Transforming Growth Factor-β Function Blocking Prevents Long-Term Intensive Exercise Training-induced Cardiac Fibrosis in Rat Model

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Directed by Professor Jong-Won Ha

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

Jeonggeun Moon

December 2016

연세대학교
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December 2016

ACKNOWLEDGEMENTS

I would like to express my heartfelt thanks to my supervisor, Professor Jong-Won Ha, who directed me in every step of this thesis and provided me with encouragement and solid advice, which enabled me to complete my thesis.

I also appreciate Professor Boyoung Joung, Professor Cheol-Koo Lee, Professor Yong-Ho Ahn and Professor Hoguen Kim for their sound advice and support. I sincerely thank Ah Reum Oh, Jewook Shin, Eun Ji Shin, Jung Hoon Lee and Professor Ji-Young Cha for their great help and advice.

Finally, I would like to give my dearest thanks and deepest respect to my parents. I could not have finished this thesis without their love and wholehearted support. This thesis is dedicated to them.

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ABSTRACT

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Background: Long-term intensive exercise training induces myocardial fibrosis, which acts as an arrhythmogenic substrate. Transforming growth factor (TGF)-β pathway causes myocardial fibrosis in various cardiac diseases. The purposes of this study were to: 1) confirm vigorous exercise-induced cardiac fibrosis and 2) examine the effect of TGF-β function blocking on cardiac structure/function and pathologic collagen deposition in a chronic intensive exercise rat model.

Methods: Male Wistar rats weighing 100 to 125 g were randomly assigned to three groups: time-matched sedentary control (S-control, n=10), exercise+dimethyl sulfoxide (DMSO) [exercise control (E-control, n=5; one dropped out)] and exercise+TGF-β antagonist (TGF-β function blocking

group, n=5). The exercise groups performed intensive exercise on a treadmill for 12 weeks after two weeks of conditioning. Transthoracic echocardiography was performed at the beginning and at the endpoint of exercise training under anesthesia. At the endpoint, the hearts were harvested after euthanasia and weighed. Collagen deposition in all cardiac chambers was quantified after Masson's Trichrome stain. Biochemical studies [ribonucleic acid (RNA) of TGF-β1, fibronectin-1, matrix metalloproteinase-2 (MMP-2), of tissue inhibitors of metalloproteinase-1 (TIMP-1), collagen-Ia1, -Ia2 and –IIIa1 in all four cardiac chambers] for pathologic collagen deposition were performed with real-time polymerase chain reaction.

Results: Chronic intensive exercise training (the E-control and TGF-β function blocking group) results in less increase in body weight and left ventricular (LV) wall thickening and dilation ($p<0.05$ for all) without significant change in ejection fraction or heart weight compared with the S-control group. Myocardial fibrosis quantity significantly increased in all cardiac chambers in the E-control group ($p<0.001$ compared to S-control). Of note, in the TGF-β function blocking group, pathologic collagen deposition was significantly lower than the E-control group ($p<0.001$) in all cardiac chambers. RNA analysis results were variable: TGF-β did not differ significantly among the three groups; MMP-2 values from left ventricle (LV) and right atrium (RA) were significantly lower in the S-control compared with the E-control ($p<0.001$ in LV and $p=0.006$ in RA) and TGF-β function

blocking group (p=0.005 in LV and p=0.006 in RA), whereas other values were did not differ in intergroup comparison. Figronectin-1 values were similar in all cardiac chambers and TIMP values from LV and RA were significantly lower in the S-control group than the E-control (p=0.020 in LV and p=0.002 in RA) and TGF-β function blocking group (p=0.045 in LV and p=0.004 in RA), while other values were not remarkable. Collagen-Ia1, -Ia2 and IIIa1 values from LV and RA were significantly lower in the S-control group than the E-control group (p=0.019, p<0.001 and p=0.005 for LV and p=0.004, p<0.001 and p=0.010 for RA, respectively). When comparing values between the E-control and TGF-β function blocking group, no collagen subtypes differed significantly. Comparing the S-control and TGF-β function blocking group, collagen-Ia1 from RA (p=0.005), collagen-Ia2 from RA (p=0.001) and collagen-IIIa1 from LV (p=0.010) were significantly lower.

Conclusion: TGF-β function blocking ameliorates the heart fibrosis induced by long-term intensive exercise training in animals, without impact on cardiac structure and function.

Key words: intensive exercise, myocardial fibrosis, transforming growth factor (TGF)-β, echocardiography.

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I. INTRODUCTION

Traditionally, physical activity is considered healthful based on broad epidemiologic evidence[1, 2], and thus regular exercise is recommended for prevention and/or treatment for cardiac[3] and metabolic diseases[4]. However, long-term intensive exercise training is observationally associated with various forms of cardiac rhythm disorder[5-10] and sudden cardiac arrest has been repeatedly reported, especially in young professional athletes. The exact mechanism for this unexpected phenomenon, which is recently more often diagnosed, is still not fully understood.

Sustained high-level exercise training often results in gross adaptive changes in the heart. With recent advances in cardiac imaging studies such as echocardiography, cardiac computed tomography or magnetic resonance imaging, those are more frequently recognized, especially in veteran athletes[10].

The so-called "athlete's heart" comprises complex cardiac structural/functional adaptive changes developing in athletes undergoing long-term intensive physical training[11, 12]. Despite striking structural changes including cardiac chamber dilation and/or hypertrophy of myocardium, athlete's heart has not been considered a pathologic condition, in contrast to hypertrophic cardiomyopathy or hypertensive left ventricular (LV) hypertrophy, because it is thought to be an adaptive remodeling to increase cardiac output during intensive exercise[13]. Corrado et al.[14], however, found that young competitive athletes bear a 2.5-fold greater risk for cardiovascular disease compared to non-athletes. A proposed explanation is that sports activity may trigger life-threatening arrhythmia during intense physical exertion in susceptible individuals.

Benito et al. reported, in a seminal animal study using a rat model, cardiac fibrosis in enlarged cardiac chambers after long-term intensive exercise training and LV functional changes with increased arrhythmia inducibility[15]. In that study, cytokine pathways, presumably including transforming growth factor (TGF)-β pathway, were suggested to play a pathophysiological role. Therefore, it can be hypothesized that TGF-β function blocking might prevent exercise-induced cardiac fibrosis in the same model; however, this hypothesis has never been tested. Gay-Jordi et al. also observed prevention of cardiac fibrosis induced by endurance exercise by Losartan, a prototype of the angiotensin receptor blocker, in same

experimental model[16]. Angiotensin II induced cardiac fibrosis reportedly requires participation of the TGF-β pathway[17]. In summation, TGF-β pathways might be part of the etiology of vigorous exercise-induced pathologic collagen deposition in myocardium. Therefore, the purposes of this study were: 1) reaffirm vigorous exercise-induced cardiac fibrosis and 2) to examine the effect of TGF-β function blocking on cardiac structure/function and pathologic collagen deposition in a long-term intensive exercise rat model.

