Additive Beneficial Effects of the Combination of a Calcium Channel Blocker and an Angiotensin Blocker on a Hypertensive Rat-Heart Failure Model

Shokei KIM-MITSUYAMA, Yasukatsu IZUMI*, Yasuhiro IZUMIYA*, Kaoru YOSHIDA*, Minoru YOSHIYAMA**, and Hiroshi IWAO*

The present study was undertaken to examine the effects of a calcium channel blocker, azelnidipine (1 mg/kg/day), an angiotensin converting enzyme (ACE) inhibitor, temocapril (10 mg/kg/day), an angiotensin II type 1 (AT1) receptor blocker (ARB), olmesartan (5 mg/kg/day), and their combination on Dahl salt-sensitive rats (DS rats) developing heart failure with preserved systolic function. DS rats were fed a high-salt diet (8% NaCl) from 7 weeks of age and progressively developed hypertension. Although monotherapy with azelnidipine lowered the blood pressure of DS rats to a greater extent than monotherapy with temocapril or olmesartan, the three drugs had similar effects on cardiac hypertrophy, cardiac fibrosis, the expressions of brain natriuretic peptide, transforming growth factor-β1, collagen I, collagen III and monocyte chemoattractant protein-1 mRNA (as estimated by Northern blot analysis), and cardiac diastolic dysfunction (as estimated by echocardiography). These results show that ACE and AT1 receptor, as well as hypertension, are involved in the development of heart failure with preserved systolic function in DS rats. The combination of azelnidipine with olmesartan or temocapril produced no additive hypotensive effect in DS rats and no additive effect on cardiac hypertrophy or gene expressions. However, the combination therapy prolonged the survival rate of DS rats more than azelnidipine (p < 0.01) or temocapril alone (p < 0.05), and this additive beneficial effect by the combination therapy was associated with a greater reduction of cardiac fibrosis, urinary albumin excretion and serum creatinine. Our results thus showed that the combination of a calcium channel blocker with an ARB or an ACE inhibitor had additive preventive effects on a rat model of hypertensive heart failure with preserved systolic function. Thus, combination therapy with these agents seems to be a useful therapeutic strategy for the prevention of hypertensive heart failure. (Hypertens Res 2004; 27: 771–779)

Key Words: hypertension, heart failure, calcium blocker, angiotensin II type 1 receptor blocker, combination therapy

Introduction

Despite advances in pharmacological therapy for congestive heart failure, the pathophysiology and treatment of heart failure, particularly diastolic heart failure (1–3), remains to be fully defined. Hypertension plays a critical role in cardiac hypertrophy and remodeling, cardiac diastolic dysfunction,
and congestive heart failure. Dahl salt-sensitive rats (DS rats), when fed an 8% NaCl diet from 7 weeks of age, develop progressive hypertension and cardiac diastolic dysfunction with preserved systolic function, and die as a result of congestive heart failure. For this reason, DS rats fed a high-salt diet are regarded as a useful model of hypertensive diastolic heart failure (4).

Angiotensin II type 1 (AT1) receptor blockers (ARB), angiotensin-converting enzyme (ACE) inhibitors, and long-acting calcium channel blockers have been well established as the most popular and useful drugs for the treatment of hypertension. However, it remains to be determined which drug is most useful for preventing hypertensive heart failure with preserved systolic function. Furthermore, in the clinical treatment of hypertensive patients, combination therapy with different types of anti-hypertensive drugs is very frequently used in an effort to enhance the effects than monotherapy. In particular, the combination of a calcium channel blocker with either an ARB or ACE inhibitor is very popular in the treatment of hypertensives. Therefore, it is also important to resolve the question of whether combination treatment with a calcium channel blocker and an ARB or ACE inhibitor leads to additive preventive effects on hypertensive heart failure.

In the present study, we compared the efficacy of a calcium channel blocker (azelnidipine), an ACE inhibitor (temocapril), and an ARB (olmesartan), both singly and in combination, at preventing hypertensive heart failure in a DS rat model with significant diastolic dysfunction. We found that the renin-angiotensin system, as well as hypertension, participated in the development of heart failure with preserved systolic function in DS rats, and that the calcium channel blocker combined with the ARB or the ACE inhibitor exerted additive preventive effects on hypertensive heart failure with preserved systolic function.

