

Original Article



Endurance Exercise Training Prevents Elevation of Soluble ST2 in Mice with Doxorubicin-Induced Myocardial Injury

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Conflict of Interest

The authors have no financial conflicts of interest.

ABSTRACT

Background and Objectives: Endurance exercise training (ET) can improve outcomes for patients with heart failure (HF). We investigated the preventive effects of ET on serum biomarkers for HF in mice treated with doxorubicin (DOX).

Methods: A cohort of male wild-type mice were randomly assigned to 3 groups: sedentary control (CON), DOX-treated sedentary (DOX), and DOX-treated endurance ET (ET-DOX) groups. ET groups performed moderate intensity endurance ET on a motor treadmill for 8 weeks. After 8 weeks, the DOX and ET-DOX groups were treated with DOX via weekly intraperitoneal injections of 8 mg/kg for a total of 4 weeks. We compared M-mode echocardiography, histology, and biomarkers for HF between groups.

Results: A total of 30 mice survived during the study period and were analyzed: CON (n=9), DOX (n=9) and ET-DOX (n=12). There was no significant difference in left ventricular ejection fraction (LVEF) or fractional shortening (FS) between DOX and ET-DOX groups. The ET-DOX group had a significantly lower soluble ST2 level (176.6 ± 44.1 vs. 225.4 ± 60.5 pg/mL, $p=0.021$) compared to the DOX group. Also similar between the ET-DOX and the DOX groups were the serum N-terminal prohormone of brain natriuretic peptide (30.3 ± 12.5 vs. 34.0 ± 21.7 pg/mL, $p=0.849$), troponin I (685.7 ± 99.2 vs. 722.5 ± 126.7 pg/mL, $p=0.766$), and neutrophil gelatinase-associated lipocalin (324.3 ± 82.4 vs. 312.7 ± 68.2 pg/mL, $p=0.922$) levels. Histologically, there was no significant difference in degree of perivascular fibrosis between DOX and ET-DOX groups.

Conclusions: Endurance ET is effective for preventing increases in serum soluble ST2 in mice treated with DOX.

Keywords: Heart failure; Exercise training; Doxorubicin; Biomarkers; Prevention

INTRODUCTION

Heart failure (HF) is a progressive disease and shows worse prognosis after every acute decompensation episode. The prevalence of HF in Korea, which has been gradually increasing, was reported to be about 1.53% in 2013.¹⁾ Among the many causes of HF, chemotherapy is associated with cardiotoxicity and progression of HF. In particular, doxorubicin (DOX) treatment significantly compromises both systolic and diastolic cardiac function and can be manifested in both acute and chronic phases.²⁾³⁾ As cardiac rehabilitation

Author Contributions

Conceptualization: Kim BJ, Heo JH; Data curation: Choi JY, Oh SJ, Heo JH; Formal analysis: Kim BJ; Investigation: Kim BJ; Writing - original draft: Kim BJ, Heo JH.

becomes more common in Korea, demand for exercise training (ET) in patients with HF has increased, and the importance of exercise to prevent HF has been emphasized.

There is convincing evidence for the benefits of ET in HF patients.⁴⁾ Improvements in peak oxygen uptake, quality of life, and functional class after ET in HF patients strongly suggest that this non-pharmacological strategy plays an important role in the treatment of HF.⁵⁾ There is also increasing interest in ET as an important opportunity for prevention of HF.⁶⁾ In particular, regular aerobic ET was approved for its preventive effect in mitigating the cardiac dysfunction that accompanies DOX treatment.⁷⁾

