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Next Generation of Spontaneously Diabetic Model of ZDSD Rats with Intact Leptin Signaling Develop Cardiac Dysfunction and Compromised Cardiac Reserve

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Abstract: Cardiomyopathy is the leading cause of morbidity and mortality among all complications of type 2 diabetes (T2D) and obese patients. Diabetic cardiomyopathy (DC) is characterized by changes in cardiac morphology with declines in both systolic and diastolic functions. No rodent models fully captured phenotypes of DC. The ZDSD rat, a new generation of T2D rat model with intact leptin signaling features with slow onset of diabetes and obesity, which closely mimics the development of the disease in patients. Age-matched male ZDSD and SD rats were monitored for blood pressure, glucose and cardiac function using echocardiography. Animals were also challenged with 1 mg/kg dobutamine for the assessment of cardiac reserve. ZDSD rats developed hypertension from age of 18 weeks with blood pressure significantly higher than controls. At resting state, ZDSD rats showed biphasic changes in left ventricular posterior wall thickness and cavity volume. Concomitantly, both ejection fraction (EF) and transmural E/A ratio of LV declined at 34 weeks old. Upon treatment with dobutamine, ZDSD lost cardiac contractility. Therefore, ZDSD rats may serve as a suitable preclinical model to study potential therapeutic approaches to treat cardiomyopathy with presence of metabolic syndromes.

Keywords: obesity, diabetes, cardiomyopathy, echocardiography, ultrasound

1. Introduction

Cardiovascular dysfunction is the leading cause of morbidity and mortality among all complications of type 2 diabetes and obesity patients. The risk of heart failure significantly increases in patients with type 2 diabetes [1]. With the prevalence of diabetes, the number of patients with diabetic cardiomyopathy also surged in the past decade. Using echocardiography, the morphological and functional changes of cardiac ventricles during the development of diabetic cardiomyopathy can be monitored longitudinally [2, 3]. At the early stage of the disease, cardiac hypertrophy is prominent with increased left ventricular (LV) mass and wall thickness. As the disease progresses, diastolic function of the heart compromises, which is characterized by the reduction in ratio of early to late (E/A) transmural flow velocities. The phenomenon is followed by declines in systolic function at the late stage, which is manifested by reductions in percentages of ejection fraction (EF) and fractional shortening (FS) of the LV. Accompanying the systolic functional changes, LV experiences enlargement in inner cardiac dimension and thinning of the cardiac wall [2, 4, 5]. Eventually, heart failure may ensue as a direct consequence of the loss of the cardiac function [3].

Although the exact cause of diabetic cardiomyopathy is not fully understood, many risk factors and molecular mechanisms have been proposed [6]. These include advanced glycated end-product (AGE) accumulation [7], intramyocardial inflammatory cytokine increase [8, 9], mitochondrial dysfunction [10], lipotoxicity [11, 12], oxidative and endoplasmic reticulum (ER) stress etc [13-15] that lead to impairment of intracellular Ca^{2+} handling [16, 17], fibrosis [18] and myocardial dysfunction [19].

In order to facilitate the understanding of the disease mechanism, pathology and the identification of drug targets for treating diabetic cardiomyopathy, multiple type 2 diabetic rodent models have been

characterized for phenotypes of cardiomyopathy [20]. *Ob/ob* and *db/db* mice develop obesity and diabetes from age of 6-8 weeks old as a result of recessive mutation of obesity gene, leptin and defects in leptin receptor respectively [21, 22]. Impaired diastolic function with reduced E/A ratios was noticed in *ob/ob* mice at the age of 11 weeks [23], while contractility of LV was mildly affected upon dobutamine stimulation at 11 weeks [23] and reductions in EF% at resting states at 22 weeks old without changes in LV mass [24]. By comparison, *db/db* mice developed cardiac hypertrophy with increased LV mass and wall thickness at the age of 22 weeks [24]. Concomitantly, the animals have shown diastolic and systolic dysfunction in LV with significant reductions in E/A ratios and EF% at age of 12 and 22 weeks respectively [24, 25]. In diabetic rat models, Zucker diabetic fatty (ZDF) rats developed impaired diastolic function with reduction in E/A ratio at age of 30 weeks [26]. This was accompanied by increases in interventricular (IV) septal wall thickness and decreases in LV dimension. At 44 weeks, reduction in end-diastolic volume (EDV) and LV mass without showing defects in systolic function with no impact on EF% at 44 weeks old were noticed [27]. Therefore, ZDF rats do not fully capture the decline of systolic function in diabetic cardiomyopathy patients.