II. MATERIALS AND METHODS

1. Animals

All animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Lee Gil Ya Cancer & Diabetes Institute, Gachon University (Incheon, Republic of Korea). Pathogen-free 4-week-old male Wistar rats weighing 100 to 125 g were purchased from Orientbio (Gyeonggi Province, Republic of Korea). Rats were maintained on a normal diet (PicoLab® Rodent Diet 20, Orientbio, Gyeonggi Province, Republic of Korea) under a 12-hr light/dark cycle for 14 weeks until sacrifice.

2. Exercise protocol

Before the main experiment, we performed a pilot experiment to induce myocardial fibrosis (intense exercise group [n=5] vs. sedentary control group [n=5]). We observed exercise-induced cardiac fibrosis, along with less

body weight gain and more increase in heart weight (Fig. 1) in rats that underwent eight-week intensive exercise training. On the other hand, collagen deposition was minimal in the sedentary control group (Fig. 2). We reached a preliminary conclusion that intense exercise for more than eight weeks would be enough to induce hypertrophy of the heart (i.e. athlete's heart model) and exercise-induced myocardial fibrosis. Therefore, we used our own exercise protocol composed of two weeks of adaptation to intensifying exercise, followed by 12 weeks of intensive exercise (Fig. 3).

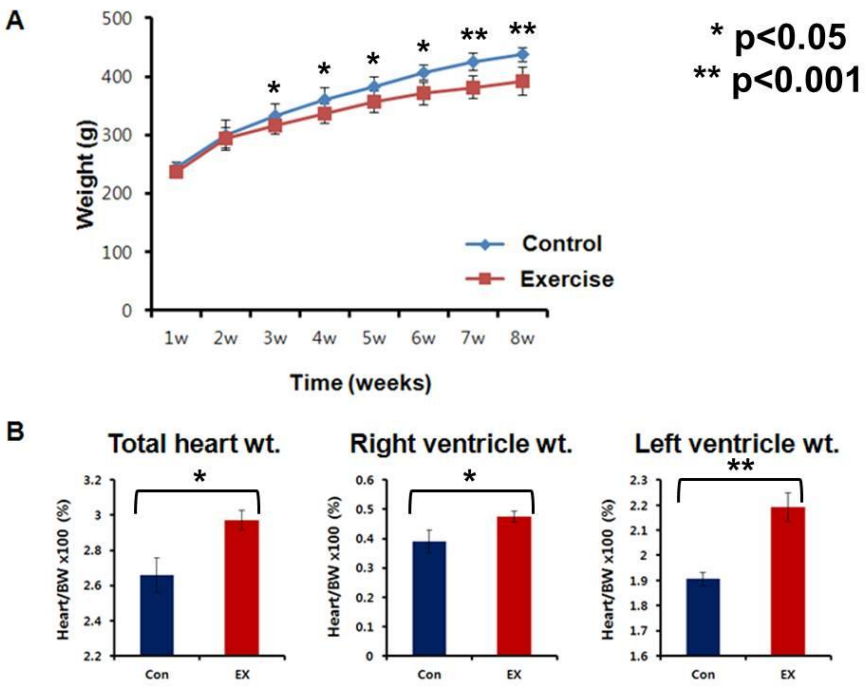

Figure 1. Pilot study to confirm eight weeks of intensive exercise-induced cardiac hypertrophy in rat model similar to the "athlete's heart." We were able to observe body-weight reduction (A) and increases in the weight of the total heart and right/left ventricle (B) in rats that underwent vigorous exercise training (n=5) compared to sedentary control group (n=5).

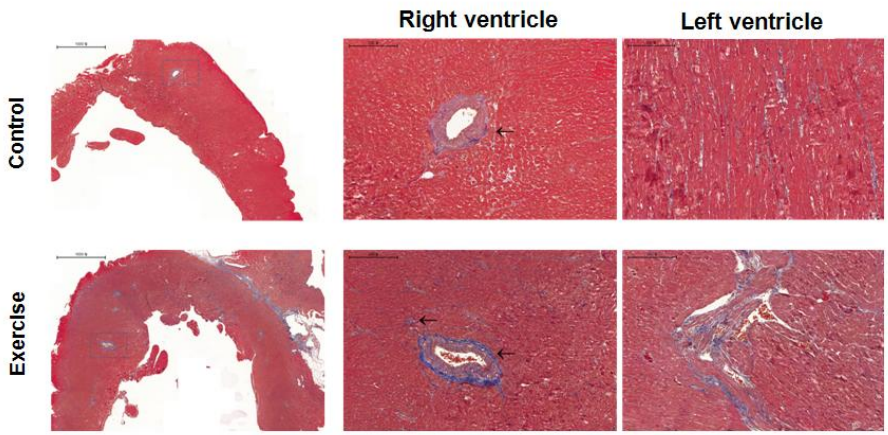

Figure 2. Pilot study to confirm intensive eight weeks of exercise-induced cardiac fibrosis in rat model using Masson's trichrome staining. We were able to observe more prominent cardiac fibrosis in the right and left ventricles in rats (arrows) that underwent vigorous exercise training (n=5) compared to sedentary control group (n=5).

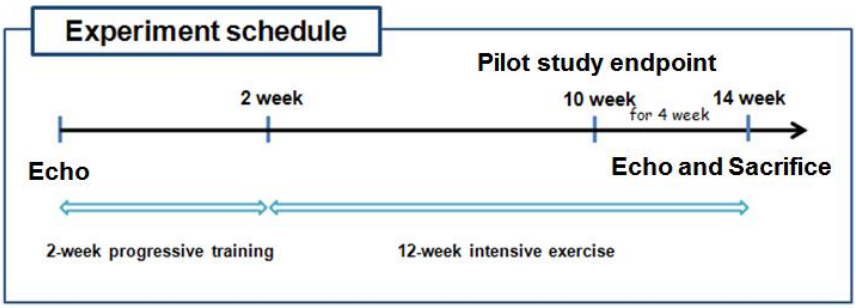

Figure 3. Exercise protocol used in the current research. After two weeks of progressive training, all rats allocated to TGF-β function blocking group (n=5) or E-control group (n=5) underwent 12 weeks of intensive exercise training. Before and after the training, two echocardiographic evaluations for cardiac structure and function evaluation was performed. The rats were then sacrificed for histologic evaluation of the heart.

Animals were randomly assigned to three experimental groups: sedentary (S-control, n=10), exercise+dimethyl sulfoxide (DMSO) [exercise control (E-control, n=5)] and exercise+TGF-β antagonist (TGF-β function blocking group, n=5). The exercise program was based on studies documenting that intensive exercise induced cardiac change[15, 16]. Exercise rats underwent daily training session on a treadmill (Treadmill CONTROL LE8710, Panlab S.I., Spain) five days a week for 14 weeks. The two-week progressive training program started with a 10 min running session at 6 m/min and increased over one hour to a speed of 36 m/min. The treadmill had separate lanes to serve as corridors for each animal, with a grid in the back that administered a small and not-physically-harmful electric shock (0.3 to 2 mA) on contact to ensure that animals ran effectively. Only rats that mastered the running training and ran spontaneously with a maximum cumulative shock time of 15 seconds per one-hour training session were included in the study. The S-control group was housed and fed in the same conditions as the E-control and TGF-β function blocking groups.