In the clinical field, brain natriuretic peptide (BNP) and N-terminal prohormone of BNP (NT-pro BNP) are currently the most widely used biomarkers for diagnosing HF and predicting prognosis. The European HF guidelines include a table of prognostic factors that includes both peptides.⁸⁾ They were also recognized to be effective in evaluating the effect of ET in HF patients in a recent meta-analysis.⁹⁾ Recently, in addition to NT-pro BNP, biomarkers such as soluble ST2 and neutrophil gelatinase-associated lipocalin (NGAL) have been used for diagnosing and predicting prognosis of HF. The ST2 gene, a member of the interleukin-1 receptor family, was recently described to be markedly upregulated in an experimental model of HF.¹⁰⁾ NGAL has also been demonstrated to be robustly expressed in HF and linked to myocardial fibrosis and remodeling in animal models.¹¹⁾¹²⁾ Some studies have analyzed the effect of ET on HF using these new biomarkers.¹³⁾ However, there is a lack of research analyzing the preventive effects of ET on the biomarkers of HF. We aimed to investigate the preventive effects of ET on several serum biomarkers for HF in mice treated with DOX.

METHODS

Animal care

All animals and procedures were handled and conducted in accordance with Southeast Medi-Chem Institute (SEMI) guidelines. Eight-week-old male C57BL6 mice were purchased from SAMTACO BIOKOREA (Korea). Before randomization, all mice were lightly exercised on the treadmill (10 m/min) for 30 min/day for 5 days to acclimatize them to the treadmill and minimize bias in the final endurance test. Mice were under a 12:12-hour light-dark cycle (06:00–18:00) and had free access to drinking water and food. The study was approved by the Institutional Animal Care and Use Committee of SEMI (SEMI-18-005).

Study protocol

Following acclimatization, at 10 weeks of age, the mice were randomly assigned to one of three groups: sedentary and treated with saline (CON, n=10), sedentary and treated with doxorubicin (DOX, n=25), and aerobic exercise training and treated with doxorubicin (DOX-ET, n=25) (**Figure 1**).

DOX treatment

Doxorubicin hydrochloride (DOX; Tokyo Chemical Industry, Tokyo, Japan) was administered via weekly intraperitoneal injections of 8 mg/kg for a total of 4 weeks.¹⁴⁾ The CON group received weekly intraperitoneal injections of 0.9% saline. The sedentary animals were handled daily.

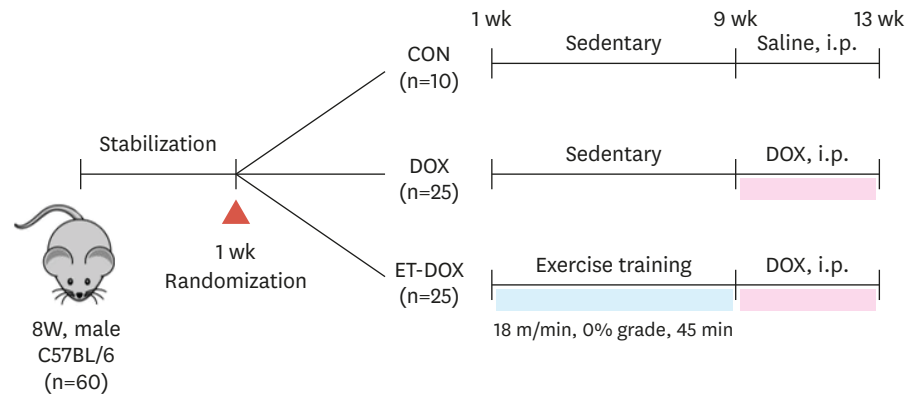


Figure 1. Study design.

Following acclimatization, at 10 weeks of age, the mice were randomly assigned to one of 3 groups: sedentary and treated with saline (CON, n=10), sedentary and treated with doxorubicin (DOX, n=25), and aerobic exercise training and treated with doxorubicin (DOX-ET, n=25).
i.p. = intraperitoneal.

