

Myocardial Protective Effect of Tezosentan, an Endothelin Receptor Antagonist, for Ischemia-Reperfusion Injury in Experimental Heart Failure Models

The myocardial protective effects of endothelin antagonist in ischemic cardiomyopathy (ICMP), doxorubicin-induced cardiomyopathy (DOX) and pressure-overload hypertrophy by transverse aortic constriction (TAC) models have been predicted to be different. The objective of this experiment, therefore, is to evaluate the myocardial protective effect of tezosentan, an endothelin receptor antagonist, in various experimental heart failure models. Sprague-Dawley rats (6-8 weeks old, 200-300 g) were randomized to three experimental groups (n=30 each): ICMP; DOX; and TAC group. Each of these groups was randomly assigned further to the following subgroups (n=10 each): sham-operated ischemia-reperfusion subgroup (SHAM); tezosentan treated ischemia-reperfusion subgroup (Tezo); and tezosentan non-treated ischemia-reperfusion subgroup (N-Tezo). Total circulatory arrest was induced for 1 hr, followed by 2 hr of reperfusion. The left ventricular developed pressure, peak positive and negative first derivatives, and coronary blood flow were significantly different ($P<0.05$) among the SHAM, Tezo, and N-Tezo subgroups of the ICMP group at 30 min of reperfusion, but there were no statistically significant differences among the subgroups of the DOX and TAC groups. In conclusion, tezosentan, an endothelin receptor antagonist, showed myocardial protection effects only on the ischemic cardiomyopathy rat model, but not in the non-ischemic heart failure rat models.

Key Words : *Cardiomyopathies; Receptors, Endothelin; Myocardial Ischemia; Myocardial Reperfusion Injury*

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INTRODUCTION

Endothelin has been identified as a neurohormone that is activated during heart failure, and also by renin-angiotensin-aldosterone, epinephrine-norepinephrine, and natriuretic peptide. In addition, a number of studies have shown that endothelin-1 and its receptors over ischemic myocardial tissue are increased during ischemia-reperfusion injury. The endothelin-1 is the principal isoform in disease states and also is the most potent vasoconstrictor. It induces myocardial injury during ischemia and reperfusion as a contributor (1).

There are numerous reports about the relationship between endothelin and congestive heart failures (CHF). Some authors showed that endothelin-1 is an independent, noninvasive predictor of functional and hemodynamic response to therapy in patients with CHF. The majority of heart failure is caused by cardiomyopathy, and the increased endothelin levels have also been observed in the cardiomyopathy heart such as cardiomyopathic Syrian hamsters (2) or in the experimental Chagas' cardiomyopathic hearts infected with *Trypanosoma cruzi* (3).

Tezosentan, the first intravenous endothelin A and B receptor antagonist, has been evaluated as the treatment for acute and chronic heart failure. There are two large randomized controlled studies about tezosentan on acute heart failure syndrome-RITZ (randomized intravenous tezosentan study) and VERITAS (value of endothelin receptor inhibition with tezosentan in acute heart failure studies) (4, 5). Although these studies demonstrated mixed results of tezosentan as the drug of treatment for decompensated heart failure, the roles of the myocardial protective effect of tezosentan in various heart failure models have not yet been defined. The objectives of this experiment are to evaluate the myocardial protective effects of tezosentan in various experimental heart failure models.

MATERIALS AND METHODS

Animals

Total 90 Sprague-Dawley male rats (6-8 weeks old, 200-

300 g) were housed at $20 \pm 2^\circ\text{C}$ and $55 \pm 20\%$ humidity with 12-hr light/dark cycles, and free access to food and water in the Animal Care Facility at the Ansan Hospital, Korea University, Korea. This study was approved by the Committee of Animal care of Korea University and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Ischemic cardiomyopathy model (ICMP)

Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), intraperitoneally. The endotracheal intubation was performed, and positive pressure ventilation was given using a mechanical ventilator (Harvard Apparatus, South Natick, MA, U.S.A.) with room air at a respiratory rate of 55-65 strokes/min and tidal volume of 10 mL/kg body weight to maintain normal PO_2 , PCO_2 and pH of blood.