Zucker Diabetic Sprague Dawley (ZDSD) rat is a new generation of diabetic rat model with intact leptin signaling [28]. ZDSD rats possess multiple metabolic disorders such as diabetes, obesity and dyslipidemia [28] etc with phenotypes highly close to T2D patients. Thus, these animals have distinctive pre-diabetic and diabetic stages, where glucose started to elevate at the age of 16-18 weeks [28]. Serum insulin levels displayed bi-phasic fluctuation where pre-diabetic insulin hyper-secretion was noticed between 13-18 weeks, after which insulin levels start to decline [28]. More importantly, the animals showed multiple diabetic late complications such as diabetic nephropathy [29], neuropathy [30] and delayed wound healing [31], which make ZDSD rat an ideal pre-clinical model for study diabetes and related complications.

The main aim of this study is to evaluate the cardiac morphology and function in ZDSD rats during the development of diabetes using non-invasive echocardiography and to assess the possibility of using ZDSD rats as a model for the development of therapeutic approaches for treating diabetic cardiomyopathy.

2. Materials and Methods

Animals

Male ZDSD (n=8) and age-matched Sprague Dawley (SD) (n=8) rats were obtained from Crown Bioscience Inc. and Envigo (Indianapolis, US) respectively. Animals were housed 2 per cage and were fed ad libitum on Purina 5008 regular rodent chow and house water. Room temperature was monitored and maintained at 20-26°C with the light cycle set at 12 hours (6:00-18:00). All procedures were included in the project (No. 17-550-352) and were approved by the local Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) on Mar 3rd 2017.

Determination of blood glucose

Morning and afternoon tail vein blood glucose levels from fed animals were determined using a hand-held glucometer (StatStrip Xpress glucometer, Nova Biomedical, Waltham MA, USA). The tail of rats will be clipped from the tail end to release a drop of blood for glucometer strips reading.

Tail cuff blood pressure measurement

Blood pressure measurements were taken on conscious rats using tail cuff method by a non-invasive rat/mouse tail cuff system (CODA high throughput system, Kent Scientific, Torrington CT, USA). 15 cycles of measurement were taken and the first 5 were performed as acclimatization not counted into the final calculation.

Pulse wave velocity measurement

Pulse wave velocity was measured as reported elsewhere [32]. In short, rats were anesthetized and maintained with isoflurane. The animal was situated in the supine position and ECG electrodes were attached to collect a lead II ECG signal. Sonoscope S8 expert system with a 12-14 MHz linear array transducer (L742) was used to scan the carotid and iliac arteries under colour-flow dopplor mode. For measuring diameters of carotid, M mode was employed. The total time for each study was approximately 10 minutes. PWV was calculated as: PWV=Distance (D)/Time (T), where D is the

distance in cm from carotid to iliac measurement sites and $T=(R \text{ to iliac foot})-(R \text{ to carotid foot})$ in msec. Three consecutive time differences from each measurement was averaged to obtain the average T for the PWV measurement.

Determination of cardiac structure and function

Echocardiography was performed on isoflurane anesthetized rats when situated on a supine position using Sonoscope S8 expert with 12-14 MHz linear array transducer. For measuring left ventricular (LV) dimension (eg: LV inner diastolic diameter (LVIDd), LV inner systolic diameter (LVIDs), end diastolic volume (EDV), end systolic volume (ESV) and stroke volume (SV)), wall thickness (eg: LV posterior wall (LVPW)) and systolic function (eg: ejection fraction (EF), fractional shortening (FS)), long axis view of the heart was applied from M mode tracing. For measuring diastolic function (eg: E/A ratio), four chamber view of the heart was applied from colour Doppler mode tracing.