Exercise protocol

Aerobic ET was performed between 09:00 and 12:00 and consisted of progressive treadmill running up to 18 m/min at 0% grade for 45 min, 5 days/wk for 8 weeks, on a motorized treadmill (DJ-344; Daejong Instrument Industry Co., Seoul, Korea).¹⁴⁾

Echocardiographic measurement

Transthoracic echocardiography was performed by an experienced and blinded animal research echocardiographer, using a 12-MHz transducer connected to a Vivid iq echocardiography machine (GE Medical, Milwaukee, WI, USA). Mice were mildly anesthetized (sedated with 3% isoflurane and 1.0 L/min oxygen and maintained at 1–1.5% isoflurane and 1 L/min oxygen). Two-dimensional M-mode recordings were obtained by transthoracic echocardiography short-axis views at the level of the papillary muscles, and standard calculations using this view were performed. Ventricular dimensions such as left ventricular (LV) end-diastolic interventricular septal and posterior wall thickness, LV end-diastolic dimension (LVEDD), and LV end-systolic dimension (LVESD) were calculated from the M-mode measurements of at least three to 6 cardiac cycles. Ejection fraction (EF) and fractional shortening (FS) were determined. FS was calculated according to the following formula: $FS (\%) = (LVEDD - LVESD) / LVEDD$. All parameters were evaluated based on the average of three consecutive beats. One cardiologist and one specialized animal echocardiographer who were blinded to animal treatment information performed all of the data acquisition.

Analysis of biochemistry

Blood samples were collected from the abdominal (descending) aorta. Serum soluble ST2, NT-pro BNP, NGAL, troponin I, and uric acid were determined by ELISA (soluble ST2: MBS748323, NT-pro BNP: MBS2501591, NGAL: ab199083, troponin I: MBS7236840, and uric acid: ab65344; MyBioSource, San Diego, CA, USA and Abcam, Cambridge, MA, USA). Analyses were performed according to the manufacturers' instructions.

Histological analysis of myocardial tissue

After all experiments were completed, the mice were sacrificed, and the heart tissues were rapidly excised and fixed in 10% formalin for paraffin sectioning. Transverse plane samples

of the right and left ventricles were sectioned into 4- μ m-thick slices for hematoxylin and eosin (H&E) staining to visualize the cardiomyocyte architecture and evaluate the general morphology of the myocardium. Masson's trichrome staining was used to demonstrate myocardial interstitial fibrosis and perivascular fibrosis. With this stain, the normal myofiber was stained red, while the collagen was stained blue. Evaluation of the pathologic response was performed by a pathologist.

Statistical analysis

Results are presented as the mean \pm standard deviation. End points were analyzed using a one-way analysis of variance (ANOVA) to confirm differences among groups. In general, when the number of groups is small, it may be necessary to perform nonparametric tests after normality tests. However, Kolmogorov-Smirnov normality test of the groups and variables used in this study confirmed that the assumption of normal distribution was satisfied ($p>0.05$). In comparisons between groups, when differences between groups were significant according to ANOVA, the significances of differences between individual groups in Scheffé post-hoc analysis was presented. A probability value of 0.05 was considered significant.

RESULTS

Mouse care and body weight

At baseline, the three groups showed similar body weights. During the study period, there were no significant differences among groups in water and food intake. After 10 weeks, the CON group showed increased body weight compared to baseline, but the DOX and DOX-ET groups did not (**Figure 2**).

Echocardiography

Table 1 shows echocardiographic data after intervention. The DOX and ET-DOX groups showed significantly reduced LVEF and FS compared to the CON group. Between the 2 groups, there were no significant differences in LVEF (DOX vs. ET-DOX: 55.1 \pm 9.8% vs. 53.7 \pm 8.0%, $p=0.877$) or FS (DOX vs. EX-DOX: 24.6 \pm 6.2 vs. 23.6 \pm 4.7, $p=0.849$) (**Figure 3A and B**). No pericardial effusion was observed in the CON groups, but significant pericardial effusion was noted in 4 mice in the DOX group and 3 mice in the DOX-ET group.