Under the right lateral decubitus position, the left anterior descending coronary artery was ligated with a 6-0 silk suture through left anterior thoracotomy. The lungs were then inflated and the chest incision was closed. After recovery of self respiration, extubation was performed.

Doxorubicin-induced cardiomyopathy model (DOX)

Doxorubicin hydrochloride (D1515, Sigma Chemical Co, St. Louis, MO, U.S.A.) was administrated in 6 equal injections (each intraperitoneal injection containing 2.5 mg/kg in saline, for a total dose of 15 mg/kg) during 2 weeks. Rats were observed for their general appearance, behavior, and mortality for 4 weeks after the final injection.

Pressure-overload hypertrophy model by transverse aortic constriction (TAC)

Under sterile condition, the midline cervical incision was performed; 1-2 cm incision was made at the level of cricoid process and dissection down to the clavicle. After cutting of the right clavicle, the thymus was retracted gently, and the aortic arch was exposed. A constriction of the aortic arch was conducted between the both carotid arteries using a 16 gauge angiocatheter (internal diameter 0.6 mm).

Experimental design

We divided the experimental animals into three cardiomyopathy groups; ischemic dilated cardiomyopathy group (ICMP, $n=30$), doxorubicin-induced cardiomyopathy group (DOX, $n=30$) and pressure-overload hypertrophy group by transverse aortic constriction method (TAC, $n=30$). One month after the construction of experimental rat groups, each group was further randomly subdivided to a sham-operated ischemia-reperfusion subgroup (SHAM) operation (SHAM, $n=10$), tezosentan treatment ischemia-reperfusion subgroup (Tezo, $n=10$), or tezosentan non-treatment ischemia-reperfusion subgroup (N-Tezo, $n=10$) (Fig. 1A). In the SHAM subgroup, only open and closure procedures or injection of saline instead of doxorubicin were performed.

Ischemia-reperfusion protocols

Four weeks after the construction of cardiomyopathy models, the rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Hearts were rapidly excised, connected via the aorta to Langendorff apparatus (size 3, type 830, Hugo Sachs Elektronik, March-Hugstetten, Germany)