Dobutamine stress test

Rats under isoflurane anesthesia were injected intraperitoneally with dobutamine (1 mg/kg). LV structure and function were measured with echocardiography before and at 1, 5, 10, and 20 min after injection. Long axis view of the heart was applied from M mode tracing. After last time point, animals were removed from isoflurane and all animals recovered from the test.

Serum cardiac biomarker analysis

Serum BNP levels were determined with a rat Meso Scale Discovery kit (#K153KFD, Rockville MD, USA) from blood collected from the tail vein.

Histology

Heart tissues collected at termination were fixed in 10% neutral buffered formalin. H&E staining and Masson's trichrome staining were processed on paraffin embedded slides. General assessment of myocyte necrosis, fibrosis and aorta thickening/plagues were performed by a board certified pathologist.

Statistical analysis

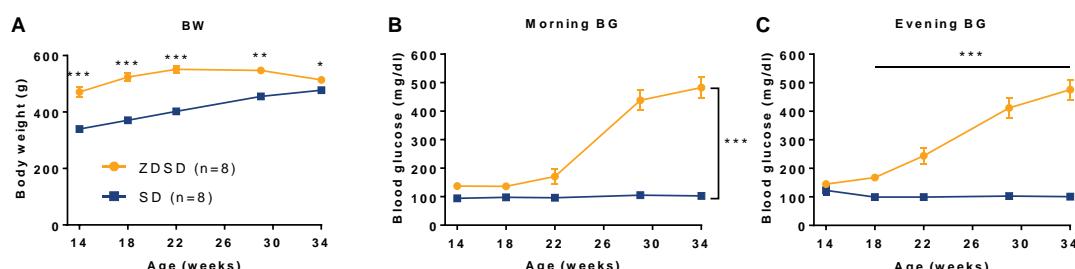
All data were expressed as mean \pm standard error (SE). Statistical analysis was performed using unpaired student t test or one-way or two-way ANOVA with post hoc comparison. P-values less than 0.05 were considered statistically significant.

3. Results

3.1 Obesity, hyperglycemia and hypertension with normal arterial stiffness

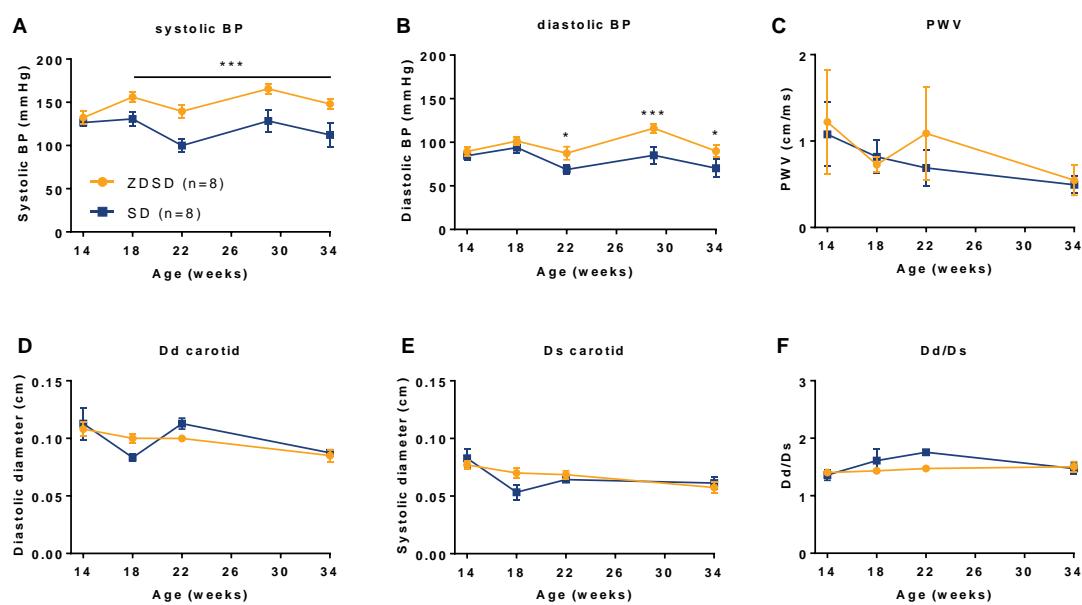
ZDSD rats were obese and heavier than SD rats from age of 14 to 30 weeks (Figure. 1A). At 34 weeks, ZDSD rats were comparable to SD in weight (Figure. 1A). By contrast, ZDSD became severely hyperglycemic at age of 30 weeks with both morning and afternoon blood glucose levels approaching 400 mg/dl (Figure. 1B, C). However, the age of elevation in blood glucose levels in ZDSD rats showed differences depending on the time of measurement, thus ZDSD rats started to show significant increases in morning blood glucose levels at 22 weeks, while the age for increases in afternoon levels were 4 weeks earlier (Figure. 1B, C).

