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Effect of miR-203 expression on myocardial fibrosis

Q. HE[1], C.-M. WANG[2], J.-Y. QIN[3], Y.-J. ZHANG[1], D.-S. XIA[1], X. CHEN[1], S.-Z. GUO[1], X.-D. ZHAO[1], Q.-Y. GUO[1], C.-Z. LU[1]

[1]Department of Cardiology, Tianjin First Center Hospital, Tianjin, China
[2]Department of Human Anatomy & Histology, Tianjin Medical University, Tianjin, China
[3]Department of Physiology and Pathophysiology, Tianjin Medical University, Tianjin, China

Qiang He and Chunmei Wang contributed equally to this paper

Abstract. – OBJECTIVE: Cardiovascular disease is one of the diseases threatening human health. Myocardial fibrosis is a major cause of cardiovascular diseases. Studies have shown that over expression of miR-203 can inhibit the fibrosis. Therefore, in this study, the effect of differential expression of miR-203 on fibrosis of cultured mouse cardiomyocytes was investigated.

MATERIALS AND METHODS: Activators and inhibitors of miR-203 were designed according to the sequence of miR-203, synthesized, and transfected into mouse cardiomyocytes to establish activator group, inhibitor group, and control group. The expression levels of fibrosis-related factors including FN, CTGF, and TGF-β1 were measured by Western blot and RT-PCR 24 h and 36 h after transfection.

RESULTS: Over-expression of miR-203 in mouse cardiomyocytes significantly decreased the expression levels of TGF-β1, CTGF, and FN in a time-dependent manner, compared with that in the control group (p <0.05). Inhibition of miR-203 expression in mouse cardiomyocytes significantly increased the expression levels of TGF-β1, CTGF, and FN 36 h after transfection, compared with that in the control group (p < 0.05). No significant differences were seen in the expression levels of TGF-β1, CTGF, and FN 24 h after transfection, compared with that in the control group (p >0.05).

CONCLUSIONS: Over-expression of miR-203 in mouse cardiomyocytes significantly decreased the expression levels of TGF-β1, CTGF, and FN, which might be used as a detection index for prediction of fibrosis.

Key Words:
Mouse, Myocardial cells, Myocardial fibrosis, miR-203.

Introduction

Myocardial fibrosis mainly refers to the excessive deposition of collagen in myocardial interstitium, which can lead to local collagen imbalance and metabolic disorders. It is usually complicated by a variety of cardiovascular diseases, such as arrhythmia, heart failure, atherosclerosis sclerosis[1,2]. A variety of factors, such as oxidative stress, inflammation, viral or parasitic infections, apoptosis, have been related to myocardial fibrosis. Because sustained myocardial fibrosis can cause chronic ischemic heart disease, the study of the regulatory mechanism of myocardial fibrosis is of great significance[3]. Studies[4,5] have shown that the expression levels of FN, CTGF, and TGF-β were increased during the process of fibrosis, suggesting that these factors might be used as a detection index for prediction of fibrosis.

miRNA involves in the regulation of various genes mainly through two different ways, fully complementary and partially complementary. It can either directly cause the degradation of mRNA of target genes, or reduce the transcription of target genes by inhibition of gene transcription[6,7]. One protein may be regulated by a variety of miRNAs. It has been shown that miRNA plays an important regulatory role in many human diseases. Some miRNAs have been used as markers for early cancer detection or clinical targets[8-10]. Studies[11-13] have shown that several miRNAs including miR-101a, miR-101b also play a regulatory role in the pathogenesis and development of myocardial fibrosis. Studies of the relationship between miRNA and disease associated genes not only can reveal the molecular mechanism of pathogenesis, but also can provide potential targets for clinical treatments.

It has been shown that over-expression of miR-203 suppresses hepatic fibrosis[14]. However, the effect of miR-203 on myocardial fibrosis remains unclear. Therefore, the expression levels of miR-

Corresponding Author: Chengzhi Lu, MD; e-mail: chengzhiluasd@sina.com

203 in mouse cardiomyocytes were manipulated to investigate the effect of miR-203 on the expression of TGF-β1, CTGF, and FN as well as the role of miR-203 in myocardial fibrosis.