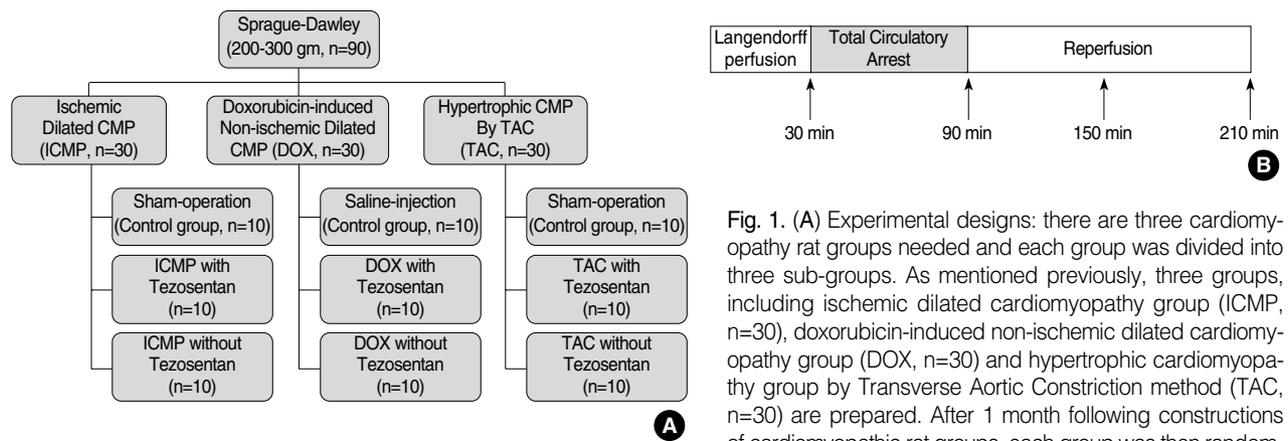


Fig. 1. (A) Experimental designs: there are three cardiomyopathy rat groups needed and each group was divided into three sub-groups. As mentioned previously, three groups, including ischemic dilated cardiomyopathy group (ICMP, $n=30$), doxorubicin-induced non-ischemic dilated cardiomyopathy group (DOX, $n=30$) and hypertrophic cardiomyopathy group by Transverse Aortic Constriction method (TAC, $n=30$) are prepared. After 1 month following constructions of cardiomyopathic rat groups, each group was then randomly

assigned to a SHAM operation (SHAM; control group, $n=10$) subgroup or tezosentan treatment ischemia-reperfusion subgroup (Tezo, $n=10$) or tezosentan non-treatment ischemia-reperfusion subgroup (N-Tezo, $n=10$). (B) Ischemia-reperfusion injury experimental protocol (isolated heart perfusion via Langendorff technique): after the stabilization period of 20-30 min, total circulatory arrest for ischemia about 1 hr was performed. After then, reperfusion was performed during 2 hr. In Tezo subgroup, the tezosentan, each 10^5 M/L concentrations, is added to the cardioplegic solution and the perfusate.

and perfused retrogradely at a constant pressure of 80 mmHg with the perfusate (Krebs-Henseleit solution) of the following composition: NaCl, 118.1 mM; KCl, 4.6 mM; CaCl₂, 2.5 mM; MgSO₄, 1.2 mM; KH₂PO₄, 1.2 mM; NaHCO₃, 24.8 mM; and glucose, 10 mM. The perfusate was continuously bubbled with a gas mixture of 95% O₂/5% CO₂ (pH 7.4), and temperature was maintained at 37°C throughout the experiment. A latex balloon filled with water was inserted into the left ventricle through the left atrium and attached to a pressure transducer (DX-360; Nihon Kohden, Tokyo, Japan). After 30 min of stabilization period, total circulatory arrest was performed with cardioplegia and coldness about 1 hr. After then, reperfusion was continued for 2 hr. In Tezo subgroup, tezosenan 10⁻⁵ M/L was added to the cardioplegic solution and the perfusate (Fig. 1B), but not to N-Tezo subgroup.

Drugs

Tezosenan (Ro 47-0203, Actelion Pharmaceuticals Ltd, Allschwil, Switzerland) is a non-selective specially formulated intravenous dual (endothelin-A and -B) endothelin receptor antagonist. Unlike its sister compound (bosentan), tezosenan is easily soluble in water and stable in solution at physiologic pH, therefore, usable clinically. Another drug, doxorubicin hydrochloride (D1515, Sigma Chemical Co) is well known for cardioselective toxicity and induces congestive heart failure.

Hemodynamic studies for cardiac performance

The left ventricular pressure was monitored with a pressure transducer to obtain the peak positive and negative first derivatives (dP/dT^{max} and dP/dT^{min}), and the left ventricular developed pressure (LVDP) was calculated as the difference between the left ventricular (LV) systolic and diastolic pressures. These parameters were measured by an amplifier (AP601G; Nihon Kohden) and also recorded using PowerLab/4sp software (AD Instruments, Mountain View, CA, U.S.A.). Coronary flow was also determined by collecting the coronary effluent from the hearts.

Statistical analysis

All data are presented as means ± standard error of the mean. The comparisons among three groups were carried out by Kruskal-Wallis Test. Differences were considered significant at *P*<0.05.