Figure. 1 Spontaneously developed diabetes and obesity in ZDSD rats. (A) Body weight (B)



morning (8:00) and (C) evening blood glucose (17:00). *p<0.05, **p<0.01, ***p<0.001 ZDSD vs age-matched control SD rats.

ZDSD rats were spontaneously hypertensive, with systolic blood pressure significantly higher than SD rats from age of 18 weeks while diastolic blood pressure was higher from age of 22 weeks (Figure. 2A, B). However, PWV was comparable (Figure. 2C) with similar systolic and diastolic carotid



diameters between two groups (Figure. 2D-F), suggesting that the elasticity of arterials in ZDSD rats was not compromised at the ages evaluated.

Figure. 2 Spontaneously hypertensive ZDSD rats displayed normal arterial stiffness. (A) Systolic and (B) diastolic blood pressure, (C) pulse wave velocity, (D) diastolic and (E) systolic inner diameters and (F) their ratios of carotid. *p<0.05, **p<0.01, ***p<0.001 ZDSD vs age-matched control SD rats.

3.2 Cardiac function and morphology at resting condition

3.2.1 Compromised left ventricular systolic and diastolic function

The systolic function of LV of ZDSD rats also displayed bi-phasic changes with initial increases in EF% and FS% from 18-22 weeks followed by declines to baseline at 30 weeks and below SD rats at 34 weeks onwards (Figure. 3A, B and Table 1). The diastolic function of ZDSD rats was also compromised at 34 weeks manifested by the significant reduction in E velocity and E/A ratio (Figure. 3C-E).

Table 1. Left ventricular wall thickness, volume and cardiac function at resting condition in ZDSD rats and their SD controls at age of 34 weeks' old

	SD (n=8)	ZDSD (n=8)	P value
LVPWd (cm)	0.190 ± 0.010	0.180 ± 0.007	0.468
LVPWs (cm)	0.270 ± 0.018	0.238 ± 0.012	0.181
LVIDd (cm)	0.747 ± 0.022	0.846 ± 0.023	0.014
LVIDs (cm)	0.400 ± 0.024	0.563 ± 0.021	0.000
EDV (ml)	0.934 ± 0.075	1.331 ± 0.101	0.010
ESV (ml)	0.173 ± 0.031	0.429 ± 0.039	0.000
SV (ml)	0.763 ± 0.066	0.901 ± 0.087	0.233
HR (bpm)	279.143 ± 4.954	231.285 ± 5.489	0.000