Materials and Methods

General Information

Mouse cardiomyocytes (LH-1) were purchased from Shanghai Beinuo Biotechnology Co., Ltd (Shanghai, China). Cell culture medium DMEM and trypsin were purchased from Hyclone (Logan, UT, USA). Calf serum was from Gibco (Grand Island, NY, USA). Cell culture dishes were purchased from BD (San Jose, CA, USA). Liposome cell transfection kit was from Invitrogen (Waltham, MA, USA). TGF-β1 antibody, CTGF antibody, FN antibody, and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

cDNA synthesis kit was purchased from TAKARA (Kusatsu, Shiga, Japan). RT-PCR reagents were purchased from Promega (Madison, WI, USA). Protein extraction reagents were from Thermo (Waltham, MA, USA). Primers were synthesized by Shanghai Shenggong (Shanghai, China).

Transfection of Mouse Cardiomyocytes in vitro

LH-1 cells were unfrozen and cultured in DMEM medium containing 10% calf serum for 24 h. Cells were then counted, seeded into 6-well plate at a concentration of 1 x 10^5 cells/well and were cultured for 16 h before transfection. 50 ml of opti-MEM was used to dilute miR-203 activators and inhibitors to a final concentration of 100 nM. 50 ml of opti-MEM was used to mix with 2 ml of lipofectamine. Diluted miR-203 activators or inhibitors and diluted lipofectamine were mixed and incubated at room temperature for 30 min and then drop wisely added to cell culture medium. Cell culture medium was replaced 4 h later. Each treatment was performed in triplicate.

RT-PCR Measurement of the Expression of miR-203 after Transfection

RNA was extracted and reversely transcripted into cDNA according to manufacturer's instruction. Briefly, 1 mg of RNA, 1 ml of random primers, and 8 ml of water were mixed and heated for 10 min at 70°C, then placed on ice for 5-10 min. 4 ml of reverse transcriptase buffer, 2 ml of DTT, 1 ml of dNTPs, and 1 ml of reverse transcriptase were then added to the mixture and incubated for 1 h at 42°C. The reaction was terminated by incubation at 65°C for 10 min. PCR was performed to amplify miR-203 and β-actin. PCR products were resolved on agarose gel.

Western Blot Analysis of Expression Levels of TGF-β1, CTGF, and FN

Proteins were extracted at 24 h, 36 h after transfection. Briefly, cells were collected, washed with cold PBS, lysed in protein extraction reagent, centrifuged at 13000 rpm for 15 min at 4°C. The supernatants were collected, quantified, resolved in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline solution with 0.1% Tween-20 (TBST) for 1 h at room temperature, and incubated with primary antibody (1:100 dilution) for 8-10 h at 4°C. The membranes were then washed 3 times with TBST and then incubated with secondary antibody for 2 h at room temperature. The bound complexes were detected via enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA). The bands were quantified by densitometry analysis, and the ratio to β-actin was calculated and represented as fold changes, setting the values of controls at 1.

RT-PCR Measurement of Expression Levels of TGF-β1, CTGF, and FN

RNA was extracted from cells of each group 24 h and 36 h after transfection, quantified, and stored at -80°C. All items used in this process were treated with 0.1% DEPC water. RT-PCR was performed with the following profile: an initial 5 min incubation at 94°C, 35 cycles of (35 s at 94°C, 35 s at 58.7°C, and 45 s at 68°C), and a final extension of 8 min at 68°C. Primers used in this study were listed in Table I.

Statistical Analysis

SPSS11.3 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Significant differences were analyzed using ANOVA with Soree post hoc test. $p < 0.05$ was considered statistically significant.

Results

qRT-PCR Assay of miR-203 Expression

qRT-PCR assay of miR-203 expression showed that the expression of miR-203 was

Table I. Primer sequences.

Gene	Sequence (5`-3`)	T (°C)
TGF-β1-F	CCCGCATCCCAGGACCTCTCT	60
TGF-β1-R	CGGGGGACTGGCGA	
CTGF-F	CTAAGACCTGTGGAATGGC	56
CTGF-R	CTCAAAGATGTCATTGCC	
FN-F	CAGTGACTGGAGAGACTG	58
FN-R	TGCCAGGGGAACATAGGCT	
β-actin-F	AACAGTCCGCCTAGAAGCAC	55
β-actin-R	CGTTGACATCCGTAAAGA	
Mir-203-F	GCTGGGTCCAGTGGTTCTTA	65
Mir-203-R	GCCGGGTCTAGTGGTCCTAA	

significantly increased 24 h and 36 h after transfection of miR-203 activator, compared with that of controls (p <0.05). In contrast, the expression of miR-203 was significantly decreased 24 h and 36 h after transfection of miR-203 inhibitor, compared with that of controls (p <0.05) (Figure 1). These results suggested that the manipulation of miR-203 expression was successful.

