Vildagliptin reduces cardiac ischemic-reperfusion injury in obese orchiectomized rats

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Abstract

Obesity and testosterone deprivation are associated with coronary artery disease. Testosterone and vildagliptin (dipeptidyl peptidase-4 inhibitors) exert cardioprotection during ischemic-reperfusion (I/R) injury. However, the effect of these drugs on I/R heart in a testosterone-deprived, obese, insulin-resistant model is unclear. This study investigated the effects of testosterone and vildagliptin on cardiac function, arrhythmias and the infarct size in I/R heart of testosterone-deprived rats with obese insulin resistance. Orchiectomized (O) or sham operated (S) male Wistar rats were divided into 2 groups to receive normal diet (ND) or high-fat diet (HFD) for 12 weeks. Orchiectomized rats in each diet were divided to receive testosterone (2 mg/kg), vildagliptin (3 mg/kg) or the vehicle daily for 4 weeks. Then, I/R was performed by a 30-min left anterior descending coronary artery ligation, followed by a 120-min reperfusion. LV function, arrhythmia scores, infarct size and cardiac mitochondrial function were determined. HFD groups developed insulin resistance at week 12. At week 16, cardiac function was impaired in NDO, HFO and HFS rats, but was restored in all testosterone- and vildagliptin-treated rats. During I/R injury, arrhythmia scores, infarct size and cardiac mitochondrial dysfunction were prominently increased in NDO, HFO and HFS rats, compared with those in NDS rats. Treatment with either testosterone or vildagliptin similarly attenuated these impairments during I/R injury. These findings suggest that both testosterone replacement and vildagliptin share similar efficacy for cardioprotection during I/R injury by decreasing the infarct size and attenuating cardiac mitochondrial dysfunction caused by I/R injury in testosterone-deprived rats with obese insulin resistance.

Key Words

- ▶ obese testosterone deprivation
- ▶ dipeptidyl peptidase-4 inhibitors
- ▶ ischemia–reperfusion
- ▶ cardiac mitochondrial function

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Introduction

Androgen deficiency occurs commonly in middle-aged to older men and also sometimes in younger men with underlying hypothalamopituitary or testicular disorders (Handelsman & Zajac 2004). It is a primary risk factor for many disorders including obesity, metabolic syndrome, dyslipidemia, and especially coronary artery diseases (CAD) (Traish & Kypreos 2011). Low testosterone levels have been shown to be related to CAD (Cornoldi *et al.* 2009) in which lower testosterone levels were associated with a higher prevalence of CAD (Wu & von Eckardstein 2003).

Similar to increased aging population, the prevalence of obesity has increased dramatically over the past decades (Ogden *et al.* 2015). Long-term consumption of a high-fat diet (HFD) has been shown to cause not only obesity but also insulin resistance, a state in which the insulin receptors do not respond to insulin properly leading to impaired glycemic control (Pratchayasakul *et al.* 2011). The main causes of this impairment begins at an intracellular level, in which overproduction of reactive oxygen species (ROS) leads to increased oxidative stress and impaired insulin signaling cascade (Valle *et al.* 2011). This impairment can cause energy insufficiency in the heart, leading to serious progressive impairments including coronary artery disease (Espinola-Klein *et al.* 2011), acute myocardial infarction (Ducimetiere *et al.* 1980) and chronic heart failure (Krishnamoorthy *et al.* 2016). As a large number of aging men are found to be obese (Flegal *et al.* 2012), the adverse effect on the heart could be cumulative from both obese insulin resistance and testosterone deprivation.

In this study, the cardioprotective effects of testosterone and vildagliptin (dipeptidyl peptidase-4 inhibitors) were investigated in testosterone-deprived and obese insulin-resistant model during ischemic and reperfusion (I/R) injury. The rationale for the use of vildagliptin in orchiectomized rats in this study was due to the fact that previous studies demonstrated that testosterone deprivation is associated with the development of several disorders including obesity, insulin resistance and metabolic syndrome (Traish *et al.* 2009) as well as induced cardiovascular diseases and ischemic heart diseases (Kelly & Jones 2013). Regarding the treatment, recent studies have demonstrated the beneficial effects of testosterone replacement and DPP4 inhibitor (vildagliptin) on insulin resistance and metabolic profile (Apaijai *et al.* 2014, Pintana *et al.* 2015) and on ischemic insults of

