue X, Zhang J, Wang Q, Xiao X, Han J, Yang Y, et al. Diallyl trisulfide exerts cardioprotection against myocardial ischemia-reperfusion injury in diabetic state, role of AMPK-mediated AKT/GSK-3β/HIF-1α activation. Oncotarget 2017; 8(43): 74791-74805.
14. Chen T, Wang RC, Lin SB, Su JP, Guo WF. Effect of inhibiting P38MAPK on inflammatory factors and cell apoptosis during flap ischemia-reperfusion injury. J Hainan Med Univ 2017; 23(5): 1-4.
15. Yu L, Di W, Dong X, Li Z, Xue X, Zhang J, Wang Q, Xiao X, Han J, Yang Y, et al. Diallyl trisulfide exerts cardioprotection against myocardial ischemia-reperfusion injury in diabetic state, role of AMPK-mediated AKT/GSK-3β/HIF-1α activation. Oncotarget 2017; 8(43): 74791-74805.
16. Zhang P, Xu J, Hu W, Yu D, Bai X. Effects of Pinocembrin Pretreatment on Connexin 43 (Cx43) Protein Expression After Rat Myocardial Ischemia-Reperfusion and Cardiac Arrhythmia. Med Sci Monit 2018; 24(2): 5008-5014.
17. Martins-Marques T, Catarino S, Marques C, Pereira P, Girão H. To beat or not to beat: Degradation of Cx43 imposes the heart rhythm. Biochem Soc Trans 2015; 43(3): 476-481.
18. Cai W, Shen WD. Anti-Apoptotic Mechanisms of Acupuncture in Neurological Diseases: A Review. Am J Chin Med 2018; 46(3): 1-21.