3. TGF-β antagonist and placebo administration

TGF-β antagonist "SB-505124" (Selleckchem Houston, TX, USA) was prepared in DMSO. Rats were treated with 6 mg/kg of SB-505124 or vehicle (DMSO) by intraperitoneal injection before daily training session. In E-control group, only DMSO was administered by same method.

4. Echocardiography and electrocardiography

Transthoracic echocardiography was performed with Vivid 7 system (Philips, the Netherlands) under 2% isoflurane anesthesia at baseline and after 14 weeks of the exercise training along with corresponding sedentary controls. Under the monitoring of electrocardiography (lead I), standard two-dimensional measurements LV end-diastolic dimension and LV septal/posterior wall thickness were measured. LV ejection fraction case calculated from the M-mode images. Heart rate was measured as beats/min with electrocardiography monitoring.

5. Surgical procedures, the Masson's Trichrome stain and fibrosis quantification

At the endpoint, the hearts were harvested after euthanasia and weighed. All surgical procedures were performed under 2% isoflurane anesthesia. The hearts were removed, weighed, dissected into LV, right ventricle (RV), left atrium (LA) and right atrium (RA). They were then snap-frozen in liquid nitrogen for storage at –80°C until ribonucleic acid (RNA) isolation or histology study. Each chamber was next embedded in paraffin, cut into 5-μm cross sections and stained with Masson's Trichrome (MT stain). To assess myocardial fibrosis quantitatively, a digital image analyzer was used and the percent area of myocardial collagen deposition (% myocardial fibrosis) was calculated using available software (Image J 1.48, National Institutes of Health, USA).

6. Messenger RNA (mRNA) isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from LV, RV, LA and RA of the heart using RNAiso Plus (Takara, Shiga, Japan). Purified total RNA was treated with RNase-free DNase (Roche, Penzberg, Germany) and reverse-transcribed using a QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). Gene-specific primers (Table 1) were designed using Primer Express Software (PerkinElmer Life Sciences, Waltham, MA) and validated by analysis of template titration and dissociation curves. Quantitative gene expression analyses were performed on a 7900HT Fast Real-Time PCR System (Life Technologies, Carlsbad, CA) using SYBR® Premix Ex Taq™ II, ROX Plus (Takara). Expression levels were calculated by the $2^{-\Delta\Delta}$CT method using cyclophilin (*Cyclo)* as the invariant control. [18]

Table 1. Primer sequences for quantitative real-time polymerase chain reaction used in biochemical analysis for mRNA associated with pathologic collagen deposition.

Gene symbol	Forward primers (5' to 3')	Reverse primers (5' to 3')
Tgf-b1	AAA CGG AAG CGC ATC GAA	TGG CGA GCC TTA GTT TGG A
Mmp2	CGG TTT ATT TGG CGG ACA GT	TGT TCA GCC ATC CCT TGC A
Timp	TGT TCA GCC ATC CCT TGC A	GGA TCT GAT CTG TCC ACA AGC A
Col1a1	GGA GAG TAC TGG ATC GAC CCT AAC	CTG ACC TGT CTC CAT GTT GCA
Col1a2	TGT TGC TGC TTG CAG TAA CGT	CCC TTC CGT ACA GAT CCC ATT
Col3a1	CCA ACT GGT GGC CAG AAT TAT T	CCA TTC CTC CGA CTC CAG ACT
Fibronectin	CTA TGA CAT CAG CGT TAT CAC TCT CA	AGT GTC CGG ACC GAT ATT GG

7. Statistical Analysis

Data are expressed as mean±standard error values with 95% confidence interval. Continuous data are expressed as means±SD and normality tests were performed for each variable to check the validity of the normality assumption for each variable. Differences were compared with Kruskal-Wallis test, and group comparisons with Mann-Whitney *U* test. A paired t test, two-sample t test and repeated-measure ANOVA were performed using commercially available software (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.). Null hypotheses of no difference were rejected if p-values were less than 0.05.

III. RESULTS

1. Structural and functional remodeling of the heart after 12 weeks of intense exercise training mimicking athlete's heart

During the experiment, unfortunately, one rat in the E-control group did not tolerate and complete the intense exercise training and died during the protocol. Therefore, only four rats in the E-control were eligible for data analysis. Fig. 4 shows body weight change during the 12 weeks of intense exercise training. Chronic intensive exercise causes significantly less weight gain in both E-control and TGF-β function blocking groups compared to the S-control group ($p=0.007$ and $p=0.019$, respectively). Body weight changes were similar between the E-control and the TGF-β function blocking group ($p=0.467$). Table 2 summarizes body weight data at the endpoint. Compared with the S-control group, the vigorous exercise group (E-control and TGF-β function blocking group) weighed significantly less at the end of intense exercise training ($p<0.05$ for all). However, endpoint body weight was similar between the E-control and TGF-β function blocking group.

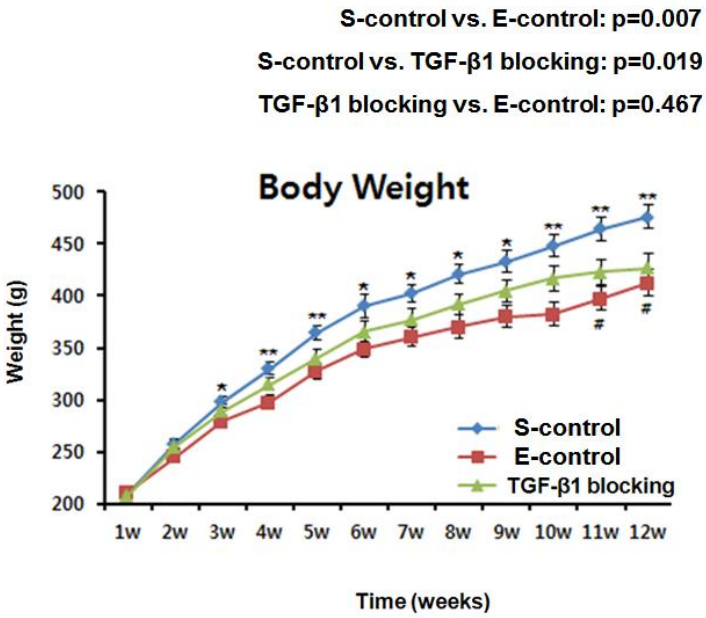

Figure 4. Body weight change during the chronic intensive work training. Male wistar rats were trained for 12 weeks beginning at 6-week-old. Body weight was monitored weekly. n=10 rats for S-control, n=4 rats for E-control, and n=5 for TGF-β function blocking group. Results are expressed as the mean±SEM. Two-sample t tests were performed for each value (*$p<0.05$ and **$p<0.001$: S-control vs. E-control/TGF-β function blocking group, #$p<0.05$: TGF-β1 function blocking group vs. E-control). In the repeated measures ANOVA test, significant weight reduction trends were observed in both the TGF-β function blocking group and E-control group compared to the sedentary control group (TGF-β function blocking group vs. S-control group: $p=0.019$ and E-control vs. S-control: $p=0.007$).