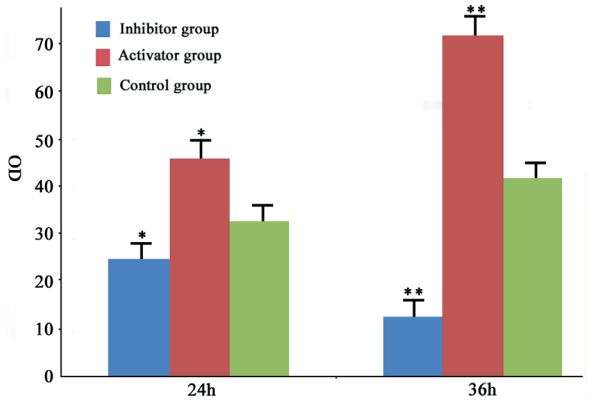

Figure 1. miR-203 expression. ***A,*** RT-PCR analysis of miR-203 expression; ***B,*** Gray value ratios of miR-203; *: p <0.05, compared with the control group, **: p < 0.01, compared with control group.

Western Blot Analysis of TGF-β1, CTGF, and FN Expression

Western blot results showed that 36 h after miR-203 overexpression, the expression levels of TGF-β1, CTGF, and FN were significantly de-

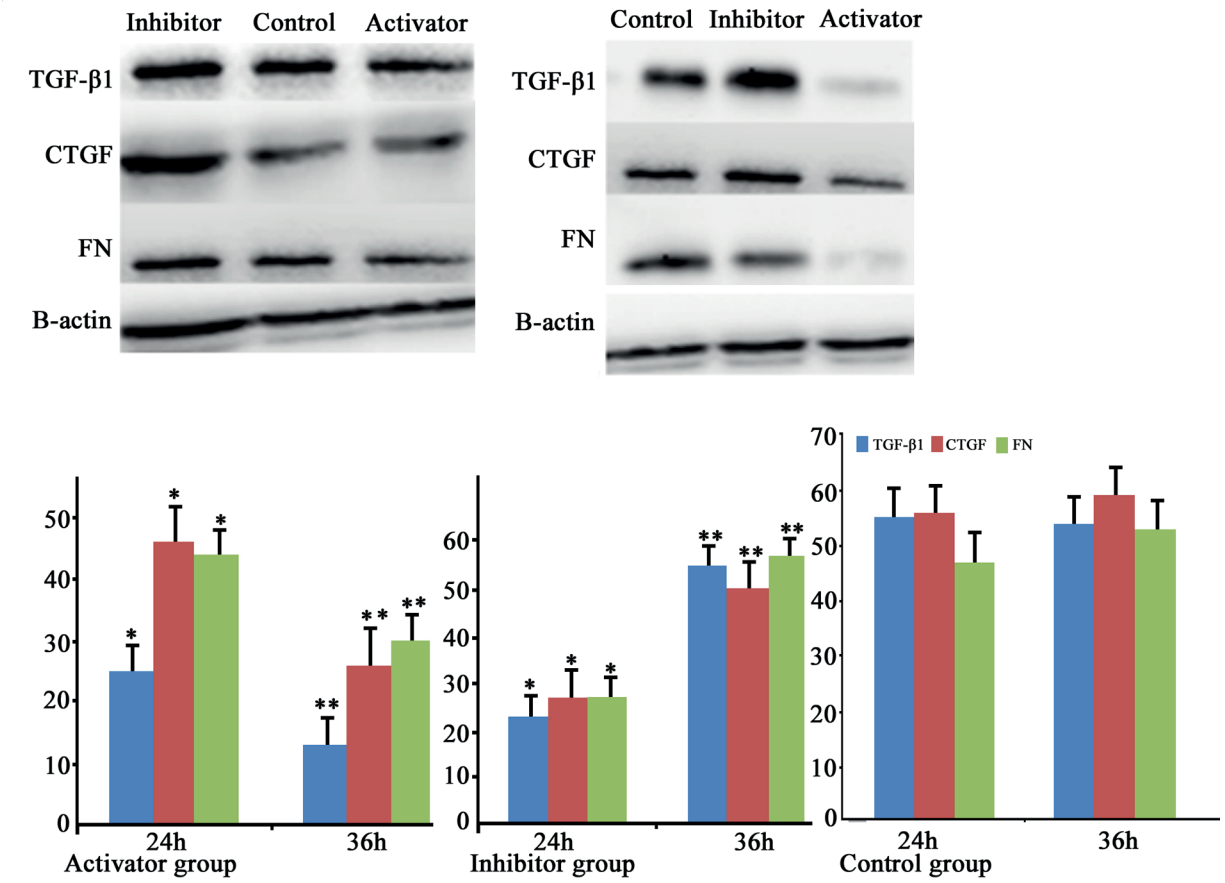

Figure 2. Western blot analysis of TGF-β1, CTGF, and FN expressions in each group.