Methods

Drugs

Azelnidipine (a calcium channel blocker) (5, 6), temocapril (an ACE inhibitor) (7, 8), and olmesartan (an ARB) (9), were obtained from Sankyo Co., Ltd. (Tokyo, Japan).

Experimental Animals

All procedures were in accordance with institutional guidelines for animal research. DS rats (DIS/Eis, Eisai, Tokyo, Japan) (10) were used in the present study. After weaning, DS rats were fed a 0.3% NaCl (low-salt) diet. At 7 weeks of age, the diet was switched to an 8% NaCl diet, and drug treatment was started from 10 weeks of age.

Effects of Azelnidipine, Olmesartan, Temocapril, or a Combination Thereof on Cardiac Hypertrophy, Gene Expressions, and Urinary Albumin Excretion (UalbV)

To examine the effects on cardiac hypertrophy and gene expressions, 10-week-old DS rats, fed an 8% NaCl diet from 7 weeks of age, were divided into 6 groups, and treated with 1) vehicle (0.5% carboxymethylcellulose solution), 2) azelnidipine (1 mg/kg/day), 3) temocapril (10 mg/kg/day), 4) olmesartan (5 mg/kg/day), 5) combined azelnidipine (1 mg/kg/day) and temocapril (10 mg/kg/day), or 6) combined azelnidipine (1 mg/kg/day) and olmesartan (5 mg/kg/day). All drugs were administered orally to DS rats by gastric gavage once a day. To examine the effects on cardiac hypertrophy and gene expressions, 16-week-old DS rats were subjected to 6 weeks of drug treatment, anesthetized with ether, and then the heart was immediately excised, the left ventricle (LV) was separated from the atria and the right ventricle, and the ventricles and atria were weighed, immediately frozen in liquid nitrogen and stored at -80 °C until use.

In another experiment, at 16 weeks of age, DS rats treated as described above were housed individually in metabolic cages, and 24-h urine was collected in a flask for measurement of UalbV. Under ether anesthesia, arterial blood was immediately collected via the abdominal aorta of rats for measurement of serum creatinine, and the heart was immediately excised for measurement of hydroxyproline content.

Effect on Survival Rate

To examine the effect on survival, 7-week-old DS rats were fed an 8% NaCl diet, and were subjected to each drug treatment from 10 weeks of age, as described above. Animals were carefully monitored and deaths were recorded every day. The survival rates at 100 days after the start of drug treatment were compared among the groups.

Preliminary experiments were undertaken to confirm that the main cause of death in DS rats fed a high-salt diet was congestive heart failure. We inspected the general conditions of animals every day, and found that all rats developed rapid and labored respiration. At the stage of severely labored respiration and loss of activity, but before death, we performed echocardiography, and then removed and weighed the lungs. We found that DS rats fed a high-salt diet had a higher E/A value than control DS rats fed a low-salt diet (5.4 ± 0.6 vs. 1.7 ± 0.1, p < 0.01), although there were no differences in LV ejection fraction (63.2 ± 4.5 vs. 64.5 ± 3.5%), LV end-diastolic volume (373 ± 25 vs. 388 ± 18 ml) or fractional shortening (34.5 ± 2.0 vs. 36.2 ± 1.5%) between the two groups. Furthermore, lung weight corrected for body weight was larger in DS rats fed a high-salt diet than those fed a low-salt diet (11.3 ± 1.4 vs. 4.2 ± 0.3 mg/g, p < 0.01), indicating pulmonary congestion. These results confirmed that DS rats showed congestive heart failure, which was consistent with previous reports (4, 11). In addition, all animals that died...
were immediately subjected to postmortem pathological examination including macroscopic examination of the intracranial cavity, and no cerebral hemorrhage or infarction was detected, indicating that stroke was not a major cause of death in DS rats in the present study.

**Effect on Cardiac Fibrosis and Functions**

To examine the effects of the various treatments on cardiac fibrosis and cardiac function, 10-week-old DS rats fed an 8% NaCl diet from 7 weeks of age were separated into six groups and treated with 1) vehicle (0.5% carboxymethylcellulose solution), 2) azelnidipine (1 mg/kg/day), 3) temocapril (10 mg/kg/day), 4) olmesartan (5 mg/kg/day), 5) combined azelnidipine (1 mg/kg/day) and temocapril (10 mg/kg/day) or 6) azelnidipine (1 mg/kg/day) and olmesartan (5 mg/kg/day). After 6 weeks of drug treatment, cardiac function was estimated by echocardiography, as described below. Cardiac fibrosis was also examined as described below.