Table 2. Body weights at the endpoint.

	Body weight (g)	p-value*	p-value†
S-control group (n=10)	488±11	-	0.002
E-control group (n=4)	412±11	0.002	-
TGF-β function blocking group (n=5)	445±14	0.035	0.121

*compared with S-control group, †compared with E-control group.

Fig. 5 and 6 compare echocardiographic data assessed at the beginning and end of the chronic exercise. In the S-control group, LV wall thickness at the interventricular septum and at the posterior wall was grossly similar between the baseline and follow-up values ($p=0.156$ and $p=0.286$). LV wall thickness at the interventricular septum and at the posterior wall were significantly larger in the E-control ($p=0.008$ and $p=0.024$) and TGF-β function blocking group ($p<0.001$ and $p=0.001$) (Fig. 5). The LV end-diastolic dimension was enlarged at the endpoint in all three groups ($p=0.001$ in S-control, $p=0.017$ in E-control and $p=0.001$ in TGF-β function blocking group) (Fig. 6). The baseline LV end-diastolic dimension was similar among the three groups (p values not significant). However, the LV end-diastolic dimension was larger in the E-control ($p=0.009$) and TGF-β function blocking group ($p=0.045$) compared to the S-control group (Fig. 6). No significant changes in the LV ejection fraction from the initial to the endpoint measurement were observed in any group ($p=0.916$ in S-control, $p=0.188$ in E-control and $p=0.139$ in TGF-β function blocking group) (Fig. 6). Figure 7 shows changes in heart rate; no significant changes were observed in intra-/inter- group comparisons (p-values not significant).

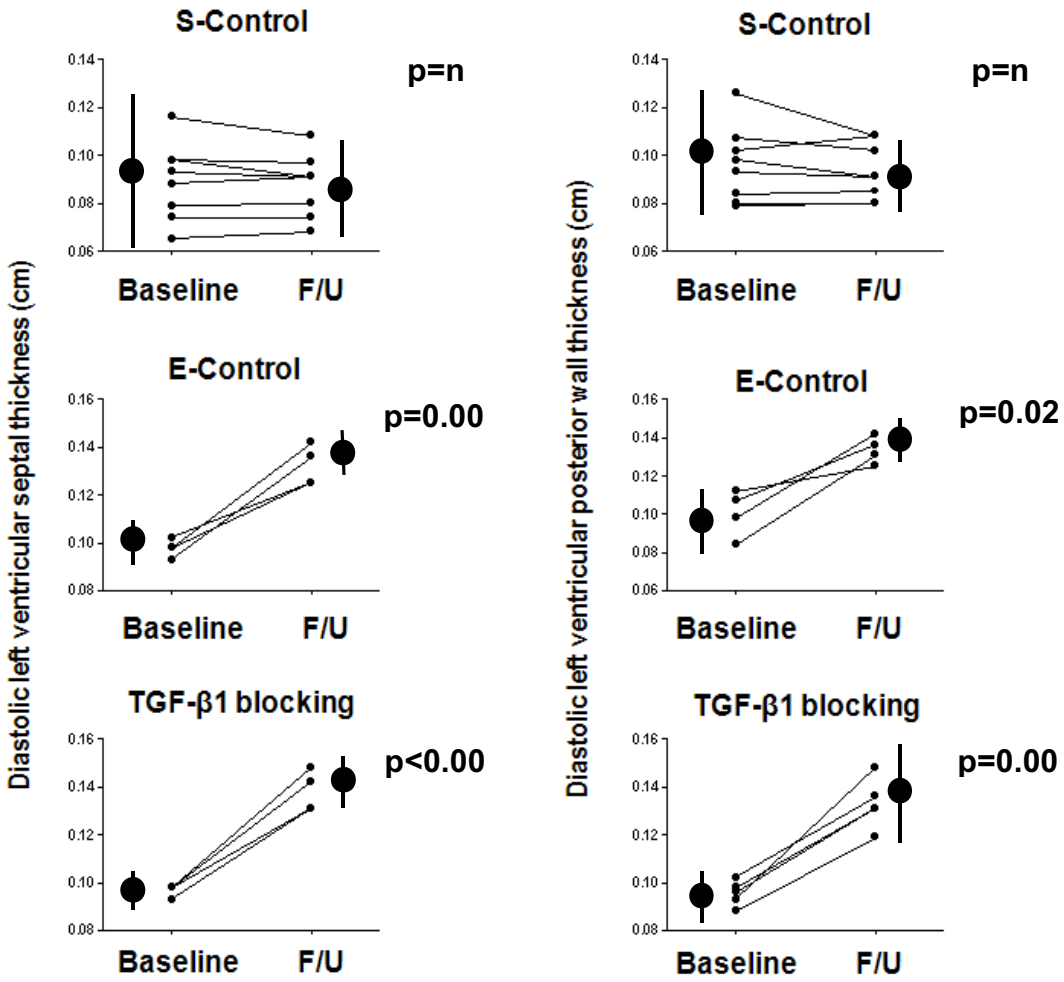

Figure 5. Changes in left ventricular wall thickness from baseline to follow-up after 12 weeks of intense exercise training assessed with transthoracic echocardiography. (S-control: n=10, E-control: n=4, TGF-β function blocking group: n=5)