the heart (Chinda *et al.* 2014, Pongkan *et al.* 2015). However, the effect of testosterone replacement and vildagliptin in the heart subjected with I/R injury with testosterone deprivation combined with obese insulin resistance has never been investigated. Therefore, if vildagliptin exerts cardioprotection in testosterone-deprived subjects similar to testosterone replacement, it would be a great benefit and provide a new alternative treatment for the heart especially in elderly men who suffer from testosterone deprivation and metabolic syndrome, whereas testosterone replacement could not ameliorate peripheral insulin resistance (Garrido *et al.* 2014) and may cause undesirable effects (Reckelhoff *et al.* 2005). In this study, we tested the hypothesis that during cardiac I/R injury in obese insulin-resistant rats with testosterone deprivation, both testosterone and vildagliptin share similar efficacy in improving LV function and decreasing the incidence of arrhythmias and myocardial infarction size by attenuating cardiac mitochondrial dysfunction and apoptosis.

Materials and methods

Animal preparation

All experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Male Wistar rats weighing ~200 g were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand and were used for this study. Food and water were given *ad libitum*. The experimental protocol is illustrated in Fig. 1. For orchiectomy procedure, rats were anesthetized using isoflurane 2–3%, and the orchiectomy was performed using the scrotal approach technique (Foley 2005). Rats vital signs were monitored throughout the operative time and observed for any other complications. Analgesic drugs and antibiotics were injected subcutaneously for 3 days after the operation.

Surgical procedure of myocardial I/R

Rats were anesthetized by intramuscular injection of Zoletil (zolazepam and tiletamine) 50 mg/kg combined with xylazine 3 mg/kg, and the rats were ventilated with air via a tracheotomy by Harvard Rodent Ventilator Model 683 (Harvard Apparatus, Holliston, MA, USA) (Pongkan *et al.* 2015). Lead II electrocardiogram (ECG) (PowerLab 4/25 T, AD Instrument) was recorded throughout the

Figure 1

Schematic representation of the study protocol. Orchiectomized or sham operated rats were divided to receive normal diet (ND) or high-fat diet (HFD) for 12 weeks. Then, orchiectomized rats in ND or HFD were divided to receive vehicle (castor oil; subcutaneously (s.c.)), testosterone 2 mg/kg BW (s.c. daily for 4 weeks) or vildagliptin (Galvas, Novartis) 3 mg/kg BW (intragastric gavage daily for 4 weeks), daily for 4 weeks. The cardiac function and HRV were determined at 12th and 16th week. I/R was performed by 30-min LAD ligation, followed by 120-min reperfusion. HFO, high fat + orchiectomy; HFOT, high fat + orchiectomy + testosterone; HFOVil, high fat + orchiectomy + testosterone; HFS, high fat + sham; NDO, normal diet + orchiectomy; NDOT, normal diet + orchiectomy + testosterone; NDOVil, normal diet + orchiectomy + vildagliptin; NDS, normal diet + sham; Sc., subcutaneous injection.

study. The right carotid artery was cannulated with a pressure–volume (P–V) conductance catheter. The heart was exposed as described by previous study (Chinda *et al.* 2014). Ischemia was confirmed by an ST elevation on the ECG and regional pallor of myocardial tissues of the ischemic area. I/R injury was instigated by 30-min ischemia, followed by 120-min reperfusion.

Normal diet and high-fat diet preparation

Standard laboratory chow (Mouse Feed Food No.082, C.P. Company, Bangkok, Thailand), considered as a normal diet (19.77% of energy fat), was fed to the control rats. The high-fat diet (57.60% of energy fat) consisted of the following ingredients: standard rat diet, casein, lard, cholesterol, vitamins, DL-methionine, yeast powder and sodium chloride (Pratchayasakul *et al.* 2011). All the ingredients were mixed and molded into a spherical shape. Rats were fed on these two diets for 12 weeks.

Heart rate variability determination

HRV is a noninvasive assessment of cardiac autonomic function, which is used to determine cardiac sympathovagal balance (Chattipakorn *et al.* 2007). In this experiment, HRV was analyzed at the 12th and 16th weeks of the study. An electrocardiogram (ECG) was conducted on each rat using PowerLab (PowerLab 4/25T, AD instrument, Bella Vista, NSW, Australia) with chart 5.0 continuously for 20 min while the animals were still

conscious. Data from the ECG recording were analyzed using the MATLAB program and analyzed as described previously (Pongkan *et al.* 2015). An increased LF/HF ratio is indicative of cardiac sympathovagal imbalance (Chattipakorn *et al.* 2007).