**Measurement of Blood Pressure**

Systolic blood pressure of conscious rats was periodically measured by the tail-cuff method, at 4 to 5 h after oral dosing, when these drugs exhibited the maximal hypotensive effects.

**RNA Preparation and Northern Blot Analysis**

The methods used for RNA preparation and Northern blot analysis have been described in detail in our previous report (12). In brief, 20 µg of total RNA samples from individual left ventricles were subjected to 1% agarose gel electrophoresis and transferred to nylon membranes, and hybridization was carried out with (32 P)-dCTP-labeled cDNA probe for brain natriuretic peptide (BNP), transforming growth factor-β (TGF-β), collagen I, collagen III, monocyte chemoattractant protein-1 (MCP-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The densities of individual mRNA bands were measured by using a bioimaging analyzer (BAS-2000; Fuji Photo Film Co., Tokyo, Japan), and corrected for GAPDH mRNA.

**Histological Examination**

Rats were anesthetized with ether and the heart was perfusion-fixed with 4% paraformaldehyde and rapidly removed. The heart was post-fixed in the above solution overnight and embedded in paraffin. Five-µm thick sections were cut and stained with Sirius red stain for measurement of interstitial fibrosis area. The interstitial fibrosis area was calculated as the ratio of the sum of the total area of interstitial fibrosis to the sum of the total connective tissue area plus the cardiomyocyte area in all the LV fields of the section.

**Echocardiographic Study**

Transthoracic echocardiographic studies were performed as described in our previous reports (11, 13). In brief, rats were lightly anesthetized by intraperitoneal injection of ketamine HCl (25 to 50 mg/kg) and xylazine (5 to 10 mg/kg). Echocardiograms were performed using a commercially-available echocardiographic system equipped with a 12.0-MHz phased-array transducer (SONOS 5500; Agilent Technology, USA). A two-dimensional short-axis view of the left ventricle was obtained at the level of the papillary muscles. M-mode tracings were recorded through the anterior and posterior LV walls at a paper speed of 100 mm/s. By moving the transducer toward the cardiac apex and angling anteriorly, we were able to acquire a four-chamber view. The end-diastolic area was defined as the largest left ventricular area and the end-systolic area as the smallest as described (13). Pulse-wave Doppler spectra (E and A waves) of mitral inflow were recorded from the apical four-chamber view, with the sample volume placed near the tips of the mitral leaflets and adjusted to the position at which the velocity was maximal and the flow pattern laminar.

**Biochemical Measurement**

To estimate cardiac collagen content, tissue hydroxyproline content was measured by hydrolysis of the sample with HCl, followed by high-performance liquid chromatography. Urinary albumin concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using anti-albumin antibody. Serum creatinine was measured by the kit.

**Statistics**

Results were expressed as the mean ± SEM. Statistical significance was determined by one way ANOVA followed by Duncan’s multiple range test. Survival was analyzed by standard Kaplan-Meier analysis with a log-rank test and χ2 analysis. In all tests, differences were considered statistically significant at a value of p<0.05.

**Results**

**Effect on Blood Pressure**

Figure 1 shows the blood pressure of DS rats treated with azelnidipine, temocapril, olmesartan, or a combination thereof from 10 weeks of age. DS rats fed a high-salt diet (8% NaCl) from 7 weeks of age progressively developed hyper tension. After starting each drug treatment, the blood pressures of DS rats treated with vehicle, azelnidipine (1 mg/kg/day), temocapril (10 mg/kg/day), and olmesartan (5 mg/kg/day) were 214 ±4, 194 ±2, 204 ±2 and 202 ±2 mmHg at 1 week, 225 ±7, 192 ±4, 209 ±2, and 209 ±6 mmHg at 2 weeks, 238 ±7, 198 ±4, 222 ±4, and 223 ±4 mmHg at 4 weeks, respectively.
weeks, and 251 ± 4, 209 ± 6, 229 ± 6, and 230 ± 5 mmHg at 6 weeks, respectively. Thus, the hypertensive effect of azelnidipine (1 mg/kg/day) in DS rats was greater than that of temocapril (10 mg/kg/day) or olmesartan (5 mg/kg/day) at all weeks examined (p < 0.05). The combination of azelnidipine (1 mg/kg/day) with temocapril (10 mg/kg/day) or olmesartan (5 mg/kg/day) produced a hypotensive effect comparable to that by azelnidipine (1 mg/kg/day) alone.