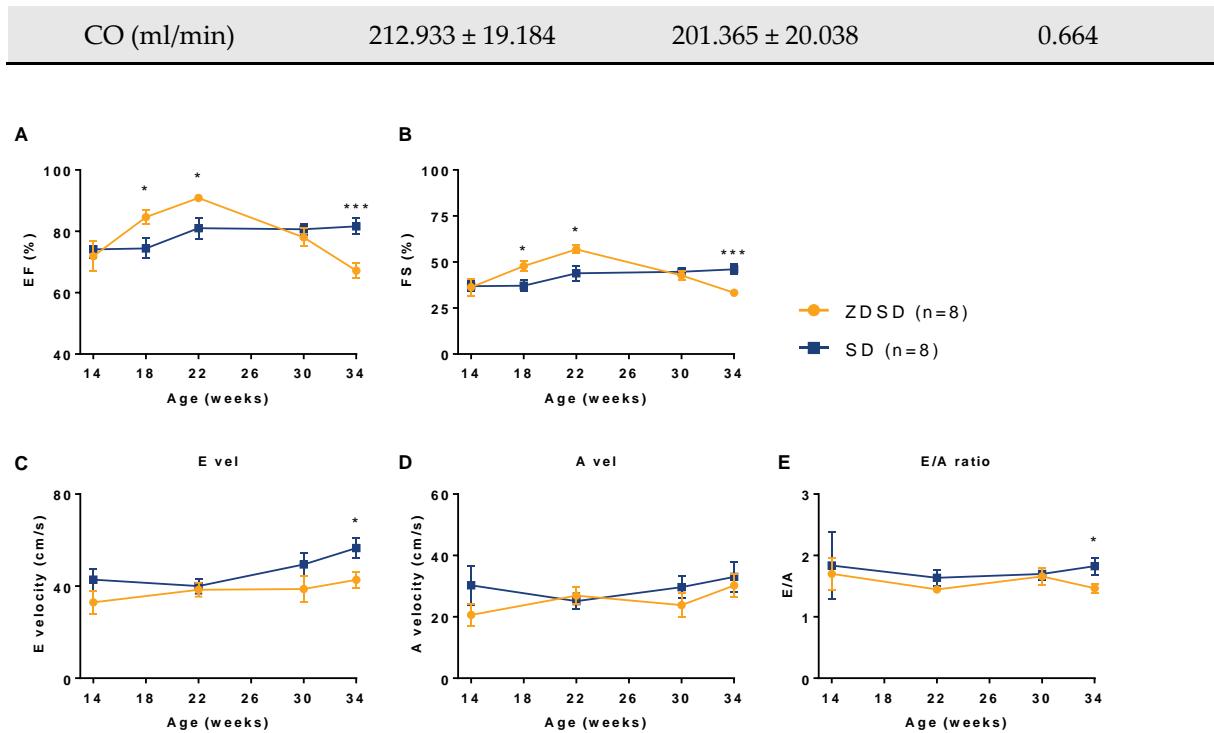


Figure. 3 Cardiac dysfunction at resting condition in ZDSD rats at 34 weeks' old. Systolic dysfunction: (A) EF% and (B) FS%. Diastolic dysfunction: (C) early, (D) late trans-mitral velocity and (E) E/A ratio. *p<0.05 ZDSD vs age-matched control SD rats.

3.2.2 Biphasic changes in left ventricular inner dimension, wall thickness

The changes in heart dimension and wall thickness of ZDSD rats appeared to be biphasic compared to SD rats. After 18 weeks of age, ZDSD rats developed thicker LVPW at both the systolic and diastolic phases vs. SD rats, which became similar between the 2 groups at 30 weeks (Figure. S1). At 34 weeks, both posterior wall thickness at systolic (LVPWs) and diastolic (LVPWd) states remained comparable to SD rats (Table 1). Systolic and diastolic LVID (LVIDs and LVIDd) were larger in ZDSD compared to SD rats at age of 34 weeks, while the sizes were smaller or comparable to control SD rats respectively before 30 weeks (Table 1 and Figure. S2A, B). As a result, EDV and ESV expanded in ZDSD rats at the age of 34 weeks (Table 1 and Figure. S2C, D). In addition, ZDSD rats were lower in heart rate with average heart rate of 62 ± 5 bpm lower than control animals (Table 1). Stroke volume and cardiac output were not significantly different between the groups (Figure. S2E, F).

3.3 Reduced left ventricular contractility under dobutamine stress test

At 30 and 34 weeks of age, animals were challenged with 1 mg/kg dobutamine, during which the heart dimension and systolic function were monitored. As shown in Figure. 4A, intraperitoneal injection of dobutamine into SD rats led to a quick surge in heart rate (<1 min) suggesting the effectiveness of the compound. In accompany with the change, the heart contracted fully with sharp decreases in ESV (Figure. 4B). As a result, the EF% and FS% in SD rats were close to 100 and 80% after dobutamine treatment (Figure. 4C, D). In comparison, despite the increase of heart rate in ZDSD rats during the test, their hearts failed to contract fully, leading to a wider gap between IVS and LVPW during each contracting cycle (Figure. S3E). As a result, the ESV in ZDSD rats were much higher than those in SD controls (Figure. 4F), and the EF% and FS% were dramatically lower in ZDSD rats (Figure. 5D, E). As the LV systolic and diastolic function of ZDSD rats at resting state deteriorated, the LV contractility worsened under dobutamine stress test at 34 weeks' old (Figure. S3A-D).

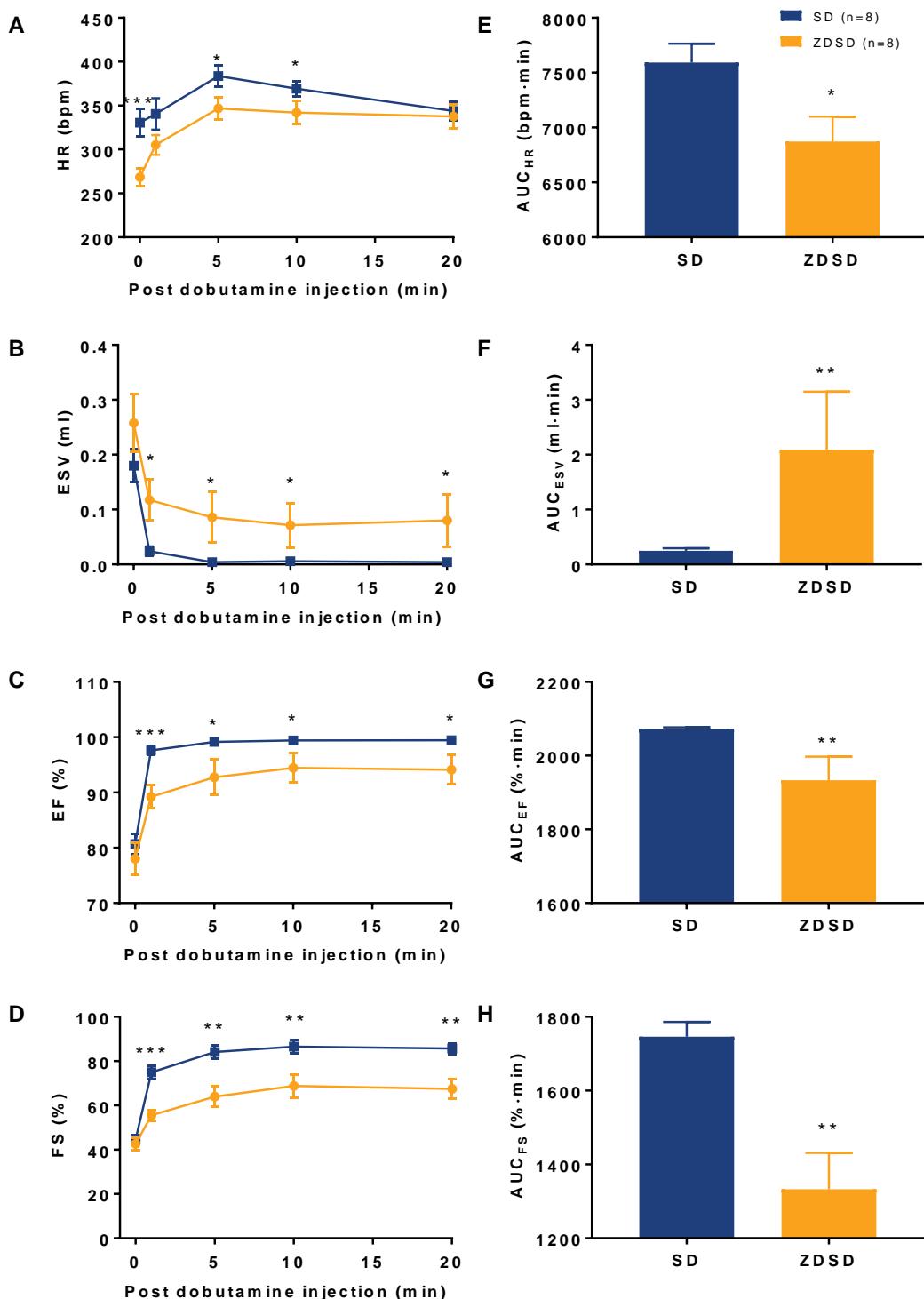


Figure. 4 Reduced cardiac functional reserve measured by dobutamine stress test in ZDSD rats at 30 weeks' old. (A-D) Response curve and (E-H) area under the curve of (A, E) HR, (B, F) ESV, (C, G) EF% and (D, H) FS% following intraperitoneally injection of 1 mg/kg dobutamine. *p<0.05, **p<0.01 ZDSD vs age-matched control SD rats.

3.4 ZDSD rats have unaltered BNP levels and normal histology of LV

The serum BNP levels during the development of the cardiac dysfunction of the ZDSD rats remained steady with comparable values between groups at 34 weeks old (2.84 ± 0.54 in ZDSD vs. 2.41 ± 0.54 pg/ml in SD). In addition, no significant histopathological changes were observed in the LV of animals examined (Data not shown).