RESULTS

Left ventricular developed pressure (LVDP)

LVDP of Tezo subgroup was significantly higher than that of N-Tezo subgroup in the ICMP group after 30 min of reperfusion (64.3 ± 7.1 mmHg vs. 41.4 ± 4.3 mmHg; *P*<0.05). In the DOX or TAC model, LVDPs were not significantly different between Tezo and N-Tezo subgroup during the entire reperfusion period (Fig. 2A, Table 1).

dP/dT^{max}

In the ICMP group, dP/dT^{max} was significantly different among the three subgroups at 30 min of reperfusion (SHAM, 3,491 ± 21 mmHg/sec; Tezo, 2,821 ± 13 mmHg/sec; N-Tezo, 2,282 ± 182 mmHg/sec; *P*<0.05). In the DOX or TAC groups, there were no statistical differences among three subgroups (Fig. 2B, Table 1).

dP/dT^{min}

In the ICMP group, dP/dT^{min} was significantly different among the three subgroups at 30 min of reperfusion (SHAM, -2,382 ± 114 mmHg/sec; Tezo, -1,638 ± 130 mmHg/sec; N-Tezo, -1,387 ± 81; *P*<0.05), similar to the above dP/dT^{max}. In the DOX or TAC groups, there were no statistically significant differences among the three subgroups (Fig. 2C, Table 1).

Coronary blood flow (CBF)

Only in the ICMP group, there were significant differences

Table 1. Variable hemodynamic parameters at the reperfusion 30 min

	ICMP*			DOX			TAC		
	SHAM	Tezo	N-Tezo	SHAM	Tezo	N-Tezo	SHAM	Tezo	N-Tezo
LVDP (mmHg)	75.2±5.3	64.3±7.1	41.4±4.3	46.8±3.2	45.1±2.9	45.6±3.8	75.2±4.2	56.2±3.2	58.7±2.9
dP/dT ^{max} (mmHg/sec)	3,491±21	2,821±13	2,282±182	2,095±11	2,320±13	2,559±18	3,491±9	2,100±11	1915±7
dP/dT ^{min} (mmHg/sec)	-2,382±114	-1,638±130	-1,387±81	-1,639±184	-1,745±97	-2,085±81	-2,382±97	-1,532±93	-1,432±76
CBF (mL/min)	33.9±4.0	26.3±3.3	19.1±2.1	32.1±2.4	24.2±2.3	20.1±1.9	33.4±2.1	24.5±4.2	22.4±3.6

*Hemodynamic variables are significantly different among SHAM, Tezo and N-Tezo subgroups at 30 min of reperfusion (*P*<0.05).

ICMP, ischemic cardiomyopathy rat group; DOX, doxorubicin treated non-ischemic dilated cardiomyopathy rat group; TAC, hypertrophic cardiomyopathy rat group by transverse aortic constriction; SHAM, Sham operated ischemia-reperfusion subgroup; Tezo, tezosenan treated ischemia-reperfusion subgroup; N-Tezo, tezosenan non-treatment ischemia-reperfusion subgroup; LVDP, left ventricular developed pressure; CBF, coronary blood flow.

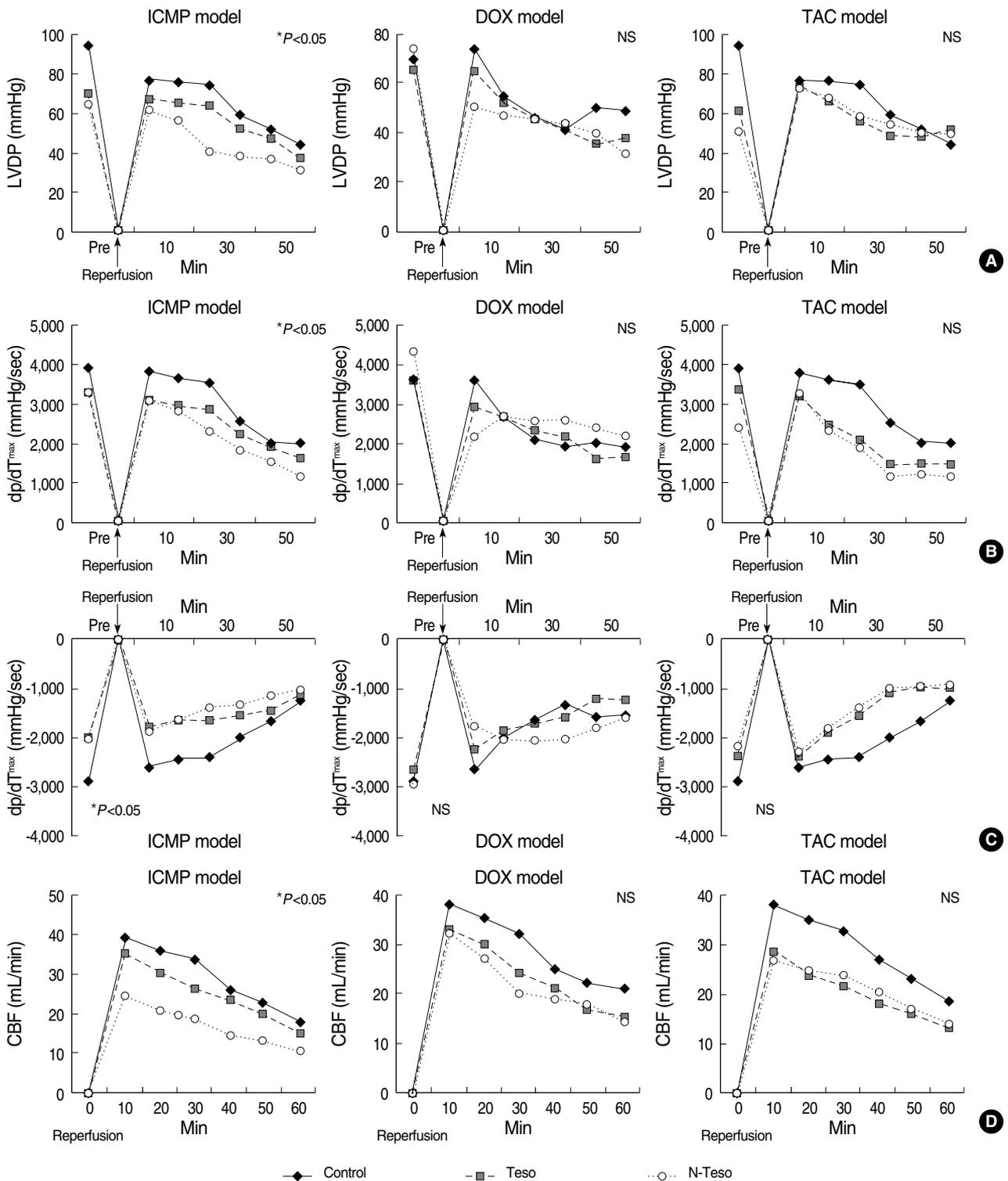


Fig. 2. The results of cardiac performance. (A) LV developed pressure (LVDP) during ischemia-reperfusion injury. (B) dp/dT^{max} during ischemia-reperfusion injury. (C) dp/dT^{min} during ischemia-reperfusion injury. (D) Coronary blood flow (CBF) during ischemia-reperfusion injury. *P<0.05 at 30 min of reperfusion among SHAM, Tezo and N-Tezo subgroups. NS, there is no statistical significance among subgroups; ICMP, ischemic cardiomyopathy rat group; DOX, doxorubicin treated non-ischemic dilated cardiomyopathy rat group; TAC, hypertrophic cardiomyopathy rat group by transverse aortic constriction; SHAM, Sham operated ischemia-reperfusion subgroup; Tezo, tezosentan treated ischemia-reperfusion subgroup; N-Tezo, tezosentan non-treatment ischemia-reperfusion subgroup.

among three subgroups after 30 min of reperfusion in the CBF (SHAM; 33.9 ± 4.0 mL/min vs. Tezo; 26.3 ± 3.3 mL/min vs. N-Tezo; 19.1 ± 1.1 mL/min; $P < 0.05$) (Fig. 2D, Table 1).

DISCUSSION

The effect of endothelin-1, the principal isoform of endothelin, is mediated by 2 types of receptor, endothelin-A and endothelin-B. The endothelin-A receptor appears to mediate vasoconstriction and stimulate the secretion of atrial natriuretic peptide, while the endothelin-B receptor mediates endothelin-induced vasodilatation and activation of the renin-angiotensin-aldosterone system. Through these effects, endothelin has been demonstrated to be not only one of the most potent vasoconstrictors known, but also to mediate pathologic hypertrophy and fibrosis of both ventricular and vascular tissues, thus promoting progression of atherosclerosis, ventricular and vascular remodeling (6-8).

Tezosentan, a dual inhibitor of endothelin A and B receptors, is weakly acidic, highly soluble in water and stable in physiologic pH solution. These properties make it an ideal candidate for intravenous use, and are the reason of why we used tezosentan in our study.

Heart failure has been associated with elevated plasma levels of endothelin-1, and the magnitude of the elevation with the severity of disease, the incidence of arrhythmia, and a worse prognosis. Increased endothelin levels have been described in patients with acute and chronic CHF that are predictive of increased mortality risk (9). Furthermore, increased endothelin levels have been suggested to play an important role in the increased systemic vascular resistance seen in CHF (10). Therefore, endothelin seems to play a major role in the pathogenesis of experimental and clinical heart failure (11, 12).

Although the endothelin receptor antagonists may have the ability to improve the outcome of heart failure by reduc-

ing preload and afterload, increasing the contractility of failing myocardium, delaying myocyte hypertrophy, and reducing the incidence of arrhythmias, some studies demonstrated no benefit of the endothelin receptor antagonists for myocardial ischemia-reperfusion heart models (13-15). Therefore, we aimed to investigate the myocardial protective effects of tezosentan for the ischemia-reperfusion injury in various heart failure models.

Before analyzing our experimental data, it was necessary to confirm the in-depth of animal heart failure models. Therefore, one month after cardiomyopathic animal models had been constructed, they were verified with pathologic specimens during euthanasia of the experimental animals. In the ICMP models, staining of triphenyltetrazolium chloride with evans blue was used for visualizing ischemia-infarct area of myocardium. In the DOX models, dilated myocardium was confirmed with pathologic specimens and hypertrophied myocardium was also confirmed in the TAC models (Fig. 3).

We also believe that hemodynamic parameters such as LVDP, dP/dT^{\max} , dP/dT^{\min} and CBF are adequate in our present study to verify myocardial protective effect of tezosentan in various rat cardiomyopathy models. It should be mentioned that these parameters have been used for proving drug efficacy in Langendorff technique (16).

As a result, our present study did not reveal any significant effect of tezosentan on DOX and TAC models. There are some hypotheses and limitations on this result in our study. First, although heart failure has been associated with elevated plasma levels of endothelin-1, the density of endothelin and endothelin binding sites had been produced in the heart has not been uniform among different animal heart failure models (17). In myocardial infarct model, it is well established that the infarcted myocardium induces the myocardial necrosis, and increases endothelin-1 levels many fold not only in the post-acute myocardial infarct scar tissues but also in the healed infarct scars after 4 and 13 weeks (18-20). Therefore, endothe-

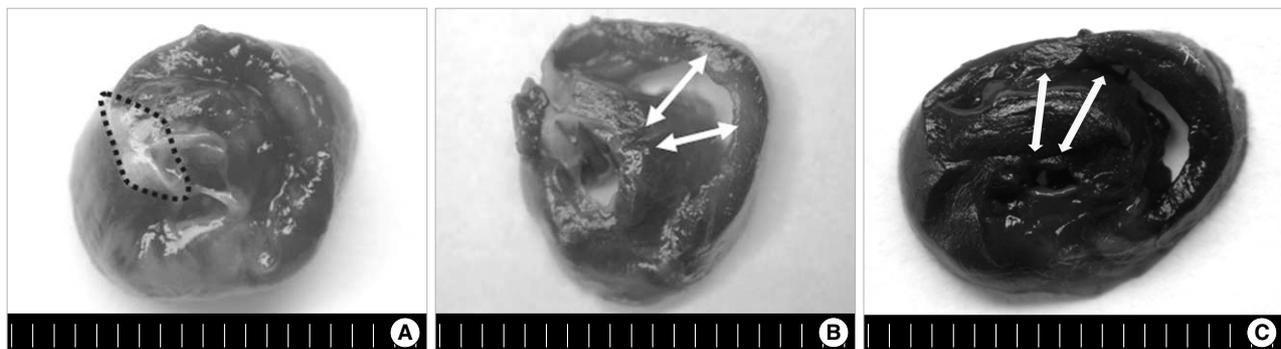


Fig. 3. (A) In the ICMP models, staining of triphenyltetrazolium chloride with evans blue was used for visualizing ischemia-infarct area of myocardium (black dash). (B) In the DOX models, dilated myocardium was confirmed with pathologic specimens (white arrow-enlarged left ventricle). (C) In the TAC models, hypertrophied myocardium was confirmed with pathologic specimens (white arrow).

*The scale of the ruler in the photo is millimeter (mm).

ICMP, ischemic cardiomyopathy rat group; DOX, doxorubicin treated non-ischemic dilated cardiomyopathy rat group; TAC, hypertrophic cardiomyopathy rat group by transverse aortic constriction.

lin-1, a recognized fibrogenic factor, plays a crucial role in the stabilization of the necrotic area and in the healing of the scar (20). However, the most prominent histological feature of DOX is not the myocyte necrosis, but the loss of myofibrils and cytoplasmic vacuolization caused by dilatation of the sarcoplasmic reticulum in the myocardial cells (21), and there is a report that high dose of endothelin antagonist is needed to block endothelin induced hypertension in the dilated cardiomyopathy rat models (22). In addition, sustained pressure overload stimulate pathological cardiac hypertrophy and dysfunction (23) and hypertrophy progresses to decompensated phase with cardiac dilatation and contractile impairment during chronic pressure overload (24, 25). Some authors showed that the production of endothelin-1 increased in the hypertrophied left ventricle (26), but it depends on the time of the disease progress. Ventricular endothelin-1 levels and the density of endothelin-1 binding sites were increased significantly, without affecting their binding affinity, on day 8 of pressure overload (27), after then, the production of endothelin-1 is decreased and endothelin receptors are down-regulated in hypertrophied ventricles 8 weeks after aortic coarctation (28). However, it is also reported that endothelin-1 levels in subjects with heart failure in whom they are found to be elevated in the late, decompensated phase of the disease (29). This time-dependant up- and down-regulations of endothelin and endothelin receptors are also another proof for our hypothesis. That is, because of the differences of pathophysiology among our study group, cardiac endothelin-1 and endothelin receptors might not uniformly be induced in various heart failure models.

Second, in addition to endothelin, renin-angiotensin-aldosterone, epinephrine-norepinephrine, and natriuretic peptide have also been identified as neurohormones to be activated in cardiomyopathy (30). It is well known that doxorubicin administration has been shown to activate the renin-angiotensin system (31, 32) and the atrial natriuretic peptide level has also been increased relative to the severity of the cardiac dysfunction in human (32). And, there is a study on angiotensin and angiotensin receptor antagonist in the hypertrophic cardiomyopathy (33), showing that stimulation of the angiotensin receptor has an important role in disease process during pressure overload. Therefore, these neurohormones might be more important disease mediators in the DOX or TAC models than in the ICMP model.

There was a limitation to our study. Endothelin is a stimulator of polymorphonuclear leukocyte (PMN) aggregation (34) and adhesion as well as a potent vasoconstrictor. Myocardial ischemia initiates an acute inflammatory response in which PMNs are of importance (35). Because we had used isolated heart perfusion using Langendorff technique, however, most of the PMNs were diluted in the buffer perfused isolated heart. In other words, the effect of PMNs might be restricted and it can affect the results in our study.

In conclusion, our result reveals that tezosentan has myocar-

dial protective effects for the ischemia-reperfusion injury in the ischemic cardiomyopathy model, but not in the doxorubicin-induced cardiomyopathy and pressure-overload hypertrophic heart failure models.

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