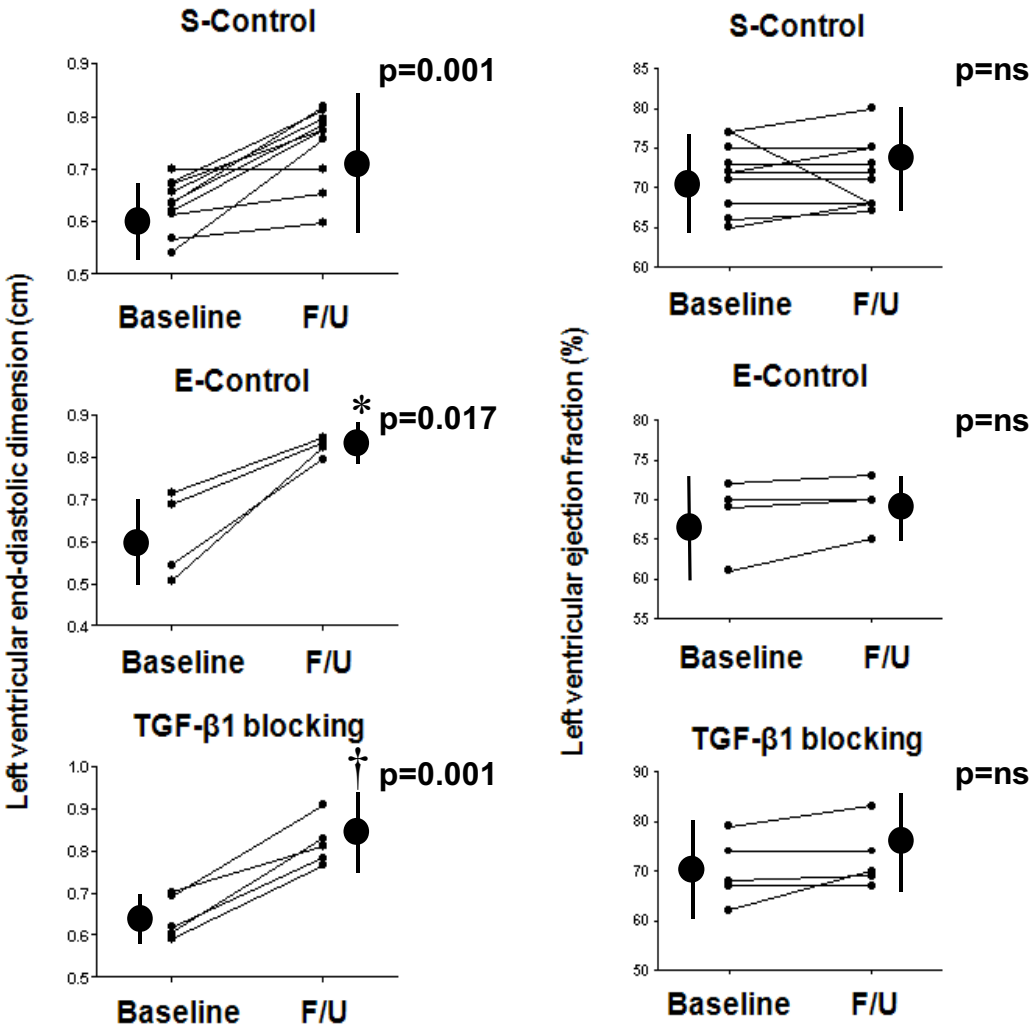

Figure 6. Changes in left ventricular end-diastolic dimension and left ventricular ejection fraction from baseline after 12 weeks of intense exercise training assessed with transthoracic echocardiography. (S-control: n=10, E-control: n=4, TGF-β function blocking group: n=5) (*p=0.009 and †p=0.045 compared to the S-control group)

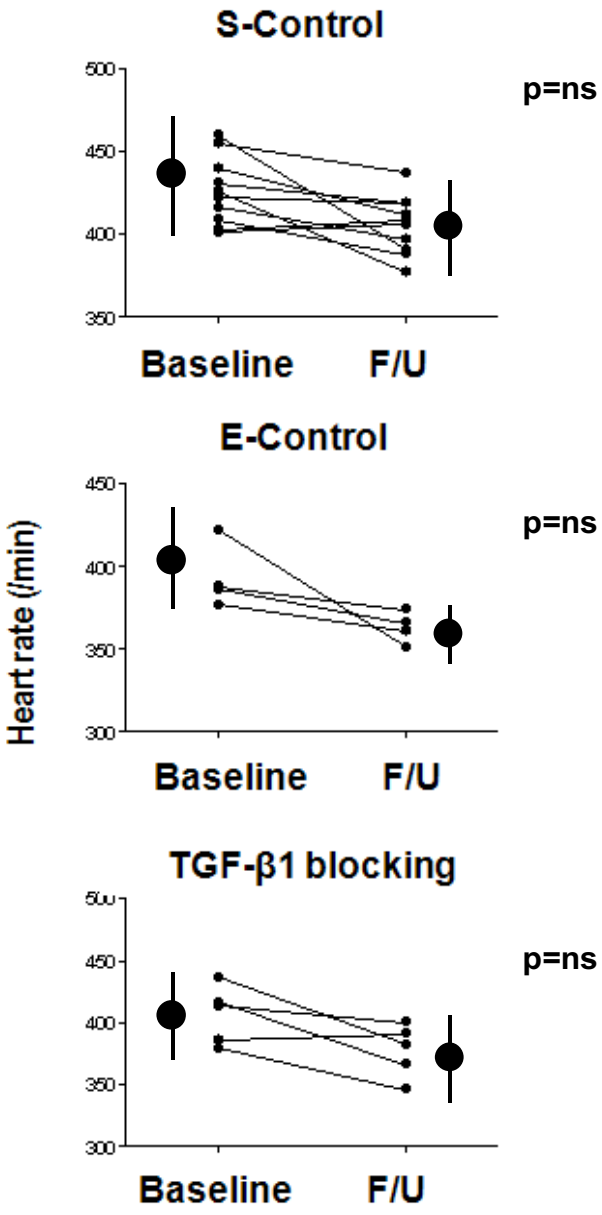

Figure 7. Changes in heart rate from baseline after 12 weeks of intense exercise training assessed with eletrocardiography. (S-control: n=10, E-control: n=4, TGF-β function blocking group: n=5)

2. Harvested heart weight and histopathology

Table 3 displays total heart, RV and LV weights. Despite aforementioned LV hypertrophy documented by echocardiography, total heart and RV/LV weights were grossly similar in intergroup comparison. Considering the significantly lower body weight of E-control and TGF-β function blocking group, however, it would be reasonable to assess the hearts of those two groups as proportionally heavier than those of S-control group.

Table 3. Total heart, right ventricle (RV) and left ventricle (LV) weights at the endpoint.

	Total heart (mg)	p-value*	p-value†
S-control group (n=10)	25.6±0.4	-	0.080
E-control group (n=4)	27.5±1.3	0.080	-
TGF-β function blocking group (n=5)	25.0±0.5	0.350	0.080
	RV weight (mg)	p-value*	p-value†
S-control group (n=10)	4.7±0.4	-	0.530
E-control group (n=4)	5.1±0.5	0.530	-
TGF-β function blocking group (n=5)	4.6±0.8	0.950	0.630
	LV weight (mg)	p-value*	p-value†
S-control group (n=10)	18.7±0.4	-	0.310
E-control group (n=4)	19.5±1.0	0.310	-
TGF-β function blocking group (n=5)	18.1±0.5	0.350	0.200

*compared with the S-contol group, †compared with the E-control group.

Fig. 8 shows images of intensive exercise-induced cardiac fibrosis. Compared to the S-control group, extensive cardiac fibrosis developed in the cardiac chambers in the E-control group. Interestingly, however, a TGF-β function blocking rat developed significantly less cardiac fibrosis than an E-control rat. As shown in Fig. 9, long-term intensive exercise training induced cardiac fibrosis in both atria and both ventricles (E-control group). Compared to the S-control group, chronic vigorous exercise (E-control group) resulted in extensive cardiac fibrosis in all cardiac chambers ($p<0.001$ for all). In the TGF-β function blocking group, significantly less pathologic collagen accumulation was observed compared with E-control group ($p<0.001$ for all). Notably, fibrosis was more heavily deposited in perivascular areas.

