Swimming training affects apoptosis-related microRNAs and reduces cardiac apoptosis in mice

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Abstract. We sought to investigate effects of exercise training on apoptosis-related microRNAs (miRs) and their validated targets, discussing molecular mechanism of the exercise-induced benefit in heart. Male C57BL/6 mice were randomly assigned to three groups: sedentary (SE), exercise training 1 (ET1) and exercise training 2 (ET2). ET1 swam for 8 weeks, once a day and 5 days *per* week with incremental load. ET2 performed the same work as ET1 and switched to twice a day by the end of the 5th week. In ET2, positive cell rate (%) tested by TUNEL assay decreased significantly ($p < 0.05$), and the load decreased miR-1 level by 29% ($p < 0.01$), also increased miR-30b and miR-21 levels by 32% ($p < 0.01$) and 18% ($p < 0.05$) respectively. In addition, Bcl-2 expression was increased by 98% ($p < 0.01$). p53, PDCD4 and Drp-1 expressions were decreased by 45% ($p < 0.01$), 6% ($p > 0.05$) and 36% ($p < 0.01$) respectively, compared with SE. In ET1, only miR-30b level was increased by 22% ($p < 0.05$) with a 48% decrease in p53 level ($p < 0.01$). Both swimming groups increased Bcl-2/Bax ratio significantly ($p < 0.01$). This study indicated that apoptosis-related miRs and their downstream proteins in heart can be influenced by swimming training that may be responsible for the exercise-induced cardioprotection.

Key words: Swimming — Exercise — Heart — MicroRNAs — Apoptosis

Introduction

The heart is an organ composed of terminally differentiated cardiomyocytes and undergoes cardiac remodeling in response to a variety of stressor. For instance, excessive apoptosis in cardiomyocytes occurs during ischemia-reperfusion (IR), heart failure, and aging (Kitsis and Mann 2005; Kwak et al. 2006), which result in abnormal cardiomyocytes loss. Since cardiomyocytes loss cannot be compensated *via* efficient cell proliferation, high levels of apoptosis in cardiomyocytes may lead to the pathophysiological disorders. The high level of apoptosis is related to cardiac diseases,

such as myocardial infarction, cardiomyopathy and cardiac hypertrophy (Crow et al. 2004).

Thus, it is urgent to develop strategies that result in cardioprotective effects, antagonizing apoptosis involved in cardiomyocytes loss. Numerous approaches to cardioprotection have been investigated, and a wide array of epidemiological studies indicates that regular exercise reduces the risk of death due to an IR insult (Ignarro et al. 2007). Animal experiments also show that consecutive bouts of endurance exercise provide cardioprotection against IR injury and downregulate the activation of the Fas-dependent and mitochondria-dependent apoptotic pathways in hypertensive hearts (Huang et al. 2012; Powers et al. 2014). In addition, regular exercise can decrease the pro-apoptotic protein and increase anti-apoptotic protein in cardiomyocytes that attenuates the extent of apoptosis in physiological condition (Siu et al. 2004).

MicroRNAs (miRs) are small noncoding single stranded RNAs molecules that downregulate their target gene expression. Growing evidence indicates that miRs have distinct expression profiles and function in regulation of gene expression during cardiac cell growth, differentiation, and

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apoptosis (Latronico et al. 2007). Recently, studies indicate that miR-1, -21 and -30 are involved in regulating cardio-myocyte. miR-1 is to be specifically expressed in cardiac muscle and has been certified the pro-apoptosis function by inhibition of Bcl-2 expression (Tang et al. 2009). On the contrary, miR-30 family members can inhibit expression of p53 directly and decrease it's downstream protein level, dynamin-related protein-1 (Drp-1), which initiates the mitochondrial fission program, thereby suppressing apoptosis in heart (Li et al. 2010). miR-21 can bind to the 3'UTR of PDCD4 mRNA and inhibit PDCD4 expression (Asangani et al. 2008), decreasing apoptotic signaling. After initiation of ischemia treatment, miR-21 is upregulated in cardiomyocytes quickly, resulting in suppression of PTEN and downregulation of PDCD4 (Kukreja et al. 2011). Although miRs regulate apoptosis, it remains unclear how they are integrated into the apoptotic program in some pathophysiological conditions. For example, whether miRs are altered during or after exercise in cardiomyocytes, and whether the alterations of miRs affected by exercise contribute to the regulation of apoptosis in heart.