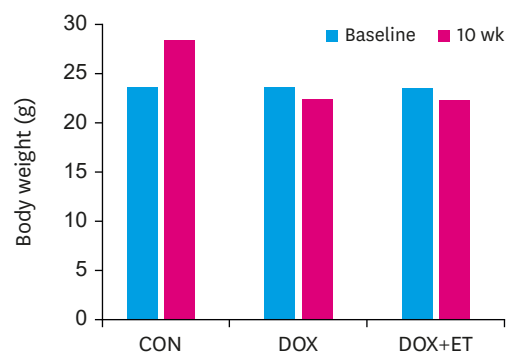


Figure 2. Baseline and final body weights. After 10 weeks, the CON group showed increased body weight compared to baseline, but the DOX and DOX-ET groups did not. CON = control; DOX = sedentary and treated with doxorubicin; DOX-ET = aerobic exercise training and treated with doxorubicin.

Table 1. Echocardiogram data

Variables	CON ^A	DOX ^B	DOX + ET ^C	F (P)	Scheffé
LVEDD	3.82±0.43	3.29±0.39	3.30±0.45	5.693 (0.006)	A>B,C
LVESD	2.39±0.63	2.50±0.31	2.43±0.61	0.144 (0.866)	
LVSWT	0.70±0.15	0.68±0.90	0.67±0.10	0.264 (0.770)	
LVPWT	0.70±0.12	0.67±0.11	0.69±0.12	0.249 (0.781)	
LVEF	68.19±6.84	55.09±9.82	53.66±8.04	9.757 (0.000)	A>B,C
FS	33.11±5.09	24.60±6.16	23.61±4.67	10.667 (0.000)	A>B,C
LVEDV	0.14±0.04	0.10±0.03	0.10±0.04	4.920 (0.012)	A>B,C
LVESV	0.05±0.02	0.04±0.02	0.04±0.02	0.342 (0.713)	

CON = control; DOX = doxorubicin; EDV = end-diastolic volume; ESV = end-systolic volume; ET = exercise training; FS = fractional shortening; LVEDD = left ventricular end-diastolic dimension; LVEF = left ventricle ejection fraction; LVESD = left ventricular end-systolic dimension; LVPWT = left ventricle posterior wall thickness; LVSWT = left ventricle septal wall thickness.

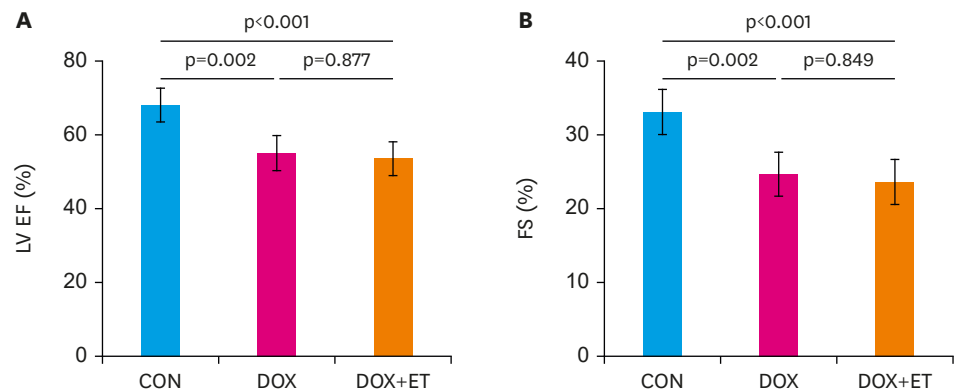


Figure 3. There were no significant differences in (A) LVEF (DOX vs. ET-DOX: 55.1±9.8% vs. 53.7±8.0%, $p=0.877$) or (B) FS (DOX vs. EX-DOX: 24.6±6.2 vs. 23.6±4.7, $p=0.849$) between the 2 groups. CON = control; DOX = sedentary and treated with doxorubicin; DOX-ET = aerobic exercise training and treated with doxorubicin; LVEF = left ventricular ejection fraction; FS = fractional shortening.

Mortality

After the first DOX injections, nine mice in the DOX group and 8 mice in the ET-DOX group died. After completing the study protocol, a total of only 30 mice survived and were finally analyzed: CON ($n=9$), DOX ($n=9$), and ET-DOX ($n=12$).