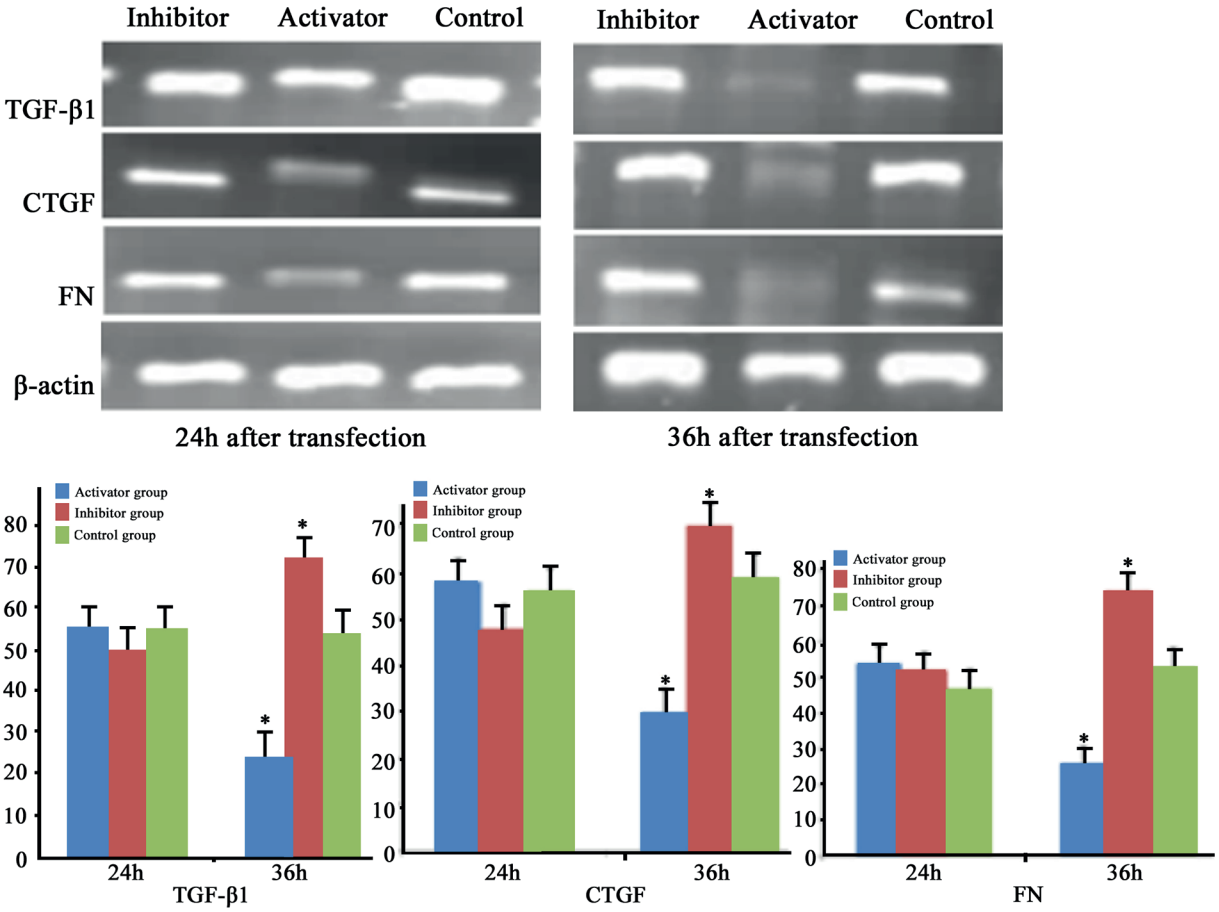

Figure 3. RT-PCR assay of TGF-β1, CTGF, and FN expression at mRNA level. *: p <0.05, compared with the control group.

creased in a time-dependent manner, compared with that of controls (p <0.05). No significant differences were found in the expression levels of TGF-β1, CTGF, and FN 24 h after miR-203 overexpression (p >0.05). These results suggested that miR-203 regulates the expression levels of TGF-β1, CTGF, and FN in a time-dependent manner.

RT-PCR Assay of TGF-β1, CTGF, and FN Expression at mRNA Level

RT-PCR results showed that after miR-203 overexpression, the mRNA levels of TGF-β1, CTGF, and FN were significantly decreased in a time-dependent manner, compared with that of controls (p <0.05). In contrast, inhibition of miR-203 for 36 h resulted in the increase of mRNA levels of TGF-β1, CTGF, and FN, compared with that of controls (p <0.05). No significant increase of mRNA levels of TGF-β1, CTGF, and FN was found at 24 h after inhibition of miR-203 (p >0.05) (Figure 3).

Discussion

Myocardial fibrosis is a continuous process in which multiple factors and signaling pathways play regulatory roles. Characterized by the increase of the number in cardiac fibroblasts, myocardial fibrosis is commonly seen in a variety of cardiovascular diseases and seriously affects patients' health[15].

Studies showed that TGF-β1, CTGF and FN have a direct link with fibrosis. TGF-β1 is positively correlated with the synthesis of extracellular matrix protein. Overexpression of TGF-β1 inhibits the degradation of extracellular matrix proteins and promotes fibrosis[16,17]. It has been shown that in the processes of hepatic fibrosis and lung fibrosis, TGF-β1 mRNA is significantly increased which leads to increased deposition of laminin and fibronectin[18]. CTGF plays the same role as TGF-β1 in the process of fibrosis. CTGF is produced mainly through autocrine and paracrine and functions

locally. Studies indicated that CTGF is regulated by TGF-β and its expression is related to cell proliferation and adsorption. Activation of CTGF can promote various types of fibrosis. Therefore, combined use of TGF-β1 and CTGF in the detection of fibrosis will give a better reliability. Studies also showed that FN expression is up-regulated in the process of fibrosis and FN plays an important role in myocardial fibrosis. The combination of the three factors will give a better result in the detection of fibrosis[19,20]. Therefore, this work used the three most representative fibrosis factors to investigate whether miR-203 plays a regulatory role in myocardial fibrosis. Results showed that overexpression of miR-203 resulted in the decrease of expression levels of TGF-β1, CTGF, and FN in a time-dependent manner. In contrast, inhibition of miR-203 expression resulted in the increase of expression levels of TGF-β1, CTGF, and FN in a time-dependent manner.

Conclusions

The transfection of miR-203 into cultured mouse cardiomyocytes in vitro affects the expression levels of fibrosis-related factors including TGF-β1, CTGF, and FN, which eventually affects the pathogenesis of myocardial fibrosis, suggesting that miR-203 can be used as a predictive indicator in the detection of myocardial fibrosis.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Accornero F, van Berlo JH, Correll RN, Elrod JW, Sargent MA, York A, Rabinowitz JE, Leask A, Molkentin JD. Genetic analysis of connective tissue growth factor as an effector of transforming growth factor beta signaling and cardiac remodeling. Mol Cell Biol 2015; 35: 2154-2164.
2) Arauz J, Rivera-Espinoza Y, Shibayama M, Favari L, Flores-Beltran RE, Muriel P. Nicotinic acid prevents experimental liver fibrosis by attenuating the prooxidant process. Int Immunopharmacol 2015; 28: 244-251.
3) Ashcroft KJ, Syed F, Bayat A. Site-specific keloid fibroblasts alter the behaviour of normal skin and normal scar fibroblasts through paracrine signalling. PLoS One 2013; 8: e75600.
4) Bagchi RA, Mozolevska V, Abrenica B, Czubryt MP. Development of a high throughput luciferase reporter gene system for screening activators and repressors of human collagen Ialpha2 gene expression. Can J Physiol Pharmacol 2015; 93: 887-892.
5) Barrios V, Gorriz JL. Atrial fibrillation and chronic kidney disease: focus on rivaroxaban. J Comp Eff Res 2015; 4: 651-664.
6) Li L, Huang W, Li K, Zhang K, Lin C, Han R, Lu C, Wang Y, Chen H, Sun F, He Y. Metformin attenuates gefitinib-induced exacerbation of pulmonary fibrosis by inhibition of TGF-beta signaling pathway. Oncotarget 2015; 6: 43605-43619.
7) Borkham-Kamphorst E, Schaffrath C, Van de Leur E, Haas U, Tihaa L, Meurer SK, Nevzorova YA, Liedtke C, Weiskirchen R. The anti-fibrotic effects of CCN1/CYR61 in primary portal myofibroblasts are mediated through induction of reactive oxygen species resulting in cellular senescence, apoptosis and attenuated TGF-beta signaling. Biochim Biophys Acta 2014; 1843: 902-914.
8) Boxma PY, van den Berg E, Geleijnse JM, Laverman GD, Schurgers LJ, Vermeer C, Kema IP, Muskiet FA, Navis G, Bakker SJ, de Borst MH. Vitamin k intake and plasma desphospho-uncarboxylated matrix Gla-protein levels in kidney transplant recipients. PLoS One 2012; 7: e47991.
9) Brampton C, Yamaguchi Y, Vanakker O, Van Laer L, Chen LH, Thakore M, De Paepe A, Pomozi V, Szabo PT, Martin L, Varadi A, Le Saux O. Vitamin K does not prevent soft tissue mineralization in a mouse model of pseudoxanthoma elasticum. Cell Cycle 2011; 10: 1810-1820.
10) Brandenburg VM, Cozzolino M, Mazzaferro S. Calcific uremic arteriolopathy: a call for action. Semin Nephrol 2014; 34: 641-647.
11) Brandenburg VM, Schurgers LJ, Kaesler N, Pusche K, van Gorp RH, Leftheriotis G, Reinartz S, Koos R, Kruger T. Prevention of vasculopathy by vitamin K supplementation: can we turn fiction into fact? Atherosclerosis 2015; 240: 10-16.
12) Brandenburg VM, Sinha S, Specht P, Ketteler M. Calcific uraemic arteriolopathy: a rare disease with a potentially high impact on chronic kidney disease-mineral and bone disorder. Pediatr Nephrol 2014; 29: 2289-2298.
13) Zhou Y, Deng L, Zhao D, Chen L, Yao Z, Guo X, Liu X, Lv L, Leng B, Xu W, Qiao G, Shan H. MicroRNA-503 promotes angiotensin II-induced cardiac fibrosis by targeting Apelin-13. J Cell Mol Med 2016; 20: 495-505.
14) Zheng L, Jian X, Guo F, Li N, Jiang C, Yin P, Min AJ, Huang L. miR-203 inhibits arecoline-induced epithelial-mesenchymal transition by regulating secreted frizzled-related protein 4 and transmembrane-4 L six family member 1 in oral submucous fibrosis. Oncol Rep 2015; 33: 2753-2760.
15) Caluwe R, Pyfferoen L, De Boeck K, De Vriese AS. The effects of vitamin K supplementation and vitamin K antagonists on progression of vascular calcification: ongoing randomized controlled trials. Clin Kidney J 2016; 9: 273-279.

16) Chang HC, Chiu YW, Lin YM, Chen RJ, Lin JA, Tsai FJ, Tsai CH, Kuo YC, Liu JY, Huang CY. Herbal supplement attenuation of cardiac fibrosis in rats with CCl(4)-induced liver cirrhosis. Chin J Physiol 2014; 57: 41-47.

17) Chen C, Han X, Fan F, Liu Y, Wang T, Wang J, Hu P, Ma A, Tian H. Serotonin drives the activation of pulmonary artery adventitial fibroblasts and TGF-beta1/Smad3-mediated fibrotic responses through 5-HT(2A) receptors. Mol Cell Biochem 2014; 397: 267-276.

18) Li HY, Ju D, Zhang DW, Li H, Kong LM, Guo Y, Li C, Wang XL, Chen ZN, Bian H. Activation of TGF-beta1-CD147 positive feedback loop in hepatic stellate cells promotes liver fibrosis. Sci Rep 2015; 5: 16552.

19) Chillon JM, Gellotte M, Ferrandon P, Lartaud I, Martin D, Armstrong JM, Atkinson J, Hicks PE. Haemodynamic effects of the calcium facilitator Bay K-8644 in rats following vascular calcium overload. J Auton Pharmacol 1992; 12: 311-319.

20) Correia E, Conceicao N, Cancela ML, Belo JA. Matrix Gla Protein expression pattern in the early avian embryo. Int J Dev Biol 2016; 60: 71-76.