Although it has been recognized that exercise can down-regulate apoptosis in cardiomyocytes, the molecular mechanism remains largely unknown and there are no publications reporting the effects of exercise on miRs and their targeted proteins involved in apoptosis of heart. Thus, we established swimming training workload in animal experiments, investigating the effects of exercise on the miR-1, -21 and -30b expressions and their validated target proteins related to apoptosis. It is a valuable strategy to study the mechanism of exercise-regulated apoptosis for the development of novel approaches in the treatment of cardiovascular diseases.

Materials and Methods

Experimental design

Animal experiments were approved by the Research Commission on Ethics of Tangshan Normal University and

Table 1. The primers of miR-1, -21 and -30b

Name	Sequence (5'-3')
mmu-miR-1 F:5'	ACGCCTGGAATGTAAAGAAGTATG
mmu-miR-21 F:5'	ACACTCCAGCTGGGTAGCTTATCA-GACTGATG
mmu-miR-30b F:5'	ACACTCCAGCTGGGTGTAAACATC-CTACACTC
miRNA-R:5'	CTCAACTGGTGTCGTGGA
U6-F:5'	CTCGCTTCGGCAGCACA
U6-R:5'	AACGCTTCACGAATTTGCGT

conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). C57BL/6 mice (male, 2 months old) were randomly assigned to one of three groups: sedentary (SE), exercise training protocol 1 (ET1) and exercise training protocol 2 (ET2), n = 7/group. All animals were housed on a 12 h light-dark cycle and fed standard mouse chow, and had ad libitum access to water.

Exercise training protocol

Animals in SE did not do any exercise. Animals in ET1 and ET2 performed increased load swimming training which consisted of swimming sessions for 8 weeks, 5 days/week. The swimming duration is 30 min at beginning, and the time was increased by 10 min *per* week. In the 7th and 8th week, swimming duration maintained at 90 min. In ET1, animals performed one time a day in whole training period. In ET2, animals performed the same work as ET1 until the end of the fifth week. Then, mice trained twice a day with an interval at least for 6 h until the end of training. All hearts were harvested 24 h after the final exercise bout. Samples were quickly frozen in liquid nitrogen and stored at –80°C for miRs, proteins and TUNEL assay.

Detection of miR-1, -21 and -30b by RT-PCR assay

Total RNA extraction

Total RNA was isolated according to the manufacturer's directions (Invitrogen). RNA was quantified by BioPhotometer plus to test its purity, $OD_{260}/OD_{280} > 1.8$.

Real-Time RT-PCR assay

After cDNA synthesis, quantitative real-time PCR (qPCR) was performed using ABI PRISM 7500 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol in triplicates. PCR was performed using the RT product and microRNA-specific PCR primers for 40 cycles. The 20 μl PCR included 5 μl RT product, 10 μl (2×) SYBR Green qPCR SuperMix (Invitrogen), 4 μl nuclease-free water and 1 μl of primers for miRs. The reactions were incubated in a 96-well optical plate at 50°C – 2 min, 95°C – 2 min, and then, followed by 40 cycles of 95°C for 15 s and 60° for 32 s. miRs expressions were normalized by evaluating U6 expression. The threshold cycle (Ct) was set within the exponential phase of the PCR. The target PCR Ct values were normalized by subtracting the U6 Ct value, which provided the ΔCt value. Relative gene expression = $2^{-(\Delta Ct sample - \Delta Ct control)}$. The primers of miRs used in PCR were shown in Table 1.

Detection of apoptosis

The heart tissue was immersion-fixed in 10% neutral formalin and embedded in paraffin. Serial sections of 4-μm thicknesses were prepared. Apoptotic cells were identified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using an apoptosis detection kit according to the manufacturer's protocol (Cat.No. G7130, Promega). Detailed materials are described in the Supplementary Methods.

Western blotting analysis

Western blotting analysis was performed using standard procedures as described previously (Duan et al. 2012). Quantification was performed in the linear absorption range by computerized densitometry using Scion Image software. GAPDH levels of heart tissues were used to normalize the results. Detailed materials are described in the Supplementary Methods.

Statistical analysis

All data are presented as mean ± SD. For relative gene expression, the mean value of the sedentary group is defined as 1. Statistical analysis was performed by one-way ANOVA for the evaluation of the data. The Pearson correlation analysis was used to analyze the correlations between parameters. $p <$ 0.05 was considered significant. All statistics were computed using PASW Statistics 18.0 software.