**Effect on Cardiac Weight**

Figure 2 indicates the body weight and the LV weight corrected for body weight in DS rats after 6 weeks of drug treatment. The corrected LV weight in DS rats (3.63 ± 0.12 mg/g) was significantly reduced by treatment with azelnidipine alone (3.20 ± 0.11 mg/g), temocapril alone (3.26 ± 0.05 mg/g), olmesartan alone (3.18 ± 0.05 mg/g), combined azelnidipine and temocapril (3.21 ± 0.13 mg/g), or combined azelnidipine and olmesartan (3.17 ± 0.10 mg/g). There was no significant difference in corrected LV weight among the drug treatments.

**Effects on Cardiac Gene Expression**

Figure 3 shows LV mRNA levels in each group of DS rats subjected to 6 weeks of drug treatment. LV mRNA levels for BNP, TGF-β1, collagen I, collagen III and MCP-1 were significantly reduced by azelnidipine alone, temocapril alone, olmesartan alone, azelnidipine with temocapril (5 mg/kg/day) or azelnidipine with olmesartan, and the degrees of reduction were similar in all these groups.

**Survival Rate**

Figure 4 indicates the survival curves in each group of DS rats. The survival rate was analyzed at 100 days after the start of drug treatment. All vehicle-treated DS rats fed a high-salt diet died of congestive heart failure at between 6 and 52 days. The Kaplan-Meier survival analysis showed that all drug treatments significantly prolonged the survival rate of DS rats (p < 0.01), and there was no significant difference in the improvement of survival between treatment with azelnidipine alone, and treatment with temocapril alone. However, olmesartan improved the survival rate of DS rats significantly more than azelnidipine (p < 0.05). No significant difference in survival rate was observed between the group treated with temocapril and that treated with olmesartan. The combination of azelnidipine with temocapril improved the survival of DS rats significantly more than azelnidipine alone (p < 0.01) and temocapril alone (p < 0.05). Furthermore, the combination of azelnidipine with olmesartan resulted in longer survival than monotherapy with either temocapril (p < 0.05) or azelnidipine (p < 0.01).

**Cardiac Fibrosis**

Figure 5 shows LV interstitial fibrosis in each group of DS rats. LV fibrosis in 16-week-old DS rats fed a high-salt diet from 7 weeks of age was significantly higher than that in DS rats of the same age fed a low-salt diet (4.99 ± 0.68 vs. 0.48 ± 0.11%, p < 0.01). LV fibrosis was significantly reduced by azelnidipine alone, temocapril alone, olmesartan alone, and azelnidipine combined with temocapril or olmesartan (p < 0.01). However, the combination of azelnidipine and either temocapril or olmesartan reduced LV fibrosis more potently than monotherapy with azelnidipine, temocapril, or olmesartan (p < 0.05). LV hydroxyproline content in DS rats was significantly more reduced by the combination of azelnidipine with either temocapril or olmesartan than by monotherapy with any of these agents.