LV function determination

LV function was determined using an echocardiograph (Vivid-i, GE) and was analyzed at the 12th and 16th weeks of the study. Rats were anesthetized and maintained with 2% isoflurane with oxygen (2 L mL⁻¹) inhalation. Signals from an M-mode echocardiogram at the level of the papillary muscles were recorded. Parameters obtained included right ventricular dimension during diastole, interventricular septum dimension during systole and diastole, LV internal dimension during systole (LVIDs) and diastole (LVIDd), and LV posterior wall thickness during systole and diastole. Fractional shortening (FS) was calculated using the formula: %FS = (LVIDd – LVIDs) × 100/LVIDd (Rottman *et al.* 2007).

At the end of the experiment (week 16), LV function during I/R injury was evaluated using a P–V catheter (Scisense Instrument, Ontario, Canada). The right carotid artery was cannulated, and the pressure–volume relationship in the LV chamber was recorded using a LabScribe2 program (iWorx Systems Inc, Dover, NH, USA) (Chinda *et al.* 2014). Heart rate (HR), LV end-systolic pressure (LVESP) and LV end-diastolic pressure (LVEDP), $P_{\max}$, $P_{\min}$, positive dp/dt, negative dp/dt and stroke volume (SV) were assessed at the

baseline, end of the ischemic period and at the end of the reperfusion period.

Arrhythmia determination during I/R period

ECG (Lead II) was monitored throughout the I/R period using a PowerLab 4/25 (AD Instruments, Inc, Colorado Springs, CO, USA). The occurrence of arrhythmia was characterized using Lambeth Conventions (Curtis *et al.* 2013), and arrhythmia scores were determined based on the following criteria as described previously (Pongkan *et al.* 2015).

Infarct size determination

At the end of the experiment, the heart was removed and the LAD was re-occluded. The heart was perfused by cold normal saline (Pongkan *et al.* 2015). Evans blue (0.5%) was retrogradely infused through the aortic root. Nonstained area by Evans blue was defined as the area at risk (AAR). The heart was cut horizontally to 7–8 slices (Chinda *et al.* 2014), and then immersed and incubated in 37°C triphenyl tetrazolium chloride (TTC) for 12–15 min to differentiate the infarct tissues (white area) from the viable myocardium (red area). The red and white areas were defined as the remote area, whereas only the white area was defined as the infarct area. The area measurements were calculated using the ImageTool software, version 3.0 (Chinda *et al.* 2014).

Cardiac mitochondrial function study

At the end of the experiment, cardiac mitochondria were isolated from nonischemic (remote area, R) and ischemic areas (I). The heart tissues were homogenized in a homogenizer as described previously (Pongkan *et al.* 2015). Cardiac mitochondrial functions were determined as mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential change ($\Delta\Psi_m$) and mitochondrial swelling. Transmission electron microscopy (TEM) was also used to determine the morphology of the isolated cardiac mitochondria (Pongkan *et al.* 2015).

Metabolic parameters, insulin resistance and testosterone level determination

Fasting plasma glucose, total cholesterol and triglyceride concentrations were determined by an enzymatic

colorimetric assay from a detection kit (Biotech, Bangkok, Thailand). Fasting plasma HDL was determined using colorimetric and fluorometric assays from a detection kit (BioVision), whereas plasma LDL was determined using Friedewald's equation using the fasting plasma HDL values (Friedewald *et al.* 1972). Fasting plasma insulin levels were determined using a sandwich ELISA (Millipore). Insulin resistance was assessed using the Homeostasis Model Assessment (HOMA) (Appleton *et al.* 2005). A higher HOMA index indicates a higher degree of insulin resistance (Pratchayasakul *et al.* 2011). Total plasma testosterone (ng/mL) was analyzed by ELISA at the Central Laboratory Service of Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University.

Western blot analysis

Myocardial protein extract was prepared by homogenization of the frozen heart tissues in a lysis buffer (containing 1% Nonidet P-40 (Calbiochem, Merck Millipore), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in 1×PBS). The total protein was mixed with a loading buffer and boiled at 95°C for 10 min. Then, it was loaded into 10% gradient SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes using transfer system (Bio-Rad). PVDF membranes were blocked with 5% nonfat skimmed milk in TBST for 1 h (Pongkan *et al.* 2015). The membranes were exposed, overnight (at 4°C), to anti- β -actin, anti-Bax (Santa Cruz Biotechnology), anti-Bcl-2, anti-caspase3, anti-total Cx43 and anti-Cx43 phosphorylated at S368 (Cell Signaling Technology) for 12 h. On the next day, bound antibody was detected by horseradish peroxidase-conjugated with anti-mouse IgG. Enhanced chemiluminescence (ECL) detection reagents were used to visualize peroxidase reaction products. The band density was analyzed using ImageJ 1.47V analysis (NIH image).