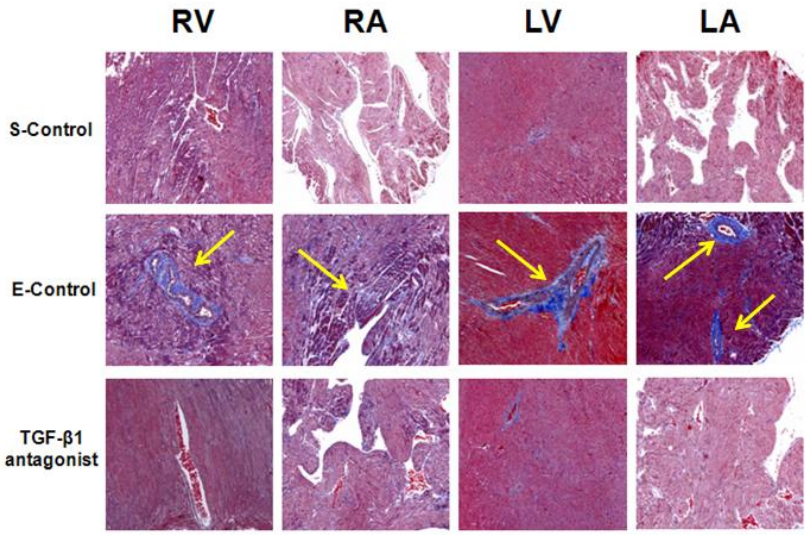

Figure 8. Representative images of intensive exercise-induced cardiac fibrosis observed in all cardiac chambers. Compared to the sedentary control group (S-control), chronic vigorous exercise [exercise control group (E-control)] resulted in extensive cardiac fibrosis in all cardiac chambers (arrows). In the TGF-β function blocking group, prominently less collagen accumulated from exercise-induced cardiac fibrosis compared to E-control.

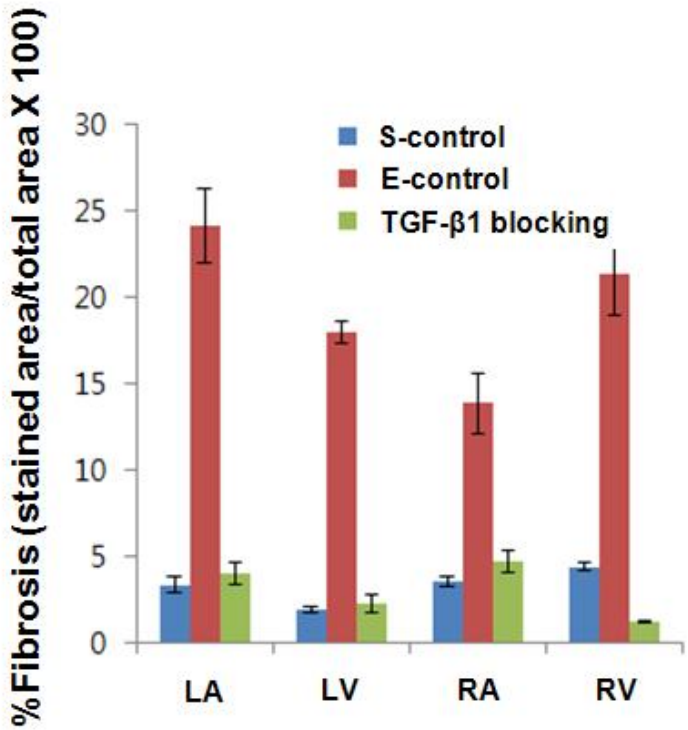

Figure 9. Long-term intensive exercise training-induced cardiac fibrosis observed in the right/left atrium (RA and LA) and right/left ventricle (RV and LV). Compared to the sedentary control group (S-control), chronic vigorous exercise [exercise control group (E-control)] resulted in extensive cardiac fibrosis in all cardiac chambers ($p<0.05$ for all). In the TGF-β function blocking group, pathologic fibrosis was observed significantly less when compared with the E-control group ($p<0.05$ for all).

3. mRNA analysis

Fig. 10 shows the results from the mRNA analysis for fibrotic markers of samples from both atria and ventricles of all rats. For TGF-β, no differences reached statistical significance among the three groups. Matrix metalloproteinase-2 (MMP-2) values from LV and RA were significantly lower in the S-control than in the E-control ($p<0.001$ in LV and $p=0.006$ in RA) and with TGF-β function blocking group ($p=0.005$ in LV and $p=0.006$ in RA), whereas other values did not differ in intergroup comparison. Figronectin-1 values were similar in all cardiac chambers and tissue inhibitor of matrix metalloproteinase (TIMP) values from LV and RA were significantly lower in S-control groups than the E-control ($p=0.020$ in LV and $p=0.002$ in RA) and with TGF-β function blocking group ($p=0.045$ in LV and $p=0.004$ in RA), while other values were not remarkable. Collagen-Ia1, -Ia2 and IIIa1 values from LV and RA were significantly lower in S-control group than the E-control group ($p=0.019$, $p<0.001$ and $p=0.005$ for LV and $p=0.004$, $p<0.001$ and $p=0.010$ for RA, respectively). When comparing values between E-control and TGF-β function blocking group, values for collagen subtypes did not differ. For the S-control versus the TGF-β function blocking group, collagen-Ia1 from RA ($p=0.005$), collagen-Ia2 from RA ($p=0.001$) and collagen-IIIa1 from LV ($p=0.010$) were significantly lower.

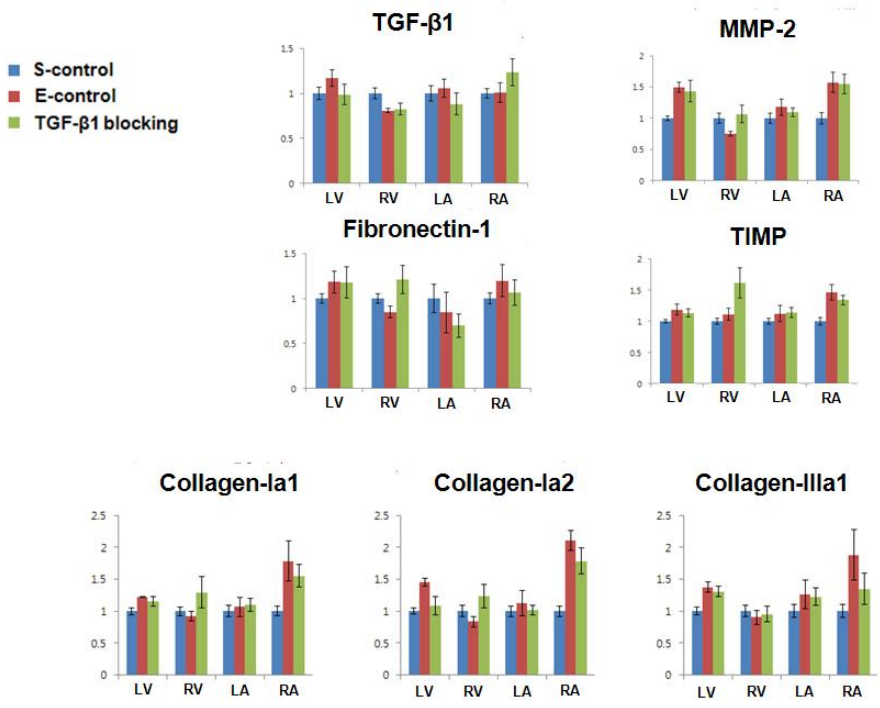

Figure 10. mRNA expression of fibrosis markers in cardiac chambers. Vertical lines mean relative mRNA levels.