Biomarkers

In the analysis of serum biomarkers, the ET-DOX group had a significantly lower soluble ST2 level compared to the DOX group (176.6±44.1 vs. 225.4±60.5 pg/mL, $p=0.021$) (**Figure 4A**). However, there was no significant difference in NT-pro BNP levels between the ET-DOX and DOX groups (30.3±12.5 vs. 34.0±21.7 pg/mL, $p=0.849$) (**Figure 4B**). Troponin I (685.7±99.2 vs. 722.5±126.7 pg/mL, $p=0.766$), NGAL (324.3±82.4 vs. 312.7±68.2 pg/mL, $p=0.922$) and uric acid (4.8±2.0 vs. 5.3±3.2 nmol/mL, $p=0.878$) levels were also similar between the two DOX-treated groups (**Figure 4C-E**).

Histologic findings

Compared to the CON group, H&E staining (**Figure 5**) of the DOX and the ET-DOX groups showed disorganization of the muscle fibers, variable degrees of interstitial edema, and multifocal areas of cytoplasmic vacuolation, which are known effects of doxorubicin (**Figure 5B and C**). However, no significant quantitative differences were observed between the DOX group and ET-DOX group. **Figure 6** illustrates microscopic examination of Masson trichrome stained tissues (×400) and reveals that the CON group showed no distinct fibrosis (**Figure 6A**), whereas perivascular fibrosis in the groups with DOX (**Figure 6B**) and ET-DOX (**Figure 6C**) was visible,