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Biochemical parameters were compared using the Mann–Whitney *U* test. VT/VF incidence was compared among groups using χ^2 test. Statistical comparisons between groups were analyzed using Student's *t*-tests and one-way ANOVAs with post hoc LSD tests using SPSS, version 10.0. A *P* value less than 0.05 was considered statistically significant.

Results

Obesity, but not testosterone deficiency, caused peripheral insulin resistance, which was attenuated by vildagliptin and testosterone treatment

Testosterone deprivation was confirmed by decreased plasma testosterone levels as well as the ratio of seminal vesicle to body weight (seminal vesicle ratio). Orchiectomized rats without/with obese insulin resistance in both dietary groups (NDO and HFO) had significantly decreased plasma testosterone levels and seminal vesicle ratios, when compared with the sham group (NDS and HFS). Testosterone replacement significantly increased plasma testosterone levels in orchiectomized rats (NDOT and HFOT) compared with those in the NDO and HFO groups, but was not different from the NDS group. However, the testosterone level in rats treated with vildagliptin (NDOVil and HFOVil) was still in the same level as that in the NDO and HFO groups (Table 1).

HFD rats showed a significant increase in body weight, compared with the ND group. Orchiectomized rats had significantly decreased body weight and visceral fat, compared with the sham rats in the same dietary groups. Interestingly, testosterone replacement significantly increased only the body weight in NDOT and HFOT rats, but not visceral fat, compared with that in NDO and

HFO groups, whereas vildagliptin treatment did not show any beneficial effects on body weight or visceral fat in NDOVil and HFOVil groups (Table 1). Moreover, peripheral insulin resistance developed only in the HFD groups (HFS and HFO), as shown by both increased plasma insulin levels and HOMA index, whereas total cholesterol levels and plasma LDL cholesterol levels were increased and plasma HDL cholesterol levels were decreased, compared with those of the NDS group (Table 1). No additive adverse effect in these parameters was found in the HFO group, compared with those in the HFS group, except in the case of plasma total cholesterol. Unlike rats in the HFD groups, NDO group did not have any significant alteration in these metabolic parameters, compared with the NDS group. These findings suggest that peripheral insulin resistance did not develop in the NDO rats.

Interestingly, testosterone replacement attenuated the severity of peripheral insulin resistance in the HFOT group, by decreasing plasma insulin levels and HOMA index, decreasing total cholesterol levels and decreasing plasma LDL cholesterol levels, whereas vildagliptin demonstrated the same beneficial effects in these parameters as well as increasing plasma HDL cholesterol levels in the HFOVil group, compared with the HFO group (Table 1). These findings suggest that testosterone replacement and vildagliptin treatment could attenuate

Table 1 Effects of testosterone and vildagliptin on metabolic parameters in testosterone-deprived rats at the end of week 16 ($n=4-6$ /group).

Parameters	Normal diet				High-fat diet			
	NDS	NDO	NDOT	NDOVil	HFS	HFO	HFOT	HFOVil
Body weight (g)	517±11	456±11 ^a	494±2 ^b	452±14 ^a	605±16 ^a	526±18 ^{b,c}	572±17 ^{a,d,e}	522±17 ^{c,f}
Visceral fat (g)	36.3±3.4	21.4±1.7 ^a	24±1.8 ^a	19.9±2.5 ^a	64.5±2.0 ^a	41.6±1.5 ^{b,c}	43.4±2.5 ^{a,c,e}	41.5±2.0 ^{c,f}
Plasma testosterone (ng/mL)	2.9±0.5	<0.025 ^a	3.4±0.5 ^b	<0.025 ^a	0.5±0.1 ^a	<0.025 ^c	3.9±0.7 ^{c,d}	<0.025 ^c
Seminal vesicle ratio (×10 ³)	4.10±0.02	0.23±0.02 ^a	4.06±0.1	0.23±0.02 ^a	2.75±0.14 ^a	0.14±0.01 ^{a,c}	4.20±0.4	0.4±0.2 ^{a,c}
Plasma insulin (ng/mL)	2.15±0.2	2.2±0.3	2.02±0.34	1.93±0.36	3.70±0.42 ^a	3.58±0.77 ^a	1.94±0.40 ^{c,d}	1.98±0.3 ^{c,d}
Plasma glucose (mg/dL)	184.2±17.6	173±7.1	174±19.8	179.4±8.7	163.5±12.4	184.7±8.8	178.7±9.0	179.6±11.3
HOMA index	18.7±2.5	19.8±3.2	19.1±3.5	19.9±1.8	32.25±3.7 ^a	35.5±9.9 ^a	16.1±2.1 ^{c,d}	18.6±1.9 ^{c,d}
Plasma total cholesterol (mg/dL)	59.2±1.6	62.7±2.3	57.1±3.9	59.3±4.1	87.5±4.5 ^a	101.2±4.8 ^{a,c}	75.1±6.3 ^{a,d}	80.8±6.6 ^{a,d}
Plasma triglyceride (mg/dL)	84.1±6.5	83.3±14.0	82.8±4.7	80.1±5.7	91.1±13.8	81.6±6.2	86.6±11.2	83.2±5.2
Plasma HDL (mg/dL)	33.3±1.6	36.4±1.4	31.4±2.1	34±1.3	23.8±2.5 ^a	25.7±2.0 ^a	25.3±2.1 ^a	33.7±2.4 ^{c,d}
Plasma LDL (mg/dL)	8.4±2.3	9.5±2.7	8.5±1.0	10.1±3.0	33.5±5.7 ^a	54.4±5.6 ^a	37.3±4.3 ^{a,d}	37.7±4 ^{a,d}