Results

Exercise training downregulates the apoptosis in cardiomyocytes

The first step is to test whether exercise training affect apoptosis in cardiomyocytes. The results obtained from TUNEL assay indicated that the rate of apoptotic cells in ET2 (1.45 ± 0.39%, $p < 0.01$) was significantly lower than that in SE (2.26 ± 0.55%) and ET1 (2.41 ± 0.56%). In addition, although the mice in ET1 undertook exercise training for 8 weeks, the apoptosis rate in their hearts did not alter significantly compared with SE ($p > 0.05$) (Fig. 1). Together, it seems that relative heavy load swimming training in ET2 can result in significant downregulation of apoptosis in heart cells tested by TUNEL assay.

miRs related to apoptosis of cardiomycoyte are changed by exercise training

Studies have shown that miRs control diverse aspects of heart disease (Divakaran and Mann 2008). Previous studies led us to consider whether long-term swimming training can affect apoptosis-related miRs. We tested the alterations
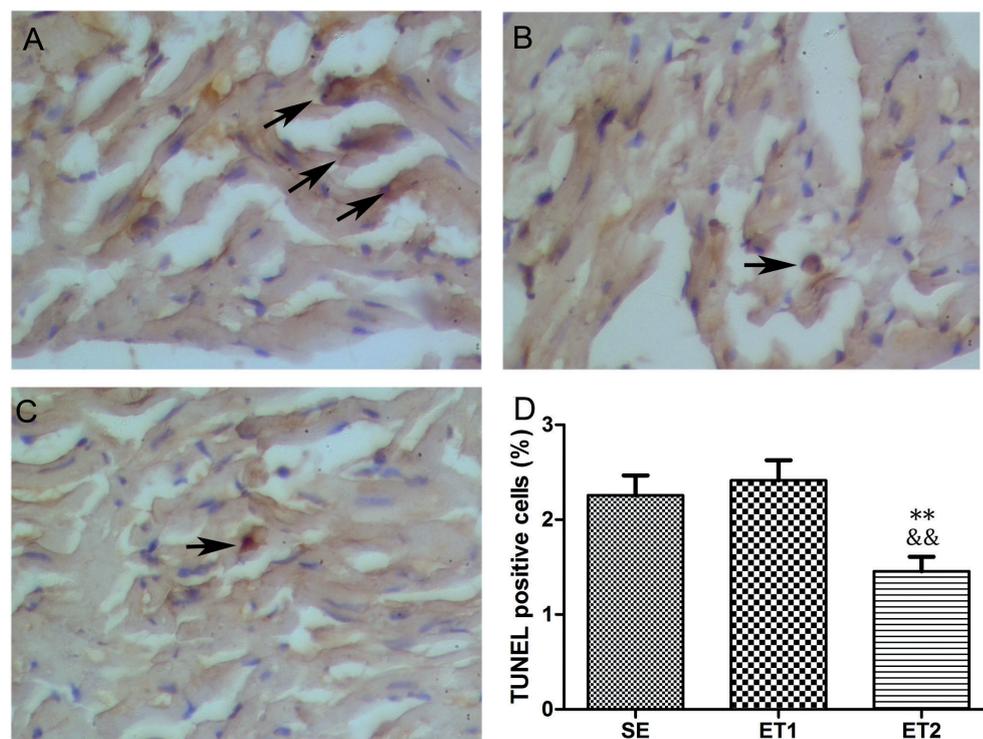