4. Discussion

ZDSD rats develop baseline diastolic and systolic cardiac dysfunction with biphasic changes in LV morphology
Despite the availability of multiple diabetic and obese rat models such as ZDF, GK and DIO rats on the market, their development in cardiomyopathy do not fully capture human disease progression [20]. We have demonstrated here that the new generation of type 2 diabetic rat, ZDSD with intact leptin signaling [28] possesses the majority of the phenotypes of diabetic cardiomyopathy during its development of diabetes. More importantly, the phenotypic changes in cardiac morphology and function resembles the disease progression in diabetic cardiomyopathy patients [4, 5].

ZDSD rats developed spontaneous hypertension and hyperglycemia after age of 18 and 24 weeks respectively (Figure. 2) with steady increases in body weight (Figure. 1) and body fat [28]. Their IVS and LVPW grew thicker compared to 14 weeks or age-matched SD controls (Figure. S1). As a result, LV ejection fraction slightly elevated probably to compensate for the hypertrophy of the LV (Figure. 3A). As the hyperglycemia of the animals persisted, the morphology of the LV further developed with LVPW becoming thinner resulting in the enlargement of LV chamber with both EDV and ESV expanded compared to SD controls at week 34 (Figures. S1, S2; Table 1). Concomitantly, both diastolic and systolic functions were compromised as manifested by reduction in E/A ratio and EF (Figures. 3, 4; Table 1). The distinctive biphasic changes in cardiac morphology and function are similarly seen in human patients.