HFO, high-fat diet + orchiectomy; HFOT, high-fat diet + orchiectomy + testosterone; HFOVil, high-fat diet + orchiectomy + vildagliptin; HFS, high-fat diet + sham surgery; HOMA, Homeostasis Model Assessment; MDA, malondialdehyde; NDO, normal diet + orchiectomy; NDOT, normal diet + orchiectomy + testosterone; NDOVil, normal diet + orchiectomy + vildagliptin; NDS, normal diet + sham surgery.

^a $P<0.05$ vs NDS, ^b $P<0.05$ vs NDO, ^c $P<0.05$ vs HFS, ^d $P<0.05$ vs HFO, ^e $P<0.05$ vs NDOT, ^f $P<0.05$ vs NDOVil.

peripheral insulin resistance in testosterone-deprived rats with obese insulin resistance by improving both insulin sensitivity and lipid profiles.

Testosterone and vildagliptin preserved both LV function and HRV in testosterone-deprived rats with obese insulin resistance before I/R injury

At week 12, LV dysfunction and sympathovagal imbalance were found in the NDO, HFS and HFO groups as indicated by decreased %fractional shortening (Fig. 2A) and increased LF/HF ratio (Fig. 2C), compared with the NDS group. At week 16, impaired LV function and cardiac sympathovagal imbalance were found in NDO, HFS and HFO groups, and these parameters were not different between groups (Fig. 2B and D). Moreover, our results demonstrated that both testosterone replacement and vildagliptin treatment could preserve LV function and HRV by increased %fractional shortening and decreased the LF/HF ratio in NDOT and NDOVil groups and HFOT and HFOVil groups, compared with that in NDO and HFO groups, respectively. No significant differences in

Figure 2

Effects of obese insulin resistance induced by long-term high-fat diet (HFD) consumption and testosterone deprivation at 12 weeks followed by testosterone and vildagliptin replacement on fractional shortening (FS) and heart rate variability (represented as low frequency/high frequency ratio: LF/HF ratio) before I/R injury. (A and B) Testosterone and vildagliptin restored the FS after treatment for 4 weeks, compared with the NDO and HFO groups. (* $P < 0.05$ vs NDS, † $P < 0.05$ vs NDO, ‡ $P < 0.05$ vs HFS, # $P < 0.05$ vs HFO). HFO, high fat + orchiectomy; HFOT, high fat + orchiectomy + testosterone; HFOVil, high fat + orchiectomy + testosterone; HFS, high fat + sham; NDO, normal diet + orchiectomy; NDOT, normal diet + orchiectomy + testosterone; NDOVil, normal diet + orchiectomy + vildagliptin; NDS, normal diet + sham.

Table 2 Effects of testosterone and vildagliptin on cardiac function (at baseline) during I/R injury ($n = 4-6$ /group).