IV. DISCUSSION

1. Principal findings

The core findings of this study are: 1) long-term intensive exercise training induces cardiac fibrosis and 2) TGF-β function blocking prevents vigorous exercise-induced collagen accumulation in the myocardium without affecting the gross cardiac structural/functional adaptations that are expected to occur during chronic high-level physical activity in a rat model. These results suggest that the TGF-β pathway is crucial - even though not the sole mechanism – in long-term intensive exercise-induced collagen deposition, as in other cardiac diseases. This is, to the best of our knowledge, the first study revealing the potential impact of TGF-β function blocking on prevention of exercise-induced cardiac fibrosis, presumably a pathologic substrate associated with potentially fatal arrhythmia. We observed that inhibition of the cytokine did not affect gross adaptational changes of the heart structure and increased cardiac function, all of which are essential parts of exercise training. If this effect of intervention with TGF-β function blocking is confirmed in humans, it would be a promising drug prevention for cardiac fibrosis and cardiac rhythm disorder in athletes, including sudden cardiac arrest, without affecting cardiac structural adaptation required in high-level exercise performance.

2. **Long-term intensive exercise training induces cardiac fibrosis; evidence from animal and human studies.**

Chronic exercise results in hemodynamic changes and volume/pressure overload of the heart, and eventually induces cardiac structural and/or functional adaptations designated "athlete's heart"[12, 19]. In this study, exercise-trained rats showed cardiac phenotypical changes similar to athlete's heart. Trained athletes commonly reveal electrical activity change in the heart including bradycardia and the early repolarization pattern that arises from an increased vagal tone and/or withdrawal of sympathetic activity[20-22]. However, we failed to observe any heart-rate lowering effect at the endpoint of the intense exercise training in our study samples. We may have been limited by our small sample size, and the much faster basal heart rate of the rats made it difficult to detect any difference in heart rate by a single measurement of transient electrocardiographic monitoring under anesthesia.

The athlete's heart is traditionally considered physiologically normal, because 1) myocardial hypertrophy is essentially a compensatory mechanism by which the heart adapts to an increased load[23] and 2) cardiac function is supra-normal in those athletes[24]. However, this concept is being challenged by a copious body of reports of disastrous cardiac events, including fatal arrhythmia and sudden death in people performing chronic high-level physical activity. Although the exact mechanism for the phenomenon is unclear, pathologic changes induced by chronic volume/pressure overload in athletes at the cardiac tissue level might be involved. Wilson et al. performed a small

but insightful study with life-long, veteran endurance athletes using cardiac magnetic resonance imaging. The authors reported an unexpectedly high prevalence of late gadolinium enhancement suggesting myocardial fibrosis[25]. Biochemical evidence of collagen synthesis, such as plasma carboxyterminal propeptide of collagen type I (PICP), carboxyterminal telopeptide of collagen type I (CITP) and TIMP-1 level, has been reported in a human study performed with veteran endurance athletes, even though it is unclear if they are cardiac specific or not[26]. Those changes are thought to result from maladaptive responses such as remodeling, fibrosis and tissue dysfunction similar to those found in cardiac disease[27, 28]. In a recent study by Benito et al., myocardial fibrosis induced by long-term intensive exercise increases arrhythmogenic susceptibility in an animal model[15] and might serve as a major pathologic substrate for fatal cardiac rhythm disorder[7]. This could provide a clue to explain the higher prevalence of arrhythmia in athletes. In the current research, we successfully confirm vigorous exercise-induced myocardial fibrosis in an animal model, with gross cardiac structural changes. One rat in the E-control group died during the exercise training in the current research. Although we still are unable to explain the animal's cause of death, it raises the suspicion that high-level exercise has a negative effect on longevity.

It is noteworthy that fibrosis was more predominantly distributed at the perivascular areas (Fig. 8). Given that the profibrotic effect of TGF-β

depends on interstitial fibroblast, this finding is somewhat unexpected; unfortunately, we are not able to explain the mechanism or clinical significance. Further study is warranted for our observation.

3. TGF-β function blocking prevents long-term intensive exercise-induced cardiac fibrosis

TGF-β, a locally generated cytokine secreted from various cells in an inactive form[29], is involved in inflammation[30] and fibrosis[31, 32] in the cardiovascular system[33]. It has a major influence on fibroblast proliferation, reducing degradation of collagen and fibronectin[32]. Pathologic accumulation of myocardial fibrosis resulting from cardiac volume and/or pressure overload[31] is a key determinant of cardiac functional impairment[34]. Of note, in a recent experimental study, increased TGF-β expression was reported in a hypertrophied heart[33, 35] and thus, it is thought to exert a major influence in collagen deposition in the organ. Therefore, we hypothesized that TGF-β function blocking might prevent cardiac fibrosis resulting from long-term endurance exercise model. In this study, we successfully prevented pathologic myocardial collagen deposition with TGF-β function blocking in a rat model. Although we observed a trend of decrease in fibrotic marker mRNA in some samples from LV and RA, we failed to demonstrate a homogenous reduction of TGF-β1, MMP-2, fibronectin-1, TIMP, collagen-Ia1, -Ia2 and IIIa1 in all cardiac chambers. Currently we do not have a clear answer for that, but we suggest several hypothetical explanations. 1) Because TGF-β is crucial but not

the only cytokine that involves in fibrosis, TGF-β function blocking might not have resulted in mRNA under-expression. 2) Because TGF-β is involved not only in fibrosis but also the inflammation process, fibrosis might be eventually prevented in the chronic volume-/pressure-overloaded heart despite lack of parallel changes in fibrotic marker mRNA. 3) The results might suggest a difference between mRNA level changes and eventual protein expressions. 4) As shown in the Figure 2 and 8, cardiac fibrosis occurs in patch-like form, especially in perivascular areas, not evenly. Therefore, the histologic sample harvested from non-fibrotic areas might have resulted in less prominent mRNA expression. 5) We also consider technical reasons such as tissue quality or small number of samples as a reason for the ambiguous results. 6) Finally, exercise duration of 12 weeks might not be enough to initiate gene level change. Further study, which includes more comprehensive quantitative analysis for protein level using the Western blot, is warranted for this unproven hypothesis.