ZDSD rats have intact leptin signaling and show leptin resistance when the animals grow obese and diabetic [28]. This creates a unique advantage over traditional ZDF rat model where leptin receptor is defective [20]. It is known that leptin is involved in the cardiac contractility and LV hypertrophy [33]. Treatment of cardiomyocytes from *ob/ob* mice enhanced phosphorylation of phospholamban (PLN) which caused sarcoplasmic reticulum Ca^{2+} sequestration from the cytosol that resulted in cardiomyocyte contraction [34]. On the other hand, neutralization of LepR using antibodies alleviated cardiac hypertrophy in coronary artery ligated rats [35]. Therefore, it is likely that depletion of downstream leptin signaling in ZDF rats might have effects on the morphology and function of the heart. Although the exact impact of lack of leptin receptor in ZDF rats on manifestation of cardiac changes is not known, ZDF rats do not exhibit impairment in systolic function with unaltered EF at age of 44 week [27], whereas ZDSD rats became defective in systolic function from age of 34 weeks (Figure. 3). Impairments in diastolic functions were noticed in both ZDSD and ZDF rats after age of 34 and 30 weeks respectively. Morphologically, both strains developed enlarged LV inner volume after 34 weeks at systolic and diastolic states possibly to increase the cardiac preload [36]. Therefore, ZDSD rats have similar morphological changes in LV and impairment of diastolic function as ZDF rats, but with additional demonstration of decline in systolic function of the LV, which makes it more relevant to diabetic cardiomyopathy.

ZDSD rats have reduced cardiac reserve

Impairment in systolic function normally occurs in the late stage of diabetic cardiomyopathy in patients with no appearance of the defects at earlier time points [6]. In clinical practice, dobutamine which stimulates $\beta 1$ receptors of sympathetic nerve to induce full contractility of the LV is sometimes used in a dobutamine stress test to evaluate the contractility reserve and other cardiac diseases in patients when coupled with echocardiography as an early diagnostic tool [37, 38]. Dobutamine stress test was also adopted in pre-clinical research for the detection of subtle changes in cardiac reserve or defects in experimental animals [39, 40]. In SD rats, 1 mg/kg can allow almost full outflow of the blood from the LV shortly after the drug administration, while the levels in ZDSD rats were significantly lower from 30 weeks old (Figure. 4). One month later, lowering of the LV contractility at resting state in ZDSD rats were evident (Figure. 2). The results suggested that dobutamine test can be used to predict declines in baseline systolic function in ZDSD rats. Moreover, the results indicated that the progression of diabetic cardiomyopathy in ZDSD rats also has clear stages that are similar to the development of the disease clinically.

Absence of atherosclerosis, serum BNP changes and fibrosis in hearts of ZDSD rats

Long term obesity is usually accompanied by atherosclerosis with accumulation of lipid and immune cell-related plaque in arterials [41]. The resulting blockade of blood flow in coronary artery is the

leading cause for myocardial infarction and heart failure [42]. However, it is rather uncommon to see the development of stiffness in arterials in rodents due to high levels of anti-atherosclerotic HDL, unless high fat diet with animals pre-disposed to atherosclerosis such as ApoE/LDLR KO mice were used [43]. Similarly, we were not able to observe the changes in arterial stiffness in ZDSD rats with comparable PWV and carotid diameters between the groups (Figure. 2C). We were also not able to detect the differences in serum BNP which are indicators for heart failure, during which the levels would normally rise. It is suggested that the ZDSD rats, though had declined systolic function did not reach the state where heart failure might occur. Concomitantly no fibrosis was seen in the heart of ZDSD rats at 48 weeks old.

5. Conclusions

Here we have evaluated and shown that spontaneously obese and diabetic ZDSD rats with intact leptin signaling can develop multiple changes in cardiac morphology with impairment in diastolic and systolic function of the LV that are similar to diabetic cardiomyopathy patients. Therefore, ZDSD rats might serve as a suitable pre-clinical model for the research of diabetic cardiomyopathy.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figureure S1: title, Table S1: title, Video S1: title.

Author Contributions: GS took charge of the study design, experiment performing, data analysis, result interpretation and manuscript writing, had full access to all the data in the study and are responsible for the integrity and accuracy of the experimental procedures. GZ performed experiments, data analysis and discussion. CJ and YW were involved in the study discussion. All authors read and approved the final manuscript for submission.

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Conflicts of Interest: No competing interests declared.

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