Genetics and Molecular Research

Expression of miR-98 in myocarditis and its influence on transcription of the *FAS/FASL* gene pair

B.Y. Zhang[1], Z. Zhao[2] and Z. Jin[3]

[1]Department of Cardiology, Tianjin Nankai Hospital, Nankai, Tianjin, China
[2]Department of Toxicology,
Tianjin Entry Exit Inspection and Quarantine Bureau, Dongli, Tianjin, China
[3]Department of Clinical, Tianjin Nankai Hospital, Dongli, Tianjin, China

Corresponding author: B.Y. Zhang
E-mail: zhangboyalive@126.com

Genet. Mol. Res. 15 (2): gmr.15027627
Received September 11, 2015
Accepted December 8, 2015
Published June 3, 2016
DOI http://dx.doi.org/10.4238/gmr.15027627

ABSTRACT. Myocarditis is a common cardiovascular disease and frequently occurs in children and teenagers. It is believed to be caused by both endogenous and exogenous factors, among which *FAS/FASL* gene pair-induced cell apoptosis is a major mechanism of myocardial cell injury. A previous study has detected low expression of microRNA (miR)-98 in myocarditis patients. Therefore, in this study we investigated the functional implications of miR-98 with respect to the disease. We carried out a case-control study including 50 myocarditis patients and 50 healthy individuals. Total RNA was extracted from peripheral blood plasma. Expression levels of miR-98 and the *FAS/FASL* gene pair were determined by real-time fluorescent quantitative polymerase chain reaction. The interaction between miR-98 and the *FAS/FASL* pair was visualized by dual-luciferase reporter assay. The expression of the *FAS/FASL* gene pair was further detected by transfecting with an miR-98 mimic or an miR-98 inhibitor. The content of miR-98 in the peripheral blood of the myocarditis patients was significantly lower than in the

healthy individuals. However, the *FAS/FASL* genes were upregulated by 1.68-fold in the myocarditis patients. miR-98 was shown to interact with the 3'-untranslated region of the *FAS/FASL* gene pair. The inhibition/facilitation of miR-98 expression in myocardial cells can modulate apoptosis. miR-98 was downregulated in the peripheral blood of myocarditis patients. It may interact with the *FAS/FASL* gene pair to further modulate cell apoptosis.

Key words: Myocarditis; MicroRNA-98; *FAS/FASL* gene pair; Cell apoptosis

INTRODUCTION

Myocarditis is a common cardiovascular disease. It is characterized by acute or chronic inflammation of myocardial cells, and is caused by multiple endogenous and exogenous factors (Ammirati et al., 2015). Myocarditis can occur in all age groups, but the highest incidence is in children and teenagers (van den Hoogen et al., 2015). In recent years, the average age of myocarditis sufferers has fallen, severely compromising patients' quality of life (Pereda et al., 2015). Myocarditis has various clinical severities: most patients present mild symptoms, with complete recovery of cardiac function after systematic treatment. However, certain patients present severe clinical symptoms, and may develop dilated cardiomyopathy with disease progression, resulting in an unfavorable prognosis. A few patients even have severe cardiac arrhythmia or fatal heart failure (Canter and Simpson, 2014; Pettit et al., 2014). Currently, the pathogenesis mechanism of myocarditis is still under investigation, but it is known to be related to multiple factors including viral infection, oxidative damage, and cell apoptosis (Ramachandra et al., 2010; Caforio et al., 2015; Ladani et al., 2015). *FAS/FASL*-induced cell apoptosis is one of the major mechanisms underlying myocardial cell injury (Guo et al., 2015). To further study the mechanism of myocarditis and to provide a more specific treatment strategy, better biological markers are required. A recently discovered type of regulatory molecule, microRNA (miR), is related to cellular oxidative stress, apoptosis, proliferation, and differentiation (Bartel, 2004). miR is a small single-stranded non-coding RNA molecule of about 25 nucleotides. It can exert unique biological influence by specifically binding to the 3'-untranslated region (3'-UTR) of the mRNA of target genes, thereby modulating gene expression (Chen, 2010). Therefore, in this study we investigated a novel miR and its implications in the pathogenesis of myocarditis.

MATERIAL AND METHODS

Research objects

We conducted a case-control study on 50 myocarditis patients recruited between January 2013 and January 2014 from the Tianjin Nankai Hospital, in parallel with 50 healthy individuals as the control group. Peripheral blood samples (5 mL) were collected and the plasma was separated by centrifugation. The study protocol was approved by the Research Ethics Committee of Tianjin Nankai Hospital, and all patients gave their informed consent before study commencement.

RNA extraction

Total RNA was extracted from the plasma samples using an RNA extraction kit (Invitrogen, Chicago, IL, USA) following the manufacturer instructions. In brief, each 0.5 mL plasma sample was mixed with an equal volume of 2X denaturing solution. After incubation on ice for 5 min, an equal volume of a phenol/chloroform mixture was added before centrifugation at 12,000 *g* for 5 min. The supernatant was saved and mixed with an equal volume of absolute ethanol. The mixture was separated using a spin column and centrifuged at 12,000 *g* for 1 min. The column was then washed with 0.7 mL washing buffer during centrifugation at 12,000 *g* for 30 s. In a new tube, 60 μL RNAse-free water was added to the top of the column to resuspend the RNA, and the mixture was subjected to centrifugation at 12,000 *g* for 30 s.

Real-time polymerase chain reaction (PCR)

Complementary DNA (cDNA) was synthesized using total RNA as the template. Within a real-time PCR system, cDNA was used as the template along with the appropriate primers (Table 1) and SYBR Green (Toyobo, Osaka, Japan) to detect the levels of miR-98, *FAS*, and *FASL* gene expression using a ViiA 7 cycler (ABI, Foster, City, CA, USA). The amplification conditions were: pre-denaturing at 95°C for 5 min; 40 cycles of denaturing at 95°C for 15 s and annealing at 60°C for 60 s; and elongation at 72°C for 60 s. The relative expression level was semi-quantitatively determined using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Real-time polymerase chain reaction primer sequences.

Gene	Forward primers (5'-3')	Reverse primers (5'-3')
β-actin	GAGGGAAATCGTGCGTGAC	CTGGAAGGTGGACAGTGAG
FAS	AAAAACTGGGGCTGCCCTTA	CTTTGTGGGGGATGGAACAA
FASL	ACTACCGCCACCACCTCTGA	GGCCACCAGAACCATGAAAA
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
miR-98	CGGCTGAGGTAGTAGATTGT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC

Luciferase reporter gene

The human myocardial cell line HCM (PromoCell, Heidelberg, Germany) was cultured using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, Grand Island, NE, USA). The luciferase reporter vector was generated by the insertion of a pMIR reporter vector fragment into both wild-type and mutant forms of the miR-89 interaction site of the 3'-UTR of the *FAS/FASL* genes. Lipofectamine 2000 reagent (Invitrogen) was then used to transfect those vectors into the HCM cells along with a PTK internal reference reporter vector and the miR-98 mimic. A dual-luciferase reporter gene system (Promega, Madison, WI, USA) was used to quantify the results 48 h after transfection.

Western blotting

The cultured HCM cells were lysed on ice for 30 min using a lysis cocktail containing proteinase inhibitor, followed by ultrasonic rupture for 30 s. The proteins were then collected by

centrifugation at 10,000 *g* for 10 min at 4°C. Proteins in the supernatants were quantified by the BCA method. After separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to a polyvinylidene difluoride (PVDF) membrane, blocking was performed with 5% defatted milk powder for 2 h at room temperature. Primary antibodies (1:1000) including those for FAS, FASL, and β-actin were incubated with the membrane at 4°C overnight. After rinsing in phosphate-buffered saline with Tween 20 (PBST), the secondary antibody (1:10,000) was added for another 60-min incubation at room temperature. A chromogenic substrate was then added to visualize the membrane, which was exposed for 90 s. The optical density values of the protein bands were analyzed by computer software for calculating the protein expression levels.

Flow cytometry

The cultured HCM cells were first lysed by trypsin (Gibco), then rinsed in DMEM to prepare single-cell suspensions, which were centrifuged at 1000 *g* for 5 min to remove supernatants. Blocking was then performed for 10 min, followed by addition of the buffer solution (0.5 mL) and centrifugation at 1000 *g* for 5 min. After discarding the supernatants, the cell suspensions were mixed with Annexin V solution (0.2 mL) followed by marker solution (0.3 mL) and propidium iodide (PI) dye. After incubation at room temperature for 5 min, the samples were loaded for quantifying myocardial cell apoptosis by flow cytometry.

Statistical analysis

The SPSS 16.0 software was used to process all the collected data, which are reported as means ± standard deviation (SD). Analysis of variance (ANOVA) was used to compare means across groups. Statistical significance was defined as $P < 0.05$.

RESULTS

miR-98 expression in myocarditis patients

Using real-time PCR, we found significantly depressed (~60%) miR-98 expression in the myocarditis patients compared with the controls ($P < 0.05$; Figure 1).

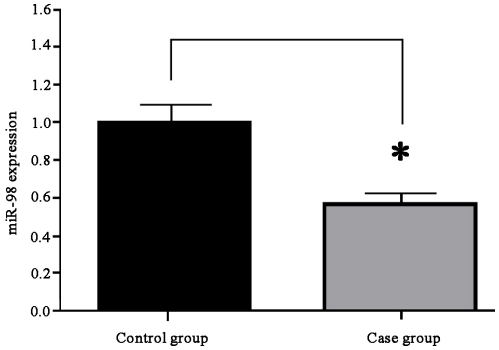

Figure 1. miR-98 expression in the myocarditis patients compared with the controls (*$P < 0.05$).

Plasma *FAS* and *FASL* expression levels

Although previous studies have suggested the involvement of *FAS/FASL* gene expression in myocardial cell damage, no direct evidence has proven a correlation between plasma *FAS/FASL* gene expression and myocardial cell injury. Therefore, in this study we detected the expression of the *FAS/FASL* genes in the plasma samples. The levels of those two genes in myocarditis patients were significantly higher than in healthy individuals ($P < 0.05$; Figure 2).

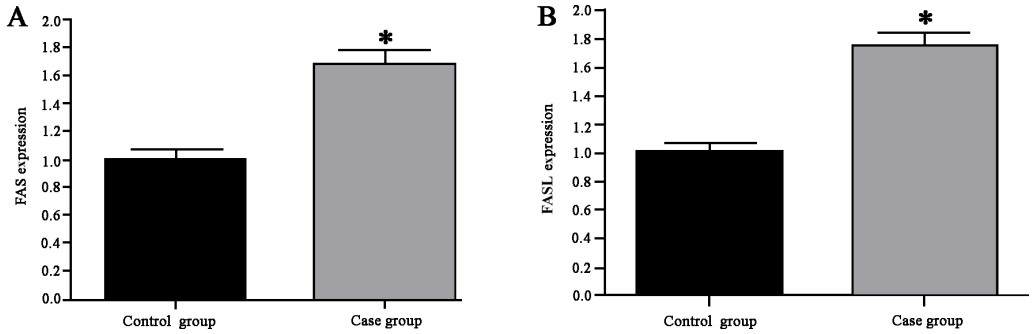

Figure 2. Expression of FAS (**A**) and FASL (**B**) gene in myocarditis patients were significantly higher than in healthy individuals (*$P < 0.05$).

Correlation between miR-98 and *FAS/FASL* genes

miR-98 is downregulated in the plasma of myocarditis patients. Software simulation using TargetScan and miRanda showed that *FAS/FASL* genes were possible targets for miR-98 (Figure 3). To directly prove the interaction between miR-98 and *FAS/FASL*, we performed a dual-luciferase reporter assay, in which both the wild-type and mutant 3'-UTRs of the *FAS* and *FASL* genes were constructed in vectors. After transfecting HCM cells with those vectors, in parallel with a PTK internal reference vector and an miR-98 mimic vector 48 h after transfection, the dual-luciferase reporter system detected significant suppression of luciferase activity in the wild-type form of the *FAS/FASL* vector-transfected cells, while those cells transfected with the mutant form showed no significant changes (Figure 4). These results suggest the action of miR-98 on the 5'-UTR of both *FAS* and *FASL* genes.

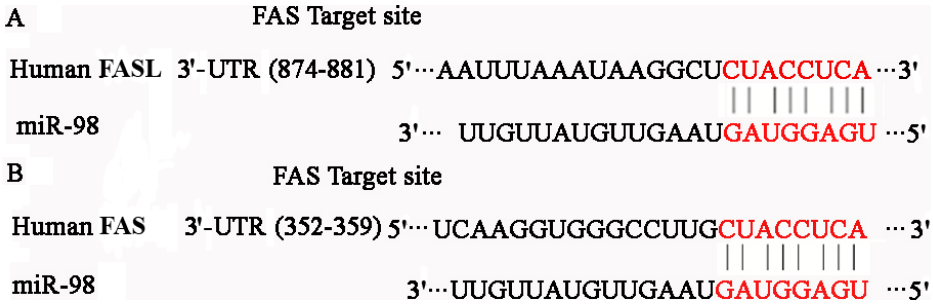

Figure 3. 3'-UTRs of the FAS (**A**) and FASL (**B**) target genes for miR-98.

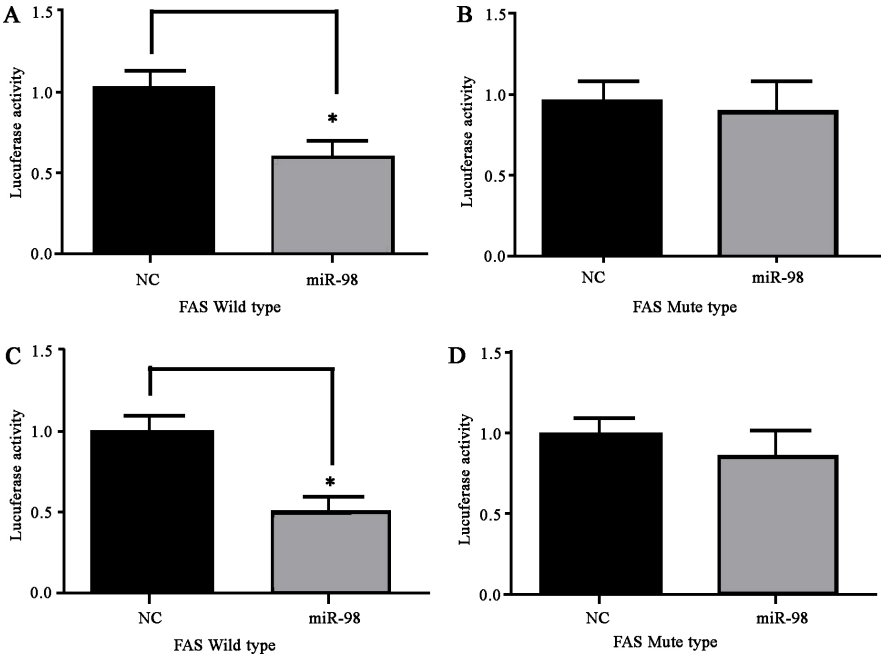

Figure 4. Detection results of luciferase reporter system (*P < 0.05). **A.** FAS wild type; **B.** FAS mute type; **C.** FASL wild type; **D.** FASL mute type. NC = normal control.

Regulatory effects of miR-98 on *FAS/FASL* gene regulation

From the dual-luciferase reporter assay mentioned above, miR-98 can bind to the 3'-UTRs of the *FAS/FASL* genes. To further explore the regulator mechanism of miR-98 underlying *FAS/FASL* gene expression, we artificially inhibited or potentiated miR-98 expression, and investigated the expression profile of the *FAS/FASL* gene pair. Forty-eight hours after transfection, *FAS/FASL* gene mRNA levels were significantly depressed (~0.60-fold) in the miR-98 mimic-transfected cells ($P < 0.05$; Figure 5A and B). When the miR-98 inhibitor was used for transfection, the mRNA levels of the *FAS* and *FASL* genes were elevated (1.65- and 1.83-fold, respectively) compared with the control transfection group ($P < 0.05$; Figure 5A and B). The western blotting results were consistent with the real-time PCR results (Figure 5C). These results collectively suggest the regulation of *FAS/FASL* gene expression by miR-98.

miR-98 and apoptosis of myocardial cells

Earlier studies have established the involvement of *FAS/FASL*-induced apoptosis in myocardial cell injury. Our results also indicated the regulatory effect of miR-98 on *FAS/FASL* gene expression. Therefore, we further investigated the effect of miR-98 expression on cell apoptosis. Using Annexin V/PI double labeling, we quantified the apoptotic cells by flow cytometry. As shown in Figure 6, there were fewer apoptotic cells among those transfected with the miR-98 mimic than those transfected with the miR-98 inhibitor ($P < 0.05$ compared

with controlled transfection cells). These results indicate the inhibitory role of miR-98 against myocardial cell apoptosis.

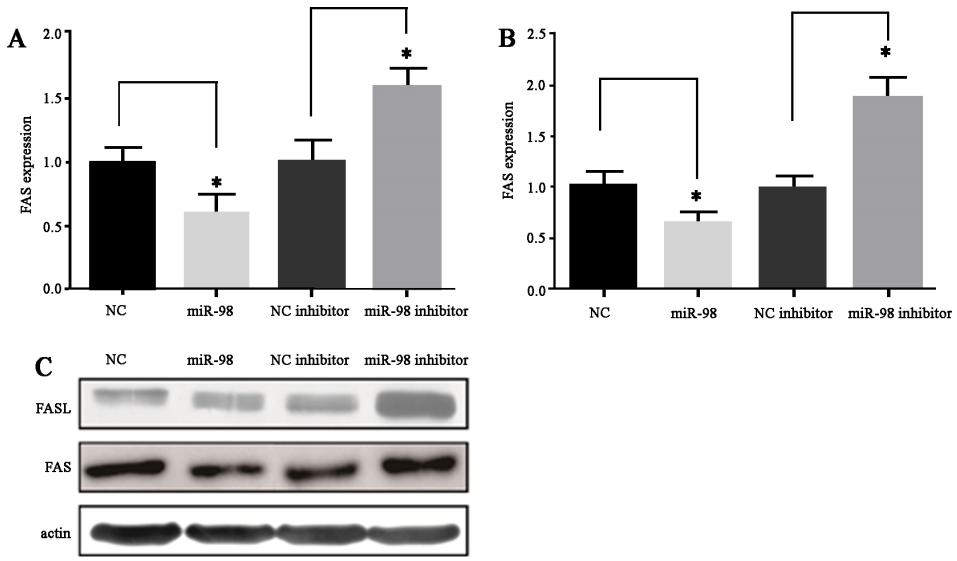

Figure 5. Expression of FAS and FASL gene in human cardiac myocytes after transfection of miR-98 (*P < 0.05). **A.** Expression level of FAS mRNA; **B.** expression level of FASL mRNA; **C.** expression level of FAS and FASL proteins. NC = normal control.

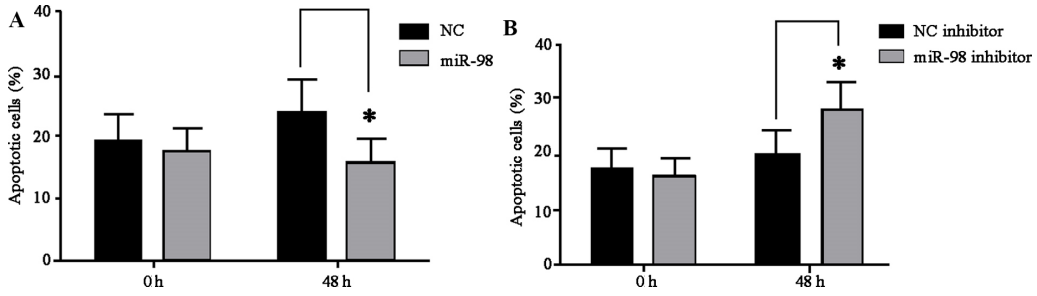

Figure 6. Apoptosis of human myocardial cells after transfection with miR-98 (*P < 0.05). **A.** Compared with control the apoptosis percentage of miR-98 mimic cells was decreased in the experimental group. **B.** Compared with control the apoptosis percentage of miR-98 inhibitor was increased in the experimental group. NC = normal control.

DISCUSSION

Myocarditis is a local or diffused inflammatory disease of the myocardial tissues, and is caused by multiple-endogenous/exogenous factors (Bergmann et al., 2015). The most common subtype is viral myocarditis, which is caused by coxsackievirus B3 (Zhang et al., 2015). Currently, the exact mechanism behind myocarditis remains unclear, but the critical role played by *FAS/FASL*-induced apoptosis in the progression of myocarditis is recognized

(Zhong et al., 2015). miR is a small non-coding RNA molecule with a sequence of ~25 highly conserved nucleotides (Wang et al., 2015). miR can regulate more than 30% of all genes by specific binding to the 3'-UTR of the target mRNA (Wendler et al., 2011). The authors of several studies have reported the involvement of miR in various biological processes including cell proliferation, differentiation, apoptosis, and tumor occurrence (Du et al., 2009; Gao et al., 2014). Therefore, this study aimed to identify a myocarditis-related miR molecule and determine its role in the pathogenesis of myocarditis.

This study found downregulation of miR-89 in the myocarditis patients' plasma samples. To explore the mechanism by which miR-98 acts in the pathogenesis of myocarditis, we further performed target sequence prediction and identified the *FAS/FASL* gene pair as a potential candidate. FAS, an important membrane surface molecule, and its ligand FASL can affect multiple signaling transduction pathways inducing cell apoptosis (Saxena et al., 2013). Real-time PCR revealed elevated plasma *FAS/FASL* gene expression in the myocarditis patients, which corroborated the results from a previous study (Wu et al., 2008). To further illustrate the relationship between miR-98 and the potential targets (the *FAS/FASL* gene pair), a dual-luciferase reporter assay was deployed. The results supported the direct binding of miR-98 with the 3'-UTR of the *FAS/FASL* gene pair to inhibit gene expression. Further correlation analysis showed a reverse relationship between miR-98 and the level of *FAS/FASL* expression. These results collectively suggest that *FAS* and *FASL* are the target genes under the direct influence of miR-98. The flow cytometry assay showed a decreased apoptotic myocardial cell number after miR-98 overexpression, while potentiated apoptosis occurred following miR-98 downregulation. In summary, our results showed that miR-98 can regulate *FAS/FASL* gene expression in myocardial cells, and modulate cell apoptosis, thereby affecting the pathogenesis of myocarditis.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

REFERENCES

Ammirati E, Roghi A, Oliva F, Turazza FM, et al. (2015). Ventricular aneurysm as a complication of giant cell myocarditis. *G. Ital. Cardiol.* 16: 389-390.

Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297. http://dx.doi.org/10.1016/S0092-8674(04)00045-5

Bergmann KR, Kharbanda A and Haveman L (2015). Myocarditis and pericarditis in the pediatric patient: validated management strategies. *Pediatr. Emerg. Med. Pract.* 12: 1-22, quiz 23.

Caforio AL, Marcolongo R, Basso C and Iliceto S (2015). Clinical presentation and diagnosis of myocarditis. *Heart* 101: 1332-1344. http://dx.doi.org/10.1136/heartjnl-2014-306363

Canter CE and Simpson KP (2014). Diagnosis and treatment of myocarditis in children in the current era. *Circulation* 129: 115-128. http://dx.doi.org/10.1161/CIRCULATIONAHA.113.001372

Chen T (2010). The role of MicroRNA in chemical carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 28: 89-124. http://dx.doi.org/10.1080/10590501.2010.481477

Du L, Schageman JJ, Subauste MC, Saber B, et al. (2009). miR-93, miR-98, and miR-197 regulate expression of tumor

suppressor gene FUS1. *Mol. Cancer Res.* 7: 1234-1243. http://dx.doi.org/10.1158/1541-7786.MCR-08-0507

Gao SY, Zhou X, Li YJ, Liu WL, et al. (2014). Arsenic trioxide prevents rat pulmonary fibrosis via miR-98 overexpression. *Life Sci.* 114: 20-28. http://dx.doi.org/10.1016/j.lfs.2014.07.037

Guo X, Chi S, Cong X, Li H, et al. (2015). Baicalin protects sertoli cells from heat stress-induced apoptosis via activation of the Fas/FasL pathway and Hsp72 expression. *Reprod. Toxicol.* 57: 196-203. http://dx.doi.org/10.1016/j.reprotox.2015.06.049

Ladani AP, Biswas A, Vaghasia N and Generalovich T (2015). Unusual presentation of listerial myocarditis and the diagnostic value of cardiac magnetic resonance. *Tex. Heart Inst. J.* 42: 255-258. http://dx.doi.org/10.14503/THIJ-14-4204

Pereda MG, López M and Mariluz M (2015). Myocarditis and complicated dengue: a case report. *Rev. Chilena Infectol.* 32: 238-239. http://dx.doi.org/10.4067/S0716-10182015000300016

Pettit MA, Koyfman A and Foran M (2014). Myocarditis. *Pediatr. Emerg. Care* 30: 832-835, quiz 836-838. http://dx.doi.org/10.1097/PEC.0000000000000272

Ramachandra G, Shields L, Brown K and Ramnarayan P (2010). The challenges of prompt identification and resuscitation in children with acute fulminant myocarditis: case series and review of the literature. *J. Paediatr. Child Health* 46: 579-582. http://dx.doi.org/10.1111/j.1440-1754.2010.01799.x

Saxena N, Yadav P and Kumar O (2013). The Fas/Fas ligand apoptotic pathway is involved in abrin-induced apoptosis. *Toxicol. Sci.* 135: 103-118. http://dx.doi.org/10.1093/toxsci/kft139

van den Hoogen P, van den Akker F, Deddens JC and Sluijter JP (2015). Heart failure in chronic myocarditis: a role for microRNAs? *Curr. Genomics* 16: 88-94. http://dx.doi.org/10.2174/1389202916999150120153344

Wang Q, Shu C, Su J and Li X (2015). A crosstalk triggered by hypoxia and maintained by MCP-1/miR-98/IL-6/p38 regulatory loop between human aortic smooth muscle cells and macrophages leads to aortic smooth muscle cells apoptosis via Stat1 activation. *Int. J. Clin. Exp. Pathol.* 8: 2670-2679.

Wendler A, Keller D, Albrecht C, Peluso JJ, et al. (2011). Involvement of let-7/miR-98 microRNAs in the regulation of progesterone receptor membrane component 1 expression in ovarian cancer cells. *Oncol. Rep.* 25: 273-279.

Wu XQ, Li YH, Zhu X, Wu MW, et al. (2008). Expression of Fas protein in myocardiac tissue of viral myocarditis and dilated cardiomyopathy. *Fa Yi Xue Za Zhi* 24: 414-416.

Zhang S, Wu T, Xia T, Rong X, et al. (2015). Regulation of the Heme Oxygenase-1/carbon monoxide system by hydrogen sulfide in murine coxsackievirus B3-induced myocarditis. *Cell. Mol. Biol. (Noisy-le-grand)* 61: 69-73.

Zhong B, Hu Z, Tan J, Lu T, et al. (2015). Hsp20 Protects against oxygen-glucose deprivation/reperfusion-induced golgi fragmentation and apoptosis through Fas/FasL pathway. *Oxid. Med. Cell. Longev.* 2015: 606934. http://dx.doi.org/10.1155/2015/606934