Cardiac function parameters	Normal diet				High-fat diet			
	NDS	NDO	NDOT	NDOVil	HFS	HFO	HFOT	HFOVil
Heart rate (bpm)	266 ± 9	229 ± 17	241 ± 4	262 ± 9	248 ± 18	266 ± 8	265 ± 20	267 ± 8
LVESP (mmHg)	96 ± 5	92 ± 4	95 ± 11	95 ± 8	95 ± 11	90 ± 9	87 ± 3	98 ± 7
LVEDP (mmHg)	9.8 ± 2	13 ± 1	12 ± 2	11.9 ± 2	14.5 ± 5	14.6 ± 3	10.9 ± 3	13.4 ± 1
Pmax (mmHg)	103 ± 4	97 ± 6	96 ± 9	103 ± 7	108 ± 9	104 ± 7	94 ± 4	129 ± 10
Pmin (mmHg)	6.3 ± 4	7.6 ± 1	11 ± 5	10.8 ± 4	8.3 ± 6	9.7 ± 3	7.3 ± 3	10.1 ± 2
dP/dtmax (mmHg/s)	224,872 ± 5629	203,491 ± 3578	181,951 ± 9509	227,987 ± 7890	233,037 ± 7611	223,466 ± 8889	235,131 ± 8404	235,582 ± 9330
dP/dtmin (mmHg/s)	-4649 ± 939	-5073 ± 319	-4461 ± 746	-5474 ± 676	-4630 ± 703	-4619 ± 670	-4724 ± 432	-4679 ± 1356
Stroke volume (μL)	542 ± 39	533 ± 15	501 ± 32	528 ± 19	511 ± 15	510 ± 6	530 ± 6	522 ± 5

BP, blood pressure; HFO, high-fat diet + orchiectomy; HFOT, high-fat diet + orchiectomy + testosterone; HFOVil, high-fat diet + orchiectomy + vildagliptin; HFS, high-fat diet + sham surgery; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; NDO, normal diet + orchiectomy; NDOT, normal diet + orchiectomy + testosterone; NDOVil, normal diet + orchiectomy + vildagliptin; NDS, normal diet + sham surgery. * $P < 0.05$ vs NDS, † $P < 0.05$ vs NDO, ‡ $P < 0.05$ vs HFS, # $P < 0.05$ vs HFO.

Table 3 Effects of testosterone and vildagliptin on cardiac function (during the ischemic period) in I/R injury ($n=4-6$ /group).

Cardiac function parameters	Normal diet				High-fat diet			
	NDS	NDO	NDOT	NDOVil	HFS	HFO	HFOT	HFOVil
Heart rate (bpm)	235 ± 10	224 ± 10	219 ± 10	235 ± 6	276 ± 30	252 ± 13	227 ± 23	268 ± 31
LVESP (mmHg)	85 ± 2	83 ± 7	76 ± 3	88 ± 12	93 ± 12	94.8 ± 4	84 ± 8	103 ± 18
LVEDP (mmHg)	11.8 ± 1	21.5 ± 1 ^a	15 ± 1 ^b	14 ± 4 ^b	17.5 ± 1 ^a	22.9 ± 2 ^{a,c}	15.8 ± 2 ^d	14.5 ± 0.3 ^{c,d}
Pmax (mmHg)	96 ± 5	104 ± 6	92 ± 8	96 ± 11	86 ± 13	84 ± 14	89 ± 6	93 ± 21
Pmin (mmHg)	8.7 ± 0.5	15.5 ± 1	9.8 ± 2	8.4 ± 5	14 ± 2	15.5 ± 2	14.1 ± 2	13.2 ± 1
dP/dtmax (mmHg/s)	219,674 ± 10,070	210,125 ± 25,306	230,472 ± 12,896	227,641 ± 11,834	250,833 ± 7793	231,060 ± 9081	245,777 ± 11,270	229,512 ± 13,480
dP/dtmin (mmHg/s)	-4321 ± 18	-3124 ± 233 ^a	-4067 ± 310 ^b	-4851 ± 352 ^b	-3455 ± 465	-3014 ± 624	-3664 ± 683	-4197 ± 1237
Stroke volume (μL)	436 ± 65	319 ± 12 ^a	467 ± 49 ^b	363 ± 12 ^b	327 ± 10 ^a	257 ± 10 ^{a,c}	341 ± 18 ^d	344 ± 41 ^d

BP, blood pressure; HFO, high-fat diet + orchiectomy; HFOT, high-fat diet + orchiectomy + testosterone; HFOVil, high-fat diet + orchiectomy + vildagliptin; HFS, high-fat diet + sham surgery; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; NDO, normal diet + orchiectomy; NDOT, normal diet + orchiectomy + testosterone; NDOVil, normal diet + orchiectomy + vildagliptin; NDS, normal diet + sham surgery.

^a $P < 0.05$ vs NDS, ^b $P < 0.05$ vs NDO, ^c $P < 0.05$ vs HFS, ^d $P < 0.05$ vs HFO.