4. Clinical perspective

As societal interest in health is increasing, more people are exercising. There are even people who enjoy extreme sports such as triathlon or marathon. There is an increasing body of evidence of potential cardiologic risk of chronic high-level sport training; however, the risk is not confined to professional athletes. In fact, a dose-response relationship between nonprofessional vigorous exercise and prevalence of atrial fibrillation was

reported in a human observational study[36]. Although the precise mechanism is unclear, proposed etiologies are cardiac chamber dilation, inflammation and/or autonomic functional changes[36, 37] all of which could result in pathologic collagen deposition in cardiac chambers, presumably via neuro-humoral pathways including TGF-β. The duration of intense exercise training in our study and previous studies was 8~16 weeks. This corresponds to 5~10 years in humans. Therefore, exercise-induced cardiac disease could be a future public health problem, especially in an advanced society where physical activity is popular and widely recommended. Based on our study, we suggest cardiologic attention should be paid not only to athletes but also to the apparently normal population who exercise regularly. Kuwahara et al. [31] reported that TGF-β function blocking prevented cardiac fibrosis and eventually resulted in less severe diastolic functional impairment, while not affecting hypertrophy in cardiac pressure overloaded rat model. That interesting data and ours raise the possibility for TGF-β function blocking to be clinically applied for cardiac fibrosis prevention in cardiac diseases associated with volume/pressure overload as a promising drug intervention. It is noteworthy that TGF-β function blocking did not result in any identifiable cardiac morphologic or functional changes in the current model. In addition, Gay-Jordi et al. reported renin-angiotensin system blockade prevents the heart fibrosis induced by endurance exercise training. If the same result were demonstrated in human “athletes’ heart”, TGF-β function blocking (and/or

renin-angiotensin system blockade) is a possible tool to prevent collagen accumulation in the myocardium, thought to be an important pathologic substrate of fatal cardiac rhythm disorder, without affecting exercise capacity.

5. Limitations

First of all, the sample size is small. As mentioned earlier, one rat in E-control group dropped out during the exercise training, and unfortunately we cannot explain the cause of death. Our study is not free from the inborn limitation of an animal study; extrapolation from bench to human should be done very cautiously. To make the animals run effectively, we administered presumably "non-physically-harmful shocks" to the rats. However, these might act as physical and emotional stress to the animals and a potential confounding factor. Regarding echocardiography, we measured only LV ejection fraction for functional assessment of the heart; however, LV ejection fraction is load-dependent parameter and reflects only systolic function. Myocardial fibrosis affects diastolic function, which is a more delicate and meticulously controlled cardiac cycle than systole. It would have been better if we had evaluated any changes in arrhythmia susceptibility associated with a different fibrosis burden in E-control and TGF-β function blocking group; unfortunately we do not have the data.

V. CONCLUSIONS

TGF-β function blocking ameliorates heart fibrosis induced by endurance exercise in training animals without impact on cardiac structure and function. This is the first report regarding the prophylactic effect of TGF-β function blocking for pathologic collagen accumulation, considered a pathologic substrate associated with cardiac rhythm disorder in animal models. If the same result were demonstrated in human “athletes’ heart”, TGF-β function blocking would be a promising tool to prevent potentially harmful myocardial fibrosis without affecting exercise capacity of the athletes.

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ABSTRACT (IN KOREAN)

Transforming Growth Factor-β 기능 억제가 장기간의 고강도 운동을 수행하는 백서 심근 섬유화 감소에 미치는 역할

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서론: 장기간 수행하는 고강도 운동이 심장 부정맥을 유발하는 심근 섬유화와 연관이 있다는 임상적, 역학적 관찰 결과들이 최근 보고되고 있다. 한편, transforming growth factor (TGF)-β pathway 는 여러 심장 질환에서 심근 섬유화에 중대한 역할을 한다. 본 연구의 목적은: 1) 고강도 운동 유발성 심근 섬유화의 발생을 백서 모델에서 확인하고, 2) 그러한 모델에서 TGF-β 의 기능 억제가 심장의 구조와 기능 및 병적인 심근 섬유화의 감소에 영향을 줄 수 있는지 확인하는 것이다.

방법: 몸무게 100~125 g 의 백서 (Wistar rat) 를 무작위로 3 군으로 배정하였다: 시간 통제의 무운동 대조군 (n=10), dimethyl sulfoxide (DMSO) 를 위약으로 투여한 운동 대조군 (n=5, 1 마리 중도 탈락) 및 TGF-β 길항제를 투여한 운동 실험군 (n=5). 운동을

시행한 군들은 2 주간의 적응기간을 거친 후 트레드밀에서 12 주간 36 m/s 의 속도로 매일 한 시간씩 주 5 일간 시행하였다. 경흉부 심초음파 검사는 운동 시작 전 및 모든 운동 종료 후 마취 하에 시행하였다. 실험 종말점에서 심장을 적출하여 무게를 측정하고, 좌/우 심방 및 심실에서 Masson's Trichrome 염색 후 심근 섬유화를 정량적으로 측정하였다. TGF-β1, fibronectin-1, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), collagen-Ia1, -Ia2 및 procollagen-III 에 대한 ribonucleic acid (RNA) 에 대한 real-time polymerase chain reaction 법을 통한 정량 검사를 좌/우 심방 및 심실 조직에서 시행하였다.

결과: 장시간에 걸친 운동을 실행한 운동 대조군 및 TGF-β 길항제 투여군은 모두 무운동 대조군에 비하여 체중 증가의 정도가 현저히 낮은 반면 좌심실 벽의 두께와 좌심실의 직경은 두드러지게 증가하였다 (모두 $p<0.05$). 단, 좌심실 구혈율과 심박수의 차이는 세군 모두 차이가 없었다. 운동 대조군에서는 심근의 섬유화가 무운동 대조군에 비하여 현격히 증가하였다. ($p<0.001$). 특히 TGF-β 길항제 투여군에서는 심근 섬유화가 운동 대조군에 비하여 유의하게 감소하였다 ($p<0.001$). 한편 RNA 분석 결과는 다양하였다: TGF-β 는 모든 군간 차이가 없었으나, 좌심실과 우심방의 MMP-2

는 무운동 대조군이 운동 대조군에 비하여 낮았고 (좌심실: p<0.001, 우심방: p=0.006) TGF-β 길항제 투여군에 비하여도 낮았다 (좌심실: p=0.005, 우심방: p=0.006); 하지만 다른 수치들은 군간 차이가 없었다. Figronectin-1 모든 군간 비교가 통계적으로 의미가 없었으나, 좌심실과 우심방의 TIMP 수치는 무운동 대조군이 운동 대조군에 비하여 (좌심실: p=0.020, 우심방: p=0.002) 그리고 TGF-β 길항제 투여군에 비하여 (좌심실: p=0.045, 우심방: p=0.004) 낮았다. Collagen-Ia1, -Ia2 과 -IIIa1 수치들은 무운동 대조군의 좌심실 및 우심방에서 측정 시 운동 대조군에 비하여 낮았고 (좌심실: p=0.019, p<0.001 및 p=0.005, 우심방: p=0.004, p<0.001 및 p=0.010). 운동 대조군과 TGF-β 길항제 투여군 사이에는 차이가 없었다. 반면, 무운동 대조군의 우심방 collagen-Ia1 (p=0.005), collagen-Ia2 (p=0.001) 및 좌심실 collagen-IIIa1 LV (p=0.010) 은 TGF-β 길항제 투여군에 비하여 낮았다.

결론: TGF-β 기능 억제는 고강도 운동을 시행하는 동물 모델에서 심장의 구조와 기능의 변화와 무관하게 심근 섬유화를 억제한다.

핵심되는 말: 고강도 운동, 심근 섬유화, transforming growth factor (TGF)-β, 심초음파.