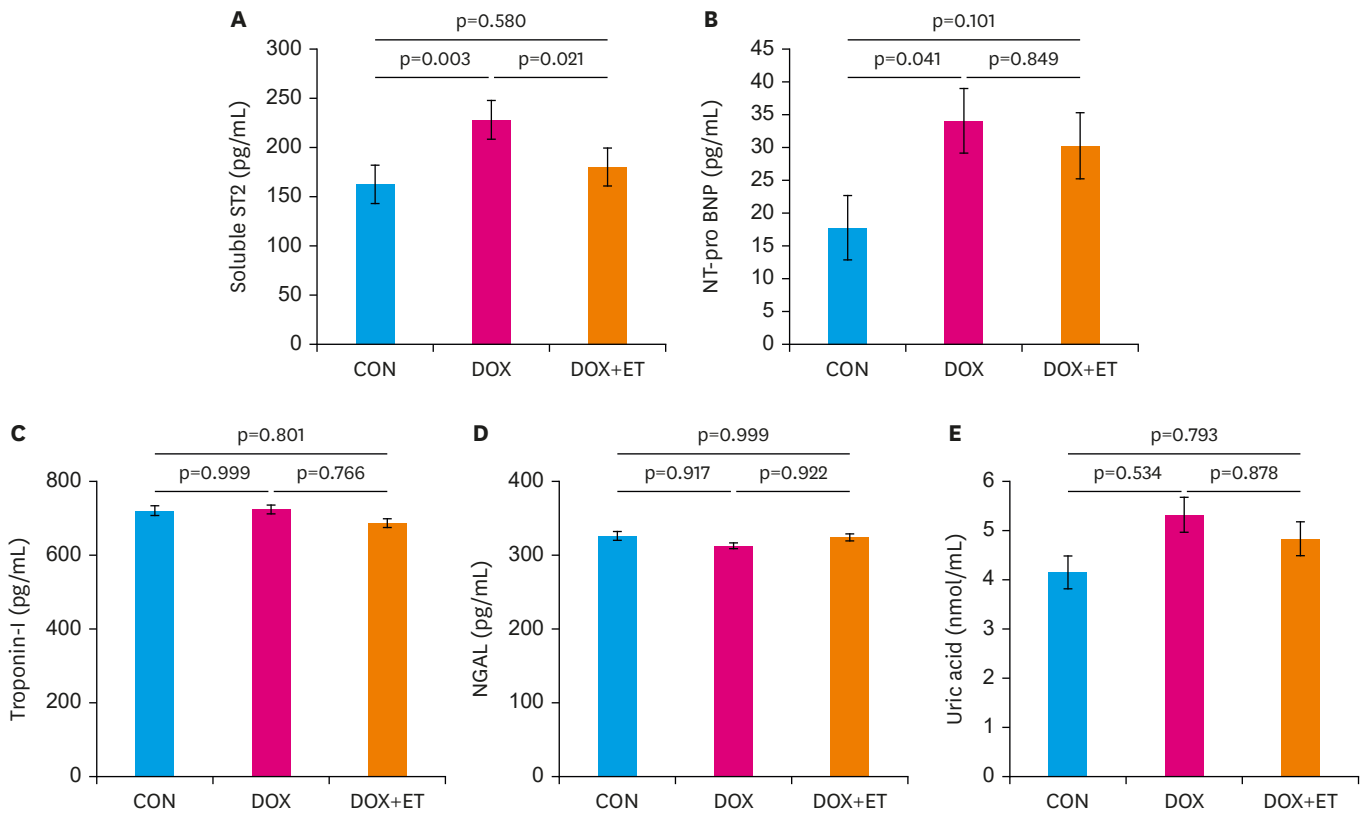


Figure 4. Analysis of serum biomarkers. (A) Soluble ST2, (B) NT-pro BNP, (C) troponin I, (D) NGAL, and (E) uric acid. CON = control; DOX = sedentary and treated with doxorubicin; DOX-ET = aerobic exercise training and treated with doxorubicin; NT-pro BNP = N-terminal prohormone of brain natriuretic peptide; NGAL = neutrophil gelatinase-associated lipocalin.

stained blue. Interstitial fibrosis (arrow) was observed infrequently in the myocardial tissue of the DOX group (**Figure 6D**). However, there was no significant difference in degree of perivascular fibrosis between the DOX group and the ET-DOX group.

DISCUSSION

The underlying mechanism by which ET prevents HF is still under investigation. In terms of hemodynamic physiology, ET helps to increase stroke volume and cardiac output^{[15][16]}

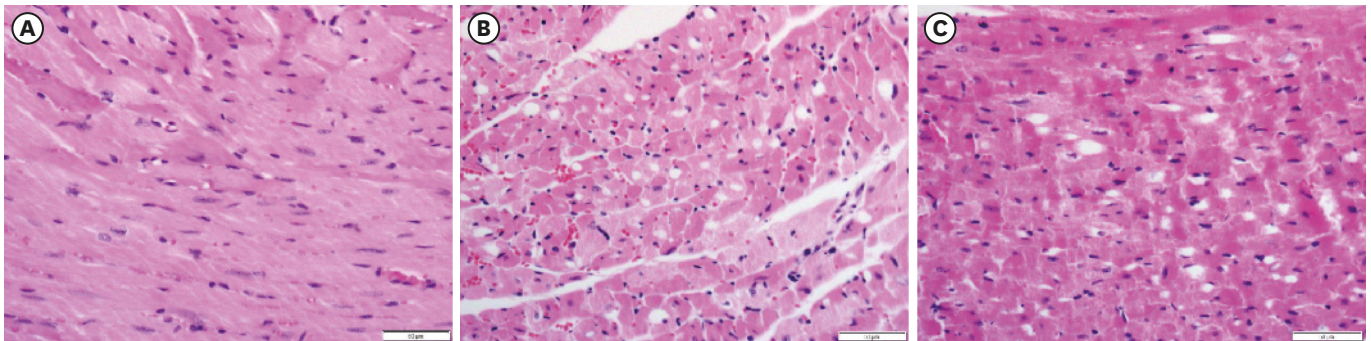


Figure 5. Representative pictures of hematoxylin and eosin staining ($\times 400$) of normal cardiac tissue in the CON group (A) and of scattered myocytes with edema and vacuolar degeneration in the DOX group (B) and the ET-DOX group (C). CON = control; DOX = sedentary and treated with doxorubicin; DOX-ET = aerobic exercise training and treated with doxorubicin.

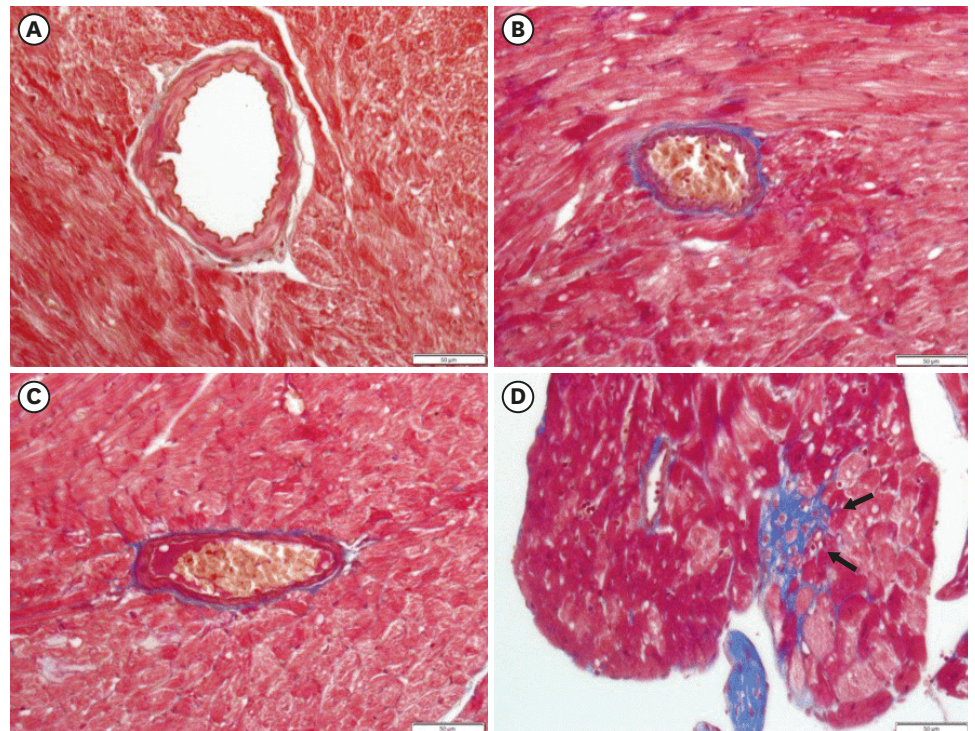


Figure 6. Masson's trichrome staining ($\times 400$) reveals the CON group had no distinct fibrosis (A), whereas perivascular fibrosis in the groups with DOX (B) and ET-DOX (C) is stained blue. Interstitial fibrosis (arrow) was observed infrequently in the myocardial tissue of the DOX group (D). CON = control; DOX = sedentary and treated with doxorubicin; DOX-ET = aerobic exercise training and treated with doxorubicin.

and so can prevent cardiac decompensation. In addition, ET has favorable effects in the myocardium such as reduction of muscle sympathetic nerve activity,⁴ increasing vascular endothelial function,¹⁷ and stabilizing by suppressing the overreaction of catecholamines.¹⁸⁾¹⁹ These neural and vascular improvements result in reduced vasoconstriction and increased peripheral oxygen supply,²⁰ resulting in increased muscle oxidative capacity.

In our study, there were significant differences in soluble ST2 according to ET in mice treated with DOX but not in NT-pro BNP. As the exact mechanism at the myocardial level is unknown, care is needed when interpreting our results. First, it is possible that ET improves the cardiorespiratory fitness of mice, and prevents myocardial injury after DOX administration. Soluble ST2 is a novel cardiovascular marker associated with myocardial hypertrophy, fibrosis, and ventricular dysfunction.²¹ Soluble ST2 is thought to reflect the stretching of cardiomyocytes and myocardial fibrosis in experimental models in particular.²² In a previous study, soluble ST2 was released in response to myocardial infarction, suggesting clinical biomarkers reflecting myocardial injury.¹⁰ NT-pro BNP is also an index that reflects myocardial stretch, and can reflect myocardial injury,²³ and the mechanism is different from soluble ST2. Therefore, it is difficult to interpret whether soluble ST is a more sensitive parameter to indicate myocardial stretch or myocardial fibrosis than NT-pro BNP and our results are less convincing because ST2 and BNP show different results. Second, as chemotherapy with DOX may cause systemic inflammation due to severe cytotoxicity, soluble ST2 levels may change. Among the limitations of soluble ST2, it has no disease specificity.²⁴ Increases in circulating concentrations of soluble ST2 in different diseases suggest that soluble ST2 is not specific to one disorder but is rather a marker of inflammatory disease in general.

NGAL is an emerging biomarker for acute kidney injury. It has been shown to be expressed in HF and linked to myocardial fibrosis and remodeling in animal models.¹¹⁾¹²⁾²⁵⁾²⁶⁾ Galectin-3 is a member of the β -galactoside-binding animal lectin family, and it interacts with various ligands located in the extracellular matrix.²⁷⁾ Thus, there is a direct implication that galectin-3 is a mediator in the development of cardiac hypertrophy and fibrosis and a potential biomarker for HF.²⁸⁾ Theoretically, ET could suppress the increase of NGAL after DOX treatment, but our results did not show this effect. As BNP and NT-pro BNP vary depending on age, sex, and accompanying diseases or drugs, these biomarkers should be judged based on the clinical situations of individual patients rather than as a sole diagnostic tool.

In the pathologic findings of our study, both DOX and ET-DOX groups showed histologic myocardial abnormalities such as interstitial edema, vacuolization of myocytes and perivascular fibrosis; however, there were no significant differences between the 2 groups. In addition, interstitial fibrosis, another characteristic of the DOX effect, was absent in both groups, perhaps because there might be insufficient time to demonstrate pathological differences between the completion of DOX injections and biopsies performed only 1 week later.

The ET group showed no significant differences in LV hypertrophy compared to ET-DOX group on echocardiography. There may be differences between functional changes of LV and pathological changes of myocardium. Myocardial damage caused by chemotherapy may appear as wall edema or hypertrophy, but wall thinning may occur as fibrosis progresses and remodeling occurs. Simple 2D echocardiography measurements may not be sufficient to visualize myocardial damage. It is also possible that ET itself increased the volume of the myocardium and led to hypertrophy, indicating that echocardiography may not be useful to distinguish it from pathological hypertrophy during treatment with HT.

The limitations of the present study should be acknowledged. First, although we followed a proven DOX treatment regimen, there were many early deaths among our sample due to DOX complications. Many mice in the DOX and ET-DOX groups were therefore not included in the study results. If their values had been included, it may have helped us produce a more well-supported HF model and validate the effectiveness of ET. Second, we did not obtain baseline serum biomarker values for each group, so the difference in biomarker values may not be due to ET. Third, in pathologic evaluations, we did not observe any significant quantitative differences between the ET-DOX and DOX groups. This finding also limits the strength of any conclusions regarding the effects of ET in preventing HF. Fourth, we did not obtain baseline echocardiograms, so there may be selection bias. Fifth, the isoflurane drug used in anesthesia may have influenced cardiac function. Sixth, NT-pro BNP levels did not show any significant changes, which indicates less reliability of the study results. Thus, further investigations are needed to determine the molecular mechanisms of exercise in preventing HF.

In conclusion, although we cannot explain the exact mechanism underlying the effects, we found that endurance ET is effective for inhibiting increases in serum soluble ST2 in mice treated with DOX. This finding suggests that soluble ST2 is an indicator of the preventive effect of ET against chemotherapy-induced myocardial injury.

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