Table 4 Effects of testosterone and vildagliptin on cardiac function (during reperfusion period) in I/R injury ($n=4-6$ /group).

Cardiac function parameters	Normal diet				High-fat diet			
	NDS	NDO	NDOT	NDOVil	HFS	HFO	HFOT	HFOVil
Heart rate (bpm)	244 ± 9	202 ± 4	208 ± 15	210 ± 23	200 ± 20	215 ± 8	210 ± 22	212 ± 16
LVESP (mmHg)	82 ± 6	90 ± 4	92 ± 4	100 ± 7	104 ± 1	94 ± 3 ^c	96 ± 4	95 ± 9
LVEDP (mmHg)	11 ± 4	21.1 ± 2 ^a	15.5 ± 1 ^b	15.3 ± 1 ^b	19 ± 2 ^a	20.7 ± 2 ^a	11.1 ± 2 ^{c,d}	13.8 ± 1 ^{c,d}
Pmax (mmHg)	97 ± 9	97 ± 4	98 ± 3	105 ± 4	107 ± 1	100 ± 5	106 ± 4	97 ± 9
Pmin (mmHg)	7.3 ± 4	15 ± 2	10.3 ± 1	13 ± 3	14.8 ± 2	16.7 ± 1	9.8 ± 4	13 ± 1
dP/dtmax (mmHg/s)	225,758 ± 12,862	241,918 ± 8443	187,639 ± 43,417	229,454 ± 11,118	246,268 ± 13,276	233,425 ± 9741	250,021 ± 11,877	225,561 ± 12,186
dP/dt min (mmHg/s)	-4270 ± 313	-2933 ± 141 ^a	-4040 ± 77 ^b	-3584 ± 139 ^b	-2051 ± 345 ^a	-1821 ± 601 ^a	-3647 ± 352 ^{c,d}	-3147 ± 452 ^{c,d}
Stroke volume (μL)	312 ± 46	207 ± 19 ^a	368 ± 8 ^b	426 ± 73 ^b	193 ± 39 ^a	191 ± 30 ^a	319 ± 24 ^{c,d}	335 ± 48 ^{c,d}

BP, blood pressure; HFO, high-fat diet + orchiectomy; HFOT, high-fat diet + orchiectomy + testosterone; HFOVil, high-fat diet + orchiectomy + vildagliptin; HFS, high-fat diet + sham surgery; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; NDO, normal diet + orchiectomy; NDOT, normal diet + orchiectomy + testosterone; NDOVil, normal diet + orchiectomy + vildagliptin; NDS, normal diet + sham surgery.

^a $P < 0.05$ vs NDS, ^b $P < 0.05$ vs NDO, ^c $P < 0.05$ vs HFS, ^d $P < 0.05$ vs HFO.

%fractional shortening and LF/HF ratio were found with testosterone and vildagliptin treatment in all groups (Fig. 2B and D). These findings suggest that testosterone replacement and vildagliptin treatment could preserve LV function and HRV by increasing %fractional shortening and decreasing the LF/HF ratio in testosterone-deprived rats with obese insulin resistance.

Testosterone and vildagliptin preserved LV function during I/R injury in testosterone-deprived rats with obese insulin resistance

The changes in LV function parameters during I/R injury are shown in Tables 2,3 and 4. At the baseline, LV functions were not different between any groups (Table 2). During I/R injury (at the end of ischemia), the NDO group had increased LVEDP and dp/dtmin as well as decreased stroke volume, compared with the NDS group. Interestingly, testosterone and vildagliptin treatment in the ND group significantly decreased LVEDP, dp/dtmin and increased stroke volume, compared with the NDO group. In addition, the HFD group (HFS and HFO group) had increased LVEDP and decreased stroke volume compared with the NDS group. However, the HFO group demonstrated greater severity in these parameters than the HFS group. Both testosterone and vildagliptin treatment in the HFD group could attenuate LV dysfunction by significantly decreasing LVEDP and increasing stroke volume, compared with that in the HFO group (Table 3). At the end of reperfusion, most parameters remained similar to those during the ischemic period except that rats in the HFO group had decreased LVESF, compared with those in the HFS group, suggesting that testosterone and vildagliptin treatment in the HFD group could attenuate LV dysfunction by significantly decreasing LVEDP, dp/dtmin and increasing stroke volume, when compared with the HFO group (Table 4). These findings suggest that testosterone replacement and vildagliptin treatment could attenuate LV dysfunction by decreasing diastolic dysfunction and increasing stroke volume in testosterone-deprived rats with obese insulin resistance.