**Echocardiographic Analysis**

Figure 6 shows the E/A value in each group of DS rats, as estimated by echocardiography. The E/A in 16-week-old DS rats fed a high-salt diet from 7 weeks of age was significantly higher than that in DS rats of the same age fed a low-salt diet (4.13 ± 0.87 vs. 1.56 ± 0.07, p < 0.01). The increase in E/A was prevented by the monotherapies with azelnidipine, temocapril, or olmesartan, as well as by the combination therapies (p < 0.01, respectively). However, there were no significant differences among DS rats treated with vehicle, azelnidipine, temocapril, olmesartan, azelnidipine with temocapril, or azelnidipine with olmesartan, and DS rats fed a low-salt, with respect to LV ejection fraction (68.3 ± 3.5, 67.4 ± 2.5, 69.1 ± 3.5, 66.5 ± 4.1, 69.2 ± 3.5, 67.3 ± 5.1, and 71.1 ± 4.0%, respectively), fractional shortening (33.4 ± 2.5, 24.8 ± 2.4, 26.2 ± 2.5, 25.7 ± 2.7, 25.1 ± 2.4, 26.1 ± 2.3, and 27.2 ± 2.5% respectively, p < 0.05) and septal thickness (1.52 ± 0.13 mm, 1.50 ± 0.11 mm, 1.51 ± 0.12 mm, 1.49 ± 0.12 mm, 1.48 ± 0.13 mm, 1.49 ± 0.13 mm, and 1.48 ± 0.12 mm respectively, p < 0.05).
35.2 ± 3.2, 34.3 ± 2.5, 34.8 ± 3.1, 33.5 ± 2.9, 32.3 ± 2.5, and 36.0 ± 3.5%, respectively), or LV end-diastolic volume (383 ± 20, 391 ± 25, 403 ± 25, 383 ± 30, 396 ± 18, 371 ± 21, and 412 ± 25 ml, respectively).

**Fig. 2.** Body weight (BW) and left ventricular (LV) weight corrected for BW in DS rats treated with each drug for 6 weeks. Abbreviations used are the same as in Fig. 1. Values are the mean ± SEM (n = 6–8).

**Fig. 3.** Left ventricular BNP, TGF-β1, collagen I, collagen III and MCP-I mRNA levels in DS rats treated with each drug for 6 weeks. The left upper panel indicates representative mRNA bands in each group of rats. In individual rats, left ventricular BNP, TGF-β1, collagen I, collagen III and MCP-I mRNA levels were corrected for GAPDH mRNA levels. The mean value in the Veh group is represented as 1. Abbreviations used are the same as in Fig. 1. Values are the mean ± SEM (n = 6–8).

**UalbV and Serum Creatinine**

There was no significant difference in UalbV among the groups treated with azelnidipine, temocapril, or olmesartan alone (Fig. 7). However, the combination of azelnidipine with temocapril or olmesartan significantly decreased UalbV compared with vehicle or azelnidipine alone. Serum creat-
nine levels were reduced only by the combination of azelnidipine with temocapril or that of azelnidipine with olmesartan.

**Discussion**

DS rats are well known to develop hypertension by high-salt loading, and the degree and characteristics of hypertension, heart failure and renal damage depend on the amount of sodium in the diet and the age of the start of the high-salt diet (4, 14–17). DS rats fed an 8% NaCl diet develop not only hypertension but also overt heart failure, and die of congestive heart failure (4, 17). Therefore, DS rats are a very useful model to study the pathophysiology of hypertensive heart failure. Interestingly, DS rats that begin the 8% NaCl diet at different ages exhibit different phenotypes of heart failure. For example, those subjected to a high-salt diet from 6 weeks of age develop heart failure due to systolic cardiac function (17). On the other hand, the initiation of an 8% NaCl diet in DS rats from 7 weeks of age produces a different phenotype of heart failure which is characterized by diastolic dysfunction with preserved systolic function (4).
In our previous study, we found that each of a calcium channel blocker and several renin-angiotensin blockers induced a similar improvement of cardiac hypertrophy, gene expressions, and survival rate under comparable hypotensive effects (18). These findings show that hypertension, rather than the renin-angiotensin system, is involved in the development of systolic heart failure in DS rats. However, it is unknown whether our previous findings on DS rats developing systolic heart failure can be applied to DS rats developing heart failure with preserved systolic function. Therefore, in the present study, we compared the effects of a calcium channel blocker, an ARB, and an ACE inhibitor in DS rats developing heart failure with preserved systolic function. In addition, combination therapy with anti-hypertensive drugs is very frequently performed in hypertensive patients. However, the cardioprotective effects of combination therapy with anti-hypertensive drugs, in comparison with those of monotherapy, remain to be determined. Therefore, in this study, we also examined the effects of the combination of a calcium channel blocker with an ARB or an ACE inhibitor in this model.

In the present work, although the hypotensive effects of temocapril and olmesartan was smaller than that of azelnidipine throughout the treatment, both agents had beneficial effects similar to those of azelnidipine with respect to the improvement of cardiac weight, cardiac BNP, TGF-β1, collagen I, collagen III and MCP-1 gene expression, cardiac fibrosis, diastolic dysfunction as estimated by echocardiography, and survival rate. These observations, taken together with the accumulating evidence of a direct role of angiotensin II in cardiac hypertrophy and remodeling (19–22), support the notion that the renin-angiotensin system itself, in addition to hypertension, participates in the progression of heart failure in DS rats, unlike our previous report (18) on systolic heart failure with preserved systolic function in DS rats.

Previously, we examined the effects of the combination of an ACE inhibitor and an ARB in DS rats developing heart failure with preserved systolic function, and found that the combination of an ACE inhibitor and an ARB exerted more beneficial effects on diastolic dysfunction and survival rate than an ACE inhibitor or ARB alone (11). In our previous study, we reported that combination treatment with an ACE inhibitor and an ARB had similar beneficial effects on stroke-prone spontaneously hypertensive rats (23) and on a rat model of balloon-injured intimal hyperplasia (24). However, it remains to be determined whether or not the combination of an ARB or an ACE inhibitor with a calcium channel blocker has additive beneficial effects in this model. Furthermore, clinically, the combination of an ARB or an ACE inhibitor with a calcium channel blocker is one of the most popular strategies for the treatment of hypertension. These findings encouraged us to examine the effects of the combination of these inhibiting agents with a calcium channel blocker in hypertensive diastolic heart failure.

In our present work, in DS rats developing heart failure with preserved systolic function, the combination of a calcium channel blocker with an ARB or an ACE inhibitor produced no additive effects on blood pressure (Fig. 1), cardiac weight (Fig. 2) or cardiac gene expression (Fig. 3). Although the reason for the lack of an additive effect by the combination therapy is unknown, these results are consistent with our previous findings on DS rats developing systolic heart failure (18). In contrast, it is noteworthy that the combination of a calcium channel blocker with an ARB or an ACE inhibitor led to more beneficial effects on survival rate in this heart failure model, compared with monotherapy with each agent (Fig. 4). These observations support the notion that the combination of these drugs has additive beneficial effects in preventing hypertensive heart failure.

The present study did not allow us to elucidate the mechanism by which the combination of these drugs resulted in a
greater prolongation of survival in DS rats than that by each drug alone. Cardiac collagen accumulation causes cardiac stiffness or cardiac diastolic dysfunction (25, 26). To determine the possible contribution of cardiac fibrosis to these additive effects, we compared combination therapy and monotherapy with respect to their effects on cardiac interstitial fibrosis. Notably, the combination of a calcium channel blocker with an ARB or an ACE inhibitor prevented LV fibrosis to a greater extent than monotherapy with each agent alone (Fig. 5). This larger reduction of cardiac fibrosis by the combination therapy, despite the lack of an additive reduction of blood pressure, might be explained by the finding that angiotensin blockades, independently of blood pressure, prevents cardiac fibrosis (19). Thus, it is plausible that additive prolongation of survival by the combination therapy may be at least partly mediated by the additive preventive effect of cardiac fibrosis. However, further study is needed to determine the possible role of diastolic function in the additive effect on survival by the combination therapy, since the E/A value, a useful marker of diastolic function, was improved by not only the combination therapy but also monotherapy with each agent alone. It is well known that DS rats develop renal dysfunction as well as heart failure (14–16). Interestingly, in the present study, the combination therapy resulted in a significantly greater reduction of UalbV and serum creatinine than monotherapy with each agent alone. These results, taken together with the fact that renal dysfunction plays an important role in the pathophysiology of heart failure, support the notion that the enhanced renal protection by combination therapy might be involved in the enhanced survival rate.

In conclusion, our present work showed that the renin-angiotensin system, as well as hypertension, is involved in the development of hypertensive heart failure with preserved systolic function in DS rats. We provided the first evidence that the combination of a calcium channel blocker with an ARB or an ACE inhibitor, independently of blood pressure or cardiac hypertrophy, had additive beneficial effects on survival and cardiac fibrosis in this model. We propose that the combination of a calcium channel blocker with an ARB or an ACE inhibitor may be a useful therapeutic strategy for prevention of hypertensive heart failure. However, further study is needed to elucidate the underlying mechanism of the additive preventive effects of the combination of these drugs on this heart failure model. It remains to be determined whether or not the rat heart failure model used in the present study can be applied to human diastolic heart failure.

References