Figure 1. Effects of swimming training on the apoptosis of cardiomyocytes by TUNEL assay. Representative images of ventricular myocardium sections in SE (**A**), ET1 (**B**) and ET2 (**C**). Black arrows point to the apoptotic cells. Quantitative analysis of apoptosis by TUNEL assay (**D**). Values are mean ± SD. $n = 7$/group, ** $p < 0.01$ *vs.* SE, && $p < 0.05$ *vs.* ET1. SE, sedentary group; ET1, exercise training 1 group; ET2, exercise training 2 group.
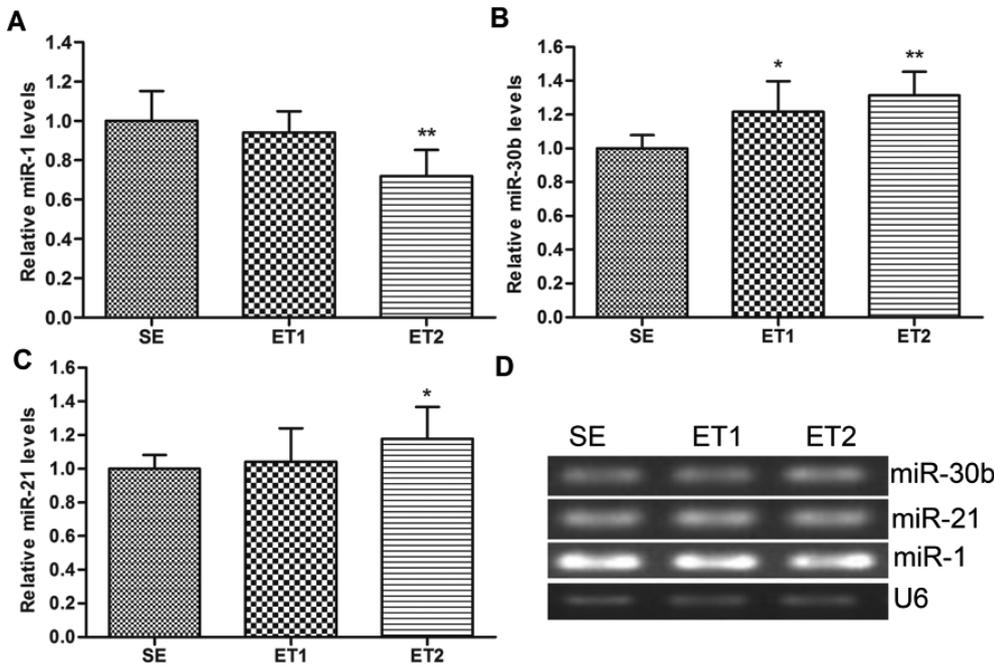
Figure 2. miRs involved in apoptotic regulation were changed by exercise training. miR-1 was down-regulated in ET1 and ET2 and only the decrease in ET2 had statistical significance (**A**); miR-30b levels were both increased significantly in ET1 and ET2 (**B**); miR-21 had a slight elavation in ET1 and a significant elavation in ET2 (**C**). Gel electrophoresis image of RT-PCR products of miRs (**D**). Values are mean ± SD. $n = 7$/group, * $p < 0.05$ *vs.* SE, ** $p < 0.01$ *vs.* SE. For abbreviations, see Fig. 1.

of miR-1, miR-21 and miR-30b in response to swimming training. As shown in Fig. 2A, miR-1 levels in ET1 and ET2 were both downregulated, and the levels were reduced by 6% in ET1 ($p > 0.05$) and 29% in ET2 ($p < 0.01$) compared with SE. Fig 2B showed significant differences in miR-30b among three groups. miR-30b levels were increased by 22%

in ET1 ($p < 0.05$) and 32% in ET2 ($p < 0.01$) compared with SE. In contrast to the sensibility of miR-30b, miR-21 levels were increased by 4% in ET1 ($p > 0.05$) and 18% in ET2 ($p < 0.05$) compared with SE (Fig. 2C), which implied that relative heavy load training in present study could influence miR-1 and miR-21 apparently.
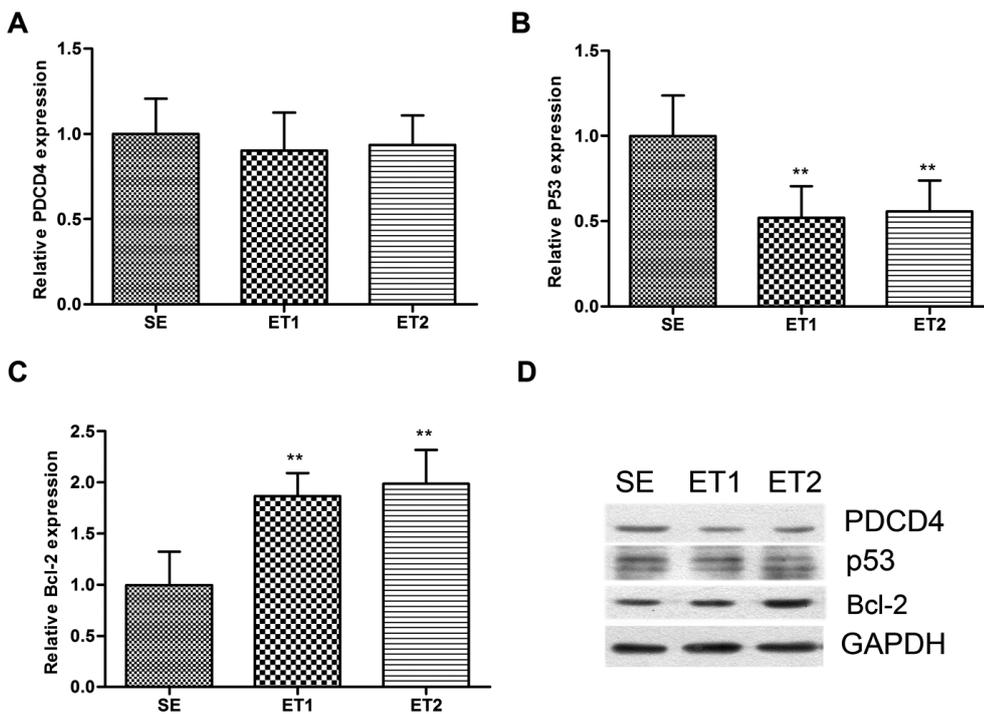