Testosterone replacement and vildagliptin decreased the occurrence of cardiac arrhythmias in testosterone-deprived rats with obese insulin resistance during I/R injury

The VT/VF incidence during I/R injury was not different between groups (Fig. 3A). However, in the NDO group,

Figure 3

Effects of testosterone and vildagliptin on the occurrence of cardiac arrhythmia during the ischemic-reperfusion period in a normal diet-fed, obese insulin-resistant with testosterone deprivation model. Testosterone and vildagliptin did not reduce: (A) VT/VF incidence in normal diet and obese insulin-resistant and testosterone-deprived rats but reduced cardiac arrhythmia by increasing (B) time to 1st VT/VF incidence and decreased (C) arrhythmia score, when compared with the NDO or HFO groups. (D) Testosterone and vildagliptin increased P-Cx43/T-Cx43 ratio when compared with the NDO and HFO groups. (* $P < 0.05$ vs NDS, † $P < 0.05$ vs NDO, # $P < 0.05$ vs HFO). HFO, high fat + orchiectomy; HFOT, high fat + orchiectomy + testosterone; HFOVil, high fat + orchiectomy + testosterone + vildagliptin; HFS, high fat + sham; I, ischemic area; NDO, normal diet + orchiectomy; NDOVil, normal diet + orchiectomy + vildagliptin; NDS, normal diet + sham; R, remote area; VF, ventricular fibrillation; VT, ventricular tachycardia.

the time to first VT/VF onset was significantly decreased, whereas the arrhythmia score was significantly greater, compared with the NDS group. Interestingly, testosterone replacement and vildagliptin treatment significantly reduced cardiac arrhythmia by increasing the time to 1st VT/VF (Fig. 3B) and reducing the arrhythmia score during I/R injury (Fig. 3C), when compared with the NDO group. The HFS group demonstrated a decreased time to 1st VT/VF and showed no difference in arrhythmia score, compared with the NDS group, whereas the HFO group demonstrated a decreased time to the 1st VT/VF and increased arrhythmia during I/R injury, compared with the NDS group. However, time to 1st VT/VF and arrhythmia score in the HFS and HFO group showed no difference. Interestingly, testosterone replacement and vildagliptin could increase the time to 1st VT/VF (Fig. 3B) and reduce arrhythmia score during I/R injury (Fig. 3C), compared with the HFO group.

Our study demonstrated that the phosphorylated Cx43 per total Cx43 ratio (P-Cx43/T-Cx43 ratio) of the ischemic-to-remote areas was significantly decreased

in NDO and HFO groups, whereas all treatment groups (NDOT, HFOT, NDOViI and HFOViI) had a significantly increased P-Cx43/T-Cx43 ratio, compared with sham rats being fed with the same diets (Fig. 3D). These findings suggest that both testosterone replacement and vildagliptin treatment can attenuate cardiac arrhythmia during I/R injury in testosterone-deprived rats with obese insulin resistance.

Testosterone replacement and vildagliptin treatment reduced the infarct size in the I/R heart of testosterone-deprived rats with obese insulin resistance

The area at risk (AAR) was not different between all groups. However, the infarct size of NDO, HFO and HFS groups were significantly greater compared with the NDS group. Interestingly, testosterone and vildagliptin reduced the infarct size by 35.7 and 44.1%, respectively, when compared with that in the NDO group and also a reduced infarct size by 55.4 and 49.5%, respectively, compared with that in the HFO group (Fig. 4A). Representative images of the infarct size are shown in Fig. 4B.

Western blot analysis demonstrated that the expression of pro-apoptotic/anti-apoptotic protein (Bax/Bcl-2 ratio) was markedly increased in NDO and HFO groups (Fig. 4C), whereas the expression of pro-caspase3 was not significantly different in the NDO and HFO groups (Fig. 4D), when compared with the NDS group. Interestingly, testosterone replacement and

vildagliptin in ND and HFD decreased Bax/Bcl-2 ratio and increased pro-caspase3 expression, compared with the NDO and HFO groups, respectively (Fig. 4C and D). These findings suggest that testosterone replacement and vildagliptin treatment effectively reduced the infarct size by attenuating apoptotic processes in testosterone-deprived rats with obese insulin resistance.

Testosterone replacement and vildagliptin treatment attenuated mitochondrial dysfunction in the I/R heart of testosterone-deprived rats with obese insulin resistance

There were no differences among groups in ROS production levels, mitochondrial depolarization or mitochondrial swelling of cardiