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MiR-808 inhibits cardiomyocyte apoptosis and expressions of caspase-3 and caspase-9 in rats with myocardial infarction by regulating TGF-β1 signaling pathway

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Abstract. – OBJECTIVE: To investigate the effects of micro ribonucleic acid (miR)-808 on cardiomyocyte apoptosis and expressions of caspase-3 and caspase-9 in rats with myocardial infarction (MI) by regulating the transforming growth factor-β1 (TGF-β1) signaling pathway.

MATERIALS AND METHODS: A total of 24 specific pathogen-free female Sprague-Dawley rats were enrolled and randomly divided into normal group, model group, and miR-808 group, 8 rats in each group. In the model group and miR-808 group, MI model was prepared by ligation of the left anterior descending coronary artery in the rats. The miR-808 group was transfected with miR-808 lentivirus after the model was established. After one week of intervention, the expression of TGF-β1 was detected by reverse transcription-polymerase chain reaction (RT-PCR). The cardiac function of rats was determined by echocardiography. The myocardium of rats was observed by Masson staining. The cardiomyocyte apoptosis of rats was examined by TdT-mediated dUTP-biotin nick end labeling (TUNEL) method. The expression levels of caspase-3 and caspase-9 were detected by Western blotting.

RESULTS: The expression of TGF-β1 mRNA was higher in the model group than that in the normal group ($p<0.05$), but compared with that in the model group, it was lower in the miR-808 group. The myocardial function and cardiomyocyte survival rate in the miR-808 group was better and higher than those in the model group ($p<0.05$). The expression levels of caspase-3 and caspase-9 in the miR-808 group were lower than those in the model group ($p<0.05$).

CONCLUSIONS: MiR-808 can inhibit cardiomyocyte apoptosis in rats with MI by down-regulating TGF-β1 expression and inhibiting the expressions of caspase-3 and caspase-9.

Key Words:
MiR-808, Myocardial infarction, Cardiomyocyte apoptosis.

Introduction

Myocardial infarction (MI) is a clinically common coronary heart disease characterized by severe myocardial ischemia. After the onset, thrombosis will be suddenly formed, resulting in vascular occlusion[1,2]. In recent years, the working environment and eating habits have changed dramatically, so the incidence of MI is increasing year by year according to epidemiological statistics. The number of MI deaths accounts for more than half of the deaths from cardiovascular diseases, so it has become the number one killer that seriously threatens the human health[3].

Micro-ribonucleic acids (miRNAs) are a class of non-coding small RNAs that are highly conserved during the evolution of higher animals[4,5]. MiRNAs are closely related to human heart diseases such as sudden MI, ischemia, abnormal hypertrophy, remodeling, and sudden heart failure[6,7]. MiR-808 is abundant in human and animal heart tissues[8]. Transforming growth factor-β (TGF-β) is a kind of cytokine that play an important role in regulating the life activities of the body. It is found that TGF-β1 is closely correlated with MI[9-12].

Caspases are a family of enzymes involved in apoptosis, which mediate and perform its functions during apoptosis. It is currently found that caspases-3 and caspases-9 are closely related to

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cardiomyocyte apoptosis[13-19]. However, whether miR-808 can ameliorate MI by regulating TGF-β1 thus affecting the expressions of caspases-3 and caspases-9 has not been reported. Therefore, this study aims to clarify the mechanism of miR-808 in MI by establishing the rat model of MI.

Materials and Methods

Experimental Animals and Grouping

A total of 24 specific pathogen-free (SPF) female Sprague-Dawley rats aged 2 months old and weighing about (200 g±20) g were enrolled and randomly divided into normal group (n=8), model group (n=8), and miR-808 group (n=8). The rats were fed in SPF animal experiment center, and they were administrated with adequate aseptic feed and water daily. This investigation was approved by the Animal Ethics Committee of Central South University Animal Center.

Main Experimental Reagents and Instruments

MiR-808 (Shanghai Genechem Co., Ltd., Shanghai, China), TGF-β1 (ABI, Applied Biosystems, Foster City, CA, USA), caspases-3 and caspases-9 (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay kit, echocardiography (Visual SonicsInc., Amsterdam, Netherlands), and polymerase chain reaction (PCR) instrument (ABI, Applied Biosystems, Foster City, CA, USA).

Animal Model Preparation and Processing

The rats were anesthetized with 7% chloral hydrate intraperitoneally. The hair was removed from the chest. After the disinfection with 75% ethanol, the skin was cut open, and subcutaneous tissues were bluntly dissected to expose the heart. The left coronary artery was ligated at 3 mm away from the origin to prepare the rat model of MI. The rats in miR-808 group were injected with miR-808 lentivirus after the model was established. No treatment was performed in normal group. The rats were fed with adequate nutrients and water in a ventilated environment.

Echocardiography

After one week of intervention, the left ventricular ejection fraction of rats was determined and calculated by echocardiography to evaluate the cardiac function.

Myocardial Masson Staining

After one week of intervention, the paraffin sections were prepared. The rat myocardium was stained with Masson staining kit, dehydrated, transparentized, and photographed.

Detection of TGF-β1 By Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The tissues were taken, ground with a grinding rod and liquid nitrogen, from which the RNA was extracted using the extractant and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) under the following conditions: reaction at 55°C for 5 min, denaturation at 95°C for 10 min, lasting for 10 s, annealing at 60°C for 40 s. The primer sequences are shown in Table I.

Examination of Cardiomyocyte Apoptosis by TUNEL Method

The TUNEL kit was applied to examine cardiomyocyte apoptosis. Under the microscope, 5 fields of view were randomly selected and photographed. The brown color was positive. The ratio (%) of the number of apoptotic cells to the total number of cells was recorded as the apoptotic index.

Detection of Expression Levels of Caspase-3 and Caspase-9 by Western Blotting

The total protein concentration was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The protein was loaded, separated *via* gel electrophoresis, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was sealed by the prepared skim milk at 4°C for 1.5 h and incubated with rabbit anti-human caspase-3 monoclonal antibody and rabbit anti-human caspase-9 monoclonal antibody at 4°C overnight. After washing by phosphate-buffered saline and tween (PBST), it was added with goat anti-rabbit second antibody for reaction at room tem-

Table I. Primer sequences.

Name	Primer sequence
TGF-β1	F: 5'-CGCCTGCAGAGATTCAAG-3' R: 5'-AGGTAACGCCAGGAATTGTTGCTA-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'

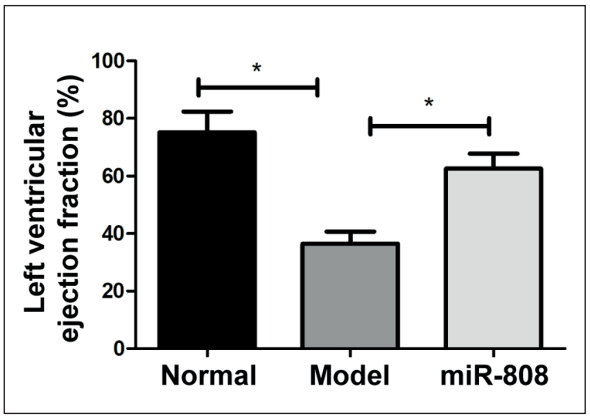

Figure 1. Left ventricular ejection fraction of each group. Note: *p<0.05 *vs.* model group.

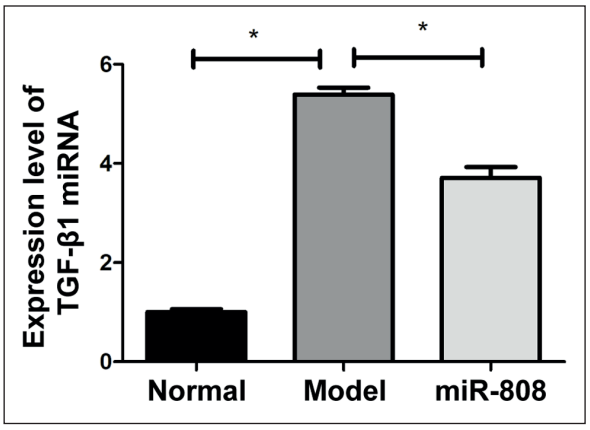

Figure 3. Expression level of TGF-β1 in the three groups of rats detected via RT-PCR. Note: *p<0.05 *vs.* model group.

perature for 1.5 h. Finally, the protein bands were exposed in the gel imaging system.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. The *t*-test was performed for data with normal distribution and equal variance, the corrected *t*-test for data with normal distribution and unequal variance, and the non-parametric test for data not meeting the normal distribution and equality of variance. Besides, the rank sum test was adopted for ordinal data, and the Chi-square test for comparison between the two groups.

Results

Echocardiography

The left ventricular ejection fraction in miR-808 group was higher than that in model group (p<0.05), and it was higher in normal group than that in model group (p<0.05; Figure 1).

Myocardial Masson Staining

The blue muscle fiber tissues were rarely observed in normal group, and cardiomyocytes were arranged neatly and orderly. There were more blue fiber tissues in model group, the collagen accumulated significantly, and cardiomyocytes were disorderly arranged. The miR-808 group had fewer blue fiber tissues that were scattered, and the cell arrangement was less disordered than that of the model group (Figure 2).

RT-PCR Results

The results of RT-PCR exhibited that the expression level of TGF-β1 in the miR-808 group was lower than that in model group (p<0.05), and compared with that in model group, its expression was lower in normal group (p<0.05; Figure 3).

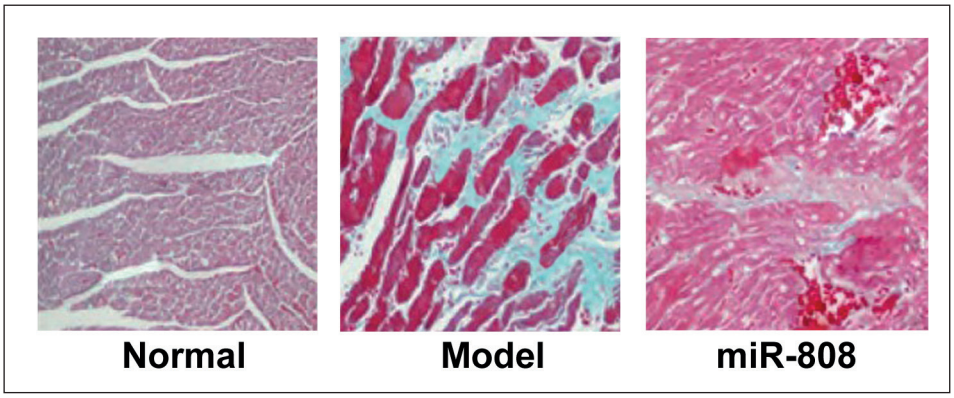

Figure 2. Masson staining result of each group (magnification: 200×).

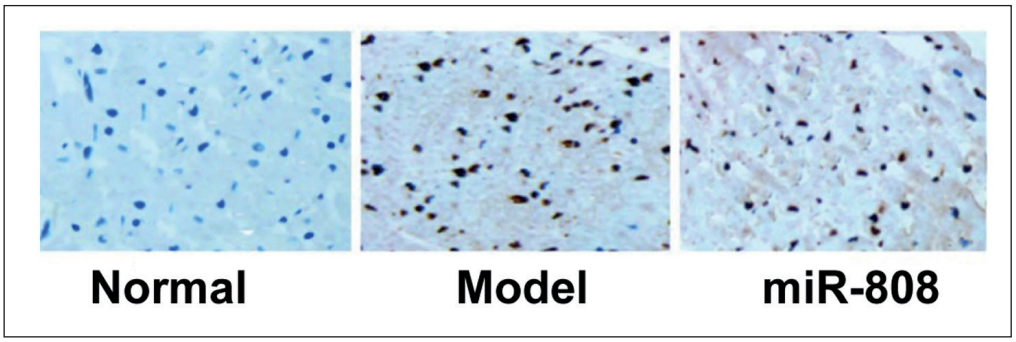

Figure 4. TUNEL staining diagrams (magnification: 400×).

TUNEL Results

The results of TUNEL demonstrated that there were a large number of apoptotic cardiomyocytes in the model group. The mortality of cardiomyocytes in the miR-808 group was lower than that in model group ($p<0.05$), and compared with that in model group, it was lower in normal group ($p<0.05$; Figures 4, 5).

Western Blotting Results

The expression levels of caspase-3 and caspase-9 in model group were higher than those in the miR-808 group ($p<0.05$), and compared with those in model group, they were lower in normal group ($p<0.05$; Figures 6, 7).

Discussion

MI is a heart disease with rapid onset and dramatic changes in the condition, and it easily induces local cardiomyocyte remodeling, which ultimately leads to heart failure. The current research shows that irreversible myocardial damage and heart failure caused by MI are related to cardiomyocyte apoptosis. The apoptosis of cardiomyocytes is complex and diverse, but the original myocardium in the heart will gradually be replaced with the continuously formed compensatory collagen fibers due to cardiomyocyte apoptosis of any reason, which will in turn aggravate the hyperplasia, hypertrophy, and fibrosis of myocardium, ventricular remodeling, and even gradual necrosis, eventually developing into whole heart failure and even death. Therefore, scholars and experts from all over the world attach great importance to cardiomyocyte death caused by MI and the related pathway mechanisms and various series of cascade reactions caused by cardiomyocyte death[20,21].

MiRNAs are a kind of conserved RNAs about 18-25 nt in length, which, by binding to related target genes, activate or inhibit the translation of target genes, thereby controlling a series of life processes of cells, including growth, division, differentiation, and even apoptosis. The previous study was limited to body tumors, metabolic abnormalities, and certain endogenous diseases. In

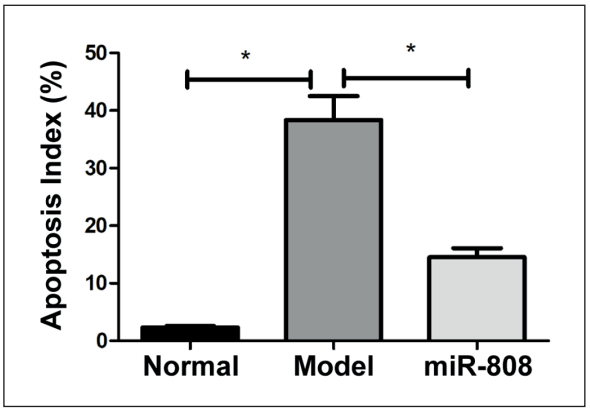

Figure 5. TUNEL staining results. Note: *$p<0.05$ *vs.* model group

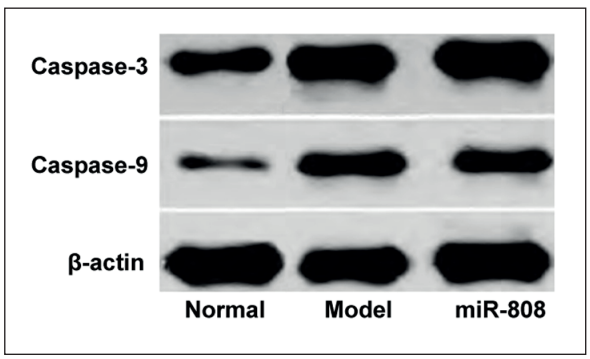

Figure 6. Protein bands detected via Western blotting.

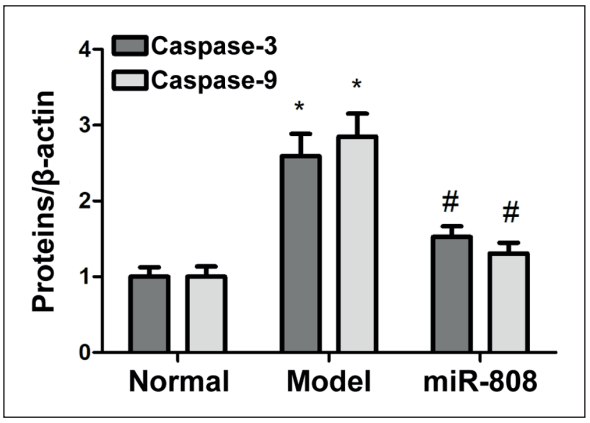

Figure 7. Protein expression results in the three groups. Note: *p<0.05 vs. normal group; #p<0.05 *vs.* miR-808 group.

recent years, research priorities have shifted to the cardiovascular system. MiRNAs are involved in the body's irreversible myocardial damage, cardiac ejection, contractile dysfunction, cardiac remodeling, heart failure and infarction, and post-ischemia perfusion[6,7]. Among many miRNAs associated with heart disease, miR-808 plays a significant role in regulating MI and cardiomyocyte apoptosis. TGF-β1 is a kind of cytokines that participate in regulating the life activities of the body. It is found that through the continuous stimulation of TGF-β1, myocardial cells will gradually become necrotic and apoptotic. TGF-β1 can play its role of mediation, causing myocardial thickening and cardiac function loss by cooperating with other signaling pathways, eventually leading to myocardial fibrosis. Therefore, it has been known as the most critical and direct endogenous cytokine that causes myocardial fibrosis. In addition, the further study verifies that TGF-β1 can induce massive abnormal activation and differentiation of cardiac tissue fibroblasts, which in turn produces excessively harmful collagen fibers and multimeric fibronectin, and inhibits extracellular matrix decomposition, thus resulting in more serious fibrosis, MI and apoptosis in the whole heart. However, when TGF-β1 is knocked out, it is found that the degree of cardiac fibrosis in rats of the same age is lower than that in the rats without TGF-β1 knockout, the MI rate is lower, and the occurrence of apoptosis is less. The degree of myocardial fibrosis, cardiac hypertrophy, ventricular remodeling, MI rate and cardiomyocyte apoptosis are higher in rats injected with TGF-β1 exogenous RNA than those in normal rats of the same age. Compared with those in rats injected with TGF-β1 antagonist, the cardiomyocyte apoptosis rate and MI rate are lower in normal rats of the same age, and the systolic and diastolic functions of rats are also better[9-12,22,23]. This study also showed that the cardiac function is poorer and cardiomyocyte apoptosis is worse in rats in the high-expression TGF-β1 group than those in normal group. Caspases are a family of enzymes involved in the regulation of apoptosis, which mediate and perform its functions during apoptosis. Its abnormal expression or massive activation will cause apoptosis in normal cells. There are many kinds of apoptotic factors, but currently it has been found that caspases-3 and caspases-9 are closely related to the apoptosis of cardiomyocytes. There are two ways to cause apoptosis. Some of the dead macromolecules in the extracellular pathway bind to apoptotic sites and signals, and after the complex reactions, caspase-3 is activated, causing apoptosis. The apoptotic factors in the intracellular pathway bind to apoptotic protein-activating enzyme to form a complex, thus activating caspases-9 in the cytoplasm. Then, caspases-9 continues to bind to the downstream caspase-3 precursor to activate caspase-3, ultimately causing apoptosis[15-19]. The novelty of our present study was that we first attempted to investigate the role of miR-808 in the development of MI and also explored its potential mechanism via *in vivo* and *in vitro* experiments.

The echocardiogram of this research showed that the cardiac function of miR-808 group was better than that of MI group, indicating that miR-808 can improve the recovery of myocardial function after MI. RT-PCR results prompted that the expression of TGF-β1 in miR-808 group was lower than that in model group, revealing that miR-808 can inhibit the expression of TGF-β1 after MI. TUNEL results exhibited that the mortality of cardiomyocytes in the miR-808 group was lower than that in model group, demonstrating that miR-808 can reduce cardiomyocyte apoptosis after MI. Western blotting results displayed that the expression levels of caspase-3 and caspase-9 in model group were higher in model group than those in miR-808 group, suggesting that miR-808 can inhibit the expressions of caspase-3 and caspase-9 after MI. In conclusion, miR-808 can inhibit the cardiomyocyte apoptosis after MI. The specific mechanism is that miR-808 inhibits the expression of TGF-β1 after MI, thereby inhibiting the expressions of apoptosis-related factors, caspases-3 and caspases-9, ultimately reducing cardiomyocyte apoptosis after MI.

Conclusions

In short we indicated that miR-808 can inhibit cardiomyocyte apoptosis in rats with MI by down-regulating TGF-β1 expression and inhibiting the expressions of caspase-3 and caspase-9.

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Conflict of Interests

The authors declare that they have no conflict of interests.

References

1) Bibas L, Thanassoulis G. Fractional flow reserve-guided multivessel angioplasty in myocardial infarction. N Engl J Med 2017; 377: 396-397.
2) Waterford SD, Di Eusanio M, Ehrlich MP, Reece TB, Desai ND, Sundt TM, Myrmel T, Gleason TG, Forteza A, de Vincentiis C, DiScipio AW, Montgomery DG, Eagle KA, Isselbacher EM, Muehle A, Shah A, Chou D, Nienaber CA, Khoynezhad A. Postoperative myocardial infarction in acute type A aortic dissection: a report from the International Registry of Acute Aortic Dissection. J Thorac Cardiovasc Surg 2017; 153: 521-527.
3) Adam M, Oh SL, Sudarshan VK, Koh JE, Hagiwara Y, Tan JH, Tan RS, Acharya UR. Automated characterization of cardiovascular diseases using relative wavelet nonlinear features extracted from ECG signals. Comput Methods Programs Biomed 2018; 161: 133-143.
4) Smirnova L, Grafe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG. Regulation of miRNA expression during neural cell specification. Eur J Neurosci 2005; 21: 1469-1477.
5) Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA unction. Dev Cell 2003; 4: 205-217.
6) Lin RC, Weeks KL, Gao XM, Williams RB, Bernardo BC, Kiriazis H, Matthews VB, Woodcock EA, Bouwman RD, Mollica JP, Speirs HJ, Dawes IW, Daly RJ, Shioi T, Izumo S, Febbraio MA, Du XJ, McMullen JR. PI3K(p110 alpha) protects against myocardial infarction-induced heart failure: identification of PI3K-regulated miRNA and mRNA. Arterioscler Thromb Vasc Biol 2010; 30: 724-732.
7) Zhao N, Yu H, Yu H, Sun M, Zhang Y, Xu M, Gao W. MiRNA-711-SP1-collagen-I pathway is involved in the anti-fibrotic effect of pioglitazone in myocardial infarction. Sci China Life Sci 2013; 56: 431-439.
8) Lin Y, Lai Z. Comparative analysis reveals dynamic changes in miRNAs and their targets and expression during somatic embryogenesis in longan (Dimocarpus longan Lour.). PLoS One 2013; 8: e60337.
9) Ko H, So Y, Jeon H, Jeong MH, Choi HK, Ryu SH, Lee SW, Yoon HG, Choi KC. TGF-beta1-induced epithelial-mesenchymal transition and acetylation of Smad2 and Smad3 are negatively regulated by EGCG in human A549 lung cancer cells. Cancer Lett 2013; 335: 205-213.
10) Zhou Y, Zhang L, Zhao W, Wu Y, Zhu C, Yang Y. Nanoparticle-mediated delivery of TGF-beta1 miRNA plasmid for preventing flexor tendon adhesion formation. Biomaterials 2013; 34: 8269-8278.
11) Festuccia C, Bologna M, Gravina GL, Guerra F, Angelucci A, Villanova I, Millimaggi D, Teti A. Osteoblast conditioned media contain TGF-beta1 and modulate the migration of prostate tumor cells and their interactions with extracellular matrix components. Int J Cancer 1999; 81: 395-403.
12) Liu F, Kong X, Lv L, Gao J. TGF-beta1 acts through miR-155 to down-regulate TP53INP1 in promoting epithelial-mesenchymal transition and cancer stem cell phenotypes. Cancer Lett 2015; 359: 288-298.
13) Fujita E, Egashira J, Urase K, Kuida K, Momoi T. Caspase-9 processing by caspase-3 via a feedback amplification loop in vivo. Cell Death Differ 2001; 8: 335-344.
14) Fan Y, Bergmann A. The cleaved-caspase-3 antibody is a marker of caspase-9-like DRONC activity in Drosophila. Cell Death Differ 2010; 17: 534-539.
15) Rodriguez-Berriguete G, Galvis L, Fraile B, de Bethencourt FR, Martinez-Onsurbe P, Olmedilla G, Paniagua R, Royuela M. Immunoreactivity to caspase-3, caspase-7, caspase-8, and caspase-9 forms is frequently lost in human prostate tumors. Hum Pathol 2012; 43: 229-237.
16) Zarnescu O, Brehar FM, Chivu M, Ciurea AV. Immunohistochemical localization of caspase-3, caspase-9 and Bax in U87 glioblastoma xenografts. J Mol Histol 2008; 39: 561-569.
17) Lou GG, Yao HP, Xie LP. Brucea javanica oil induces apoptosis in T24 bladder cancer cells via upregulation of caspase-3, caspase-9, and inhibition of NF-kappaB and COX-2 expressions. Am J Chin Med 2010; 38: 613-624.
18) Sun C, Liu H, Guo J, Yu Y, Yang D, He F, Du Z. MicroRNA-98 negatively regulates myocardial infarction-induced apoptosis by down-regulating Fas and caspase-3. Sci Rep 2017; 7: 7460.
19) Burke SP, Smith L, Smith JB. cIAP1 cooperatively inhibits procaspase-3 activation by the caspase-9 apoptosome. J Biol Chem 2010; 285: 30061-30068.
20) Thygesen K. 'Ten Commandments' for the Fourth Universal Definition of Myocardial Infarction 2018. Eur Heart J 2019; 40: 226.
21) Thygesen K, Alpert JS, Jaffe AS, Chaitman BR, Bax JJ, Morrow DA, White HD. Fourth Universal Definition of Myocardial Infarction (2018). Circulation 2018; 138: e618-e651.
22) Hanna A, Frangogiannis NG. The role of the TGF-beta superfamily in myocardial infarction. Front Cardiovasc Med 2019; 6: 140.
23) Zhou FQ, Zhao XF, Liu FY, Wang SS, Hu HL, Fang Y. MiR-101a attenuates myocardial cell apoptosis in rats with acute myocardial infarction via targeting TGF-beta/JNK signaling pathway. Eur Rev Med Pharmacol Sci 2019; 23: 4432-4438.