Figure 3. Effects of swimming training on miRs-targeted proteins in apoptotic regulation of cardiomyocytes. The expression of PDCD4 was decreased slightly by exercise training (**A**), and a remarkable decease in p53 was also observed (**B**). In contrast, Bcl-2 was increased in exercise protocols (**C**). Representative blots of PDCD4, p53 and Bcl-2 (**D**). Values are mean ± SD. $n = 7$/group, ** $p < 0.01$ *vs.* SE. For abbreviations, see Fig. 1.

The changes of miRs-targeted proteins involved in apoptosis of cardiomycoyte

Bcl-2, PDCD4 and p53 expressions can be inhibited directly by miR-1, -21 and -30b respectively (Asangani et al. 2008; Tang et al. 2009; Li et al. 2010). Western blot assay demonstrated that PDCD4 expressions were downregulated in ET1 and ET2 compared with SE (Fig. 3A). But there were no statistical significances among the three groups (9% and 6% reduction in ET1 and ET2 respectively, $p > 0.05$). p53 expressions in ET1 and ET2 were reduced by 48% ($p < 0.01$) and 45% ($p < 0.01$) in ET1 and ET2 compared with SE respectively (Fig 3B). In contrast to the reduction of PDCD4 and p53 expressions, swimming training significantly increased the Bcl-2 expressions in ET1 and ET2 (86% and 98% increase in ET1 and ET2 respectively, $p < 0.01$) (Fig. 3C). Altogether, pro-apoptotic proteins expressions such as PDCD4 and p53 can be decreased, but Bcl-2 that antagonizes apoptosis can be increased by swimming training. p53 and Bcl-2 are more sensitive than PDCD4 in response to swimming training.

The changes of pro-apoptotic proteins involved in apoptosis of cardiomycoyte

Drp1 which is induced by p53 is a GTPase that causes scission of the mitochondrial outer membrane, resulting in fission of mitochondrial tubules into fragments. It is also responsible for cytochrome c release and caspase activation (Frank et al. 2001). Bax, a member of the Bcl-2 pro-apoptotic family, can also be induced by p53. In response to stress activation, Bax forms a homodimer and releases

cytochrome c from the mitochondria, which results in caspase-9 activation (Miyashita and Reed 1995). We are interested in the alteration of Drp-1 and Bax controlled by p53. Drp-1 levels were reduced by 17% in ET1 ($p > 0.05$) and 36% in ET2 ($p < 0.01$) compared with SE (Fig. 4A). Bax levels were downregulated greatly by swimming training. Reduction in ET1 and ET2 reached to 38% ($p < 0.01$) and 60% ($p < 0.01$) compared with SE respectively (Fig. 4B). Bcl-2 and Bax represent the anti- and pro-apoptotic properties in mitochondria-dependent pathway, and the Bcl-2/Bax ratio were increased by 2 folds in ET1 and nearly 4 folds in ET2 compared with SE ($p < 0.01$) (Fig. 4C). Although significant downregulation of apoptosis by TUNEL assay was not observed in ET1, our results indicates that ET1 and ET2 both attenuate apoptotic signals estimated by apoptotic proteins expressions.

Correlations among different apoptotic regulators in present study

To further clarify the relations between the proteins and miRs in our study, we used the Pearson coefficient of correlation to analyze their correlations. Consistent with our hypothesis, undergoing different workloads in mice, miR-1 and Bcl-2 appeared a significant negative correlation (R = −0.543, $p < 0.05$), and the same result was observed between miR-30b and p53 (R = −0.633, $p < 0.01$). Interestingly, a special discovery in present study was that miR-21 and PDCD4 had no significant negative correlation due to the low correlation coefficient (R = −0.062, $p > 0.05$). Additionally, we studied the relations among these proteins in present research.
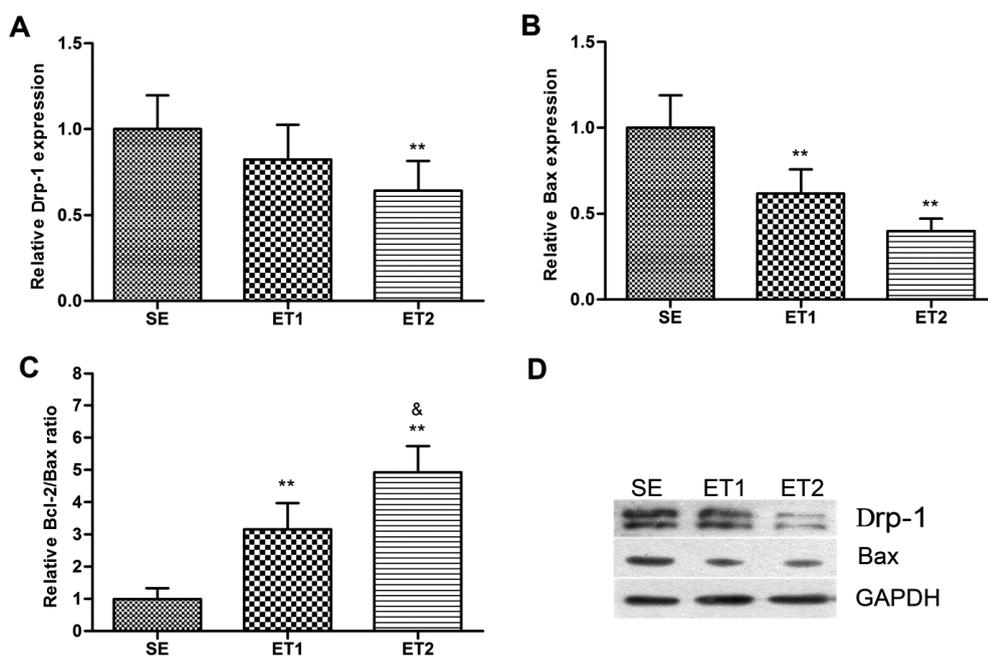

Figure 4. Effects of swimming training on the pro-apoptotic proteins involved in intrinsic apoptosis pathway. The expression of Drp-1 was decreased in ET1 and ET2 (**A**). A similar decreased change in Bax was induced by the two trainings (**B**). The Bcl-2/Bax ratio was increased in ET1 and ET2 (**C**). Representative blots of Drp-1 and Bax (**D**). Values are mean ± SD. $n = 7$/group, ** $p < 0.01$ *vs.* SE, & $p < 0.05$ *vs.* ET1. For abbreviations, see Fig. 1.

A positive correlation was found between p53 and Drp-1 (R = 0.738, *p* < 0.01), p53 and Bax also had the same relation (R = 0.52, *p* < 0.01).

Discussion

The heart may undergo an increase in apoptosis during ischemia-reperfusion, heart failure, and aging (Chen et al. 2009; Duan et al. 2012; Fannin et al. 2013). On the other hand, high apoptosis in heart is believed to contribute to impaired contractile function, cardiomyopathy and heart disease (Masri and Chandrashekhar 2008; Favaloro et al. 2012). Therefore, developing a strategy that results in a cardioprotection is important. Much evidence contends that exercise is cardioprotective. First, previous studies confirmed that exercise reduces the risk of heart diseases in pathological models. For instance, fewer Fas-dependent and mitochondria-dependent apoptotic proteins were found in exercise group in spontaneously hypertensive rats (Lee et al. 2006; Huang et al. 2012). A second, *in vitro* experiments, the exercise-induced cardioprotection is mediated through mitochondrial adaptations, because isolated mitochondria resist ROS and IR-induced damage (Kavazis et al. 2008; Lee et al. 2012). We want to investigate whether exercise leads to the same benefit in physiological condition of healthy individual. A study from Kwak et al. (2006) demonstrated that exercise training can reduce age-induced apoptosis in rats. The present study tested the effects of swimming training on the apoptosis of heart in adult mice, consistent with previous observations; our data showed that TUNEL positive cell rate in ET2 was decreased significantly, while mice in ET1 did not create the same result. The current data suggested that swimming training could lead to relatively low level cardiac apoptosis in adult mice. The reason why ET1 failed to produce the protective effect may be the exercise intensity was relatively weak.

Understanding of the cellular mechanisms responsible for this cardioprotection remains incomplete. Heart is energetically-demanding tissue requiring continuous production of ATP from mitochondria which occupy up to 25% of cell volume (Li et al. 2012). The merger of adverse events may orchestrate opening of the mitochondrial permeability transition pore, leading to cell death through necrosis or apoptosis (Kung et al. 2011). Thus, we mainly focused on mitochondria-dependent pathway in apoptosis. miRs are about 22 nucleotides long and act as negative regulators of gene expression by inhibiting mRNA translation or promoting mRNA degradation (Lee et al. 2003). miRs can regulate cardiac function, including the conductance of electrical signals, heart muscle contraction, heart growth and morphogenesis (Chien 2007). miR-1 was suggested to participate in apoptosis, and Bcl-2 was also confirmed to be

a direct target of miR-1 later (Tang et al. 2009). In current study, we evaluated changes of miR-1 and Bcl-2 in response to different swimming training protocols. The results revealed that miR-1 expressions were downregulated in ET1 and ET2, and the decreased extent in ET2 had statistical significance. In contrary to the decrease in miR-1, the Bcl-2 expressions were upregulated in the two training groups. In ET1, the significant increase of Bcl-2 level was not consistent with the change of miR-1, which suggested other upstream regulators affect Bcl-2 expression more effectively than miR-1. The Bcl-2 level also had a significant negative correlation with miR-1 level, which suggests, the exercise induced cardioprotection in current study may be partly attributed to the changes of miR-1 and Bcl-2.

The miR-30 family consists of five members, miR-30a through -30e. Recent studies identified that miR-30 family also mediates the mitochondria-dependent apoptosis in heart. A study from Shi et al. reported that apoptosis-associated genes were highly enriched among the predicted targets of miR-30 family, and the total protein and phosphorylation expressions of p53 were inhibited by miR-30 in podocytes (Shi et al. 2013). A study from Li et al. (2010) also proved that miR-30a and miR-30b directly targets p53 in heart. miR-30b appeared to be more sensitive to ROS than the other family members (Haque et al. 2012). Additionally, after fractionating the heart of mice into nonmyocyte and cardiomyocyte populations, miR-30b other than miR-30a was substantially enriched in cardiomyocytes (Ikeda et al. 2009). Thus, we study the change of miR-30b upon exercise training. p53, a down stream target of miR-30b, can initiate apoptosis by transcriptionally regulating the gene products of the pro-apoptotic proteins. For instance, it can activate Bax, which translocates from the cytosol to mitochondria and induces the release of apoptotic proteins (Miyashita and Reed 1995). Mitochondria constantly undergo fusion and fission, which are necessary for the maintenance of organelle fidelity. Abnormal mitochondrial fission is involved in the initiation of apoptosis, which requires Drp-1, a GTPase induced by p53 that causes scission of the mitochondrial outer membrane. Drp-1 accumulates on mitochondria and mediates dramatic mitochondrial fission contributing to caspase activation in cardiomyocytes (Wang et al. 2011). We evaluated miR-30b-p53-downstream proteins axis in the current study. The results showed that miR-30b level was upregulated, while p53, Drp-1 and Bax levels were downregulated significantly in both ET1 and ET2. It is of note that ET1 also resulted in significant increase in miR-30b and decrease in pro-apoptotic proteins, especially in p53 and Bax. This implied that moderate or relative heavy load swimming training could result in beneficial effects in view of the miR-30b-p53-downstream proteins axis. The further analysis also indicated there were significant negative correlation between miR-30b and p53,

positive correlation between p53 and Drp-1. Together, our study suggested that miR-30b-p53- downstream proteins axis is responsible for the cardioprotective effects induced by exercise training.

miR-21 is found to be an anti-apoptotic regulator (Buscaglia and Li 2011). Transfection with adenovirus expressing miR-21 reduced cell apoptosis in heart, decreased myocardial infarction size, and improved left ventricular remodeling (Dong et al. 2009). Cheng et al. (2009) found miR-21 downregulated PDCD4 expression, and the protective effect of miR-21 on cell death was inhibited by overexpression of PDCD4. Another study confirmed that miR-21 directly binds to PDCD4 and inhibit PDCD4 expression by the luciferase assay (Cheng et al. 2010). PDCD4 is a critical mediator for cancer cell apoptosis, PDCD4 was overexpressed, then, cardiomyocyte apoptosis induced by ischemia-reperfusion was aggravated consequently (Cheng et al. 2010). We tested the expressions of miR-21 and PDCD4. miR-21 seemed to be less susceptible than miR-30b, only the increase in ET2 had statistical significance. Change in miR-21 meant that the increase in miR-21 may be a reason for the relatively low level of cardiac apoptosis in ET2. Interestingly, we did not observe remarkable decrease in PDCD4 expressions in ET1 and ET2, which suggested swimming training can not affect the expression of PDCD4 apparently. The relation between miR-21 and PDCD4 was assessed, and there was no significant correlation. Despite the notable increased miR-21 level in ET2, the PDCD4 level in ET1 and ET2 did not change with statistical significance. It is possible that some regulating factors induced by exercise interfere with the relation between miR-21 and PDCD4. Taken together, miR-21 and PDCD4 appear more sustainable upon the exercise training than miR-1 and miR-30b regulatory pathways.

Of note that the two swimming training resulted in different effects on apoptosis in the heart. Although the load in ET1 affected some proteins levels significantly, the significant decrease in apoptosis evaluated by TUNEL assay was not observed, which meant that the molecular changes in ET1 is not enough to induce the expected effects. In contrast, load in ET2 both affected miR-1-Bcl-2, miR-30b-p53 pathways apparently. In addition, apoptotic cell rate in ET2 decreased significantly. Together, ET2 other than ET1 can lead to more notable cardioprotection in the present work. Altogether, our study suggested that the changes of miRs and their downstream proteins induced by swimming training participate in reducing the apoptotic level in hearts.

Conflict of interest. The authors have no conflict of interest to declare.

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Swimming training affects apoptosis-related microRNAs and reduces cardiac apoptosis in mice

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Supplementary Methods

Detection of apoptosis

The heart tissue was immersion-fixed in 10% neutral formalin and embedded in paraffin. Serial sections of 4-μm thicknesses were prepared. Apoptotic cells were identified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using an apoptosis detection kit according to the manufacturer's protocol (Cat.No. G7130, Promega). Briefly, the tissue sections were dewaxed twice in xylene, 5 minutes each time, and rehydrated in decreasing concentrations of ethanol (100%, 95%, 85%, 70%, 50%), 3 minutes each time. After that, sections were immersed in 0.85% NaCl for 5 minutes and in PBS for 5 minutes respectively. The sections were fixed and washed, then, added with 100 μl Equilibration Buffer for 5 minutes. 100 μl of TdT reaction were added to the tissue sections on the slides and the sections were incubated for 60 minutes at 37°C in a humidified chamber. Then, the reaction was stopped and slides were immersed in 2X SSC for 15 minutes. After washing and then immersing slides in 0.3% hydrogen peroxide for 5 minutes, 100 μl Streptavidin HRP (diluted 1:500 in PBS) was added to slides which were incubated for 30 minutes at room temperature. After washing slides in PBS, sections were added 100 μl DAB (prepared immediately prior to use). Then, slides were immersed in deionized water for washing. The percentage of myocytes with DNA nick end labeling was analyzed by counting the cells exhibiting brown nuclei at ×20 magnification in 10 randomly chosen fields (Olympus, CX41), total 1000 cells were counted in each section (4 sections for each sample, average percentage was the sample's rate of apoptotic cells).

Western blotting analysis

Total proteins were extracted from the frozen heart tissue. The samples were placed in RIPA buffer (Beyotime Inst. Biotech, China), homogenized, incubated on ice for 20 min and then centrifuged at $12,000 \times g$ for 20 min. Samples were subjected to SDS-PAGE in polyacrylamide gels (6–15%) depending on the protein molecular weight. After electrophoresis, proteins were transferred to the polyvinylidene fluoride membrane (Cat.No. IPVH00010, Millipore). Ponceau S red (Sigma) was used to confirm equal loading and transfer of proteins to the membrane in each lane. The blot membrane was then incubated in a blocking buffer (5% non-fat dry milk, 10mM Tris-HCl (pH 7.6), 100 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and then incubated overnight at 4°C with specific antibody. These antibodies included p53 polyclonal antibody, Bcl-2 polyclonal antibody, Bax polyclonal antibody and PDCD4 polyclonal antibody (Santa Cruz Biotechnology, CA, USA, 1:1,000 dilution); Dynamin-related protein-1 (Drp-1) polyclonal antibody (Cell Signaling Tech, MA, USA, 1:1,000 dilution); HRP-conjugated monoclonal GAPDH antibody (KangChen Bio-tech, China, 1:10,000 dilution). Immunodetection of the primary antibody was carried out with peroxidase-labeled secondary antibodies and enhanced chemiluminescence reagents (Engreen Biosystem Co. Ltd, China) were used to visualize the autoradiogram. Quantification was performed in the linear absorption range by computerized densitometry using Scion Image software. GAPDH levels of heart tissues were used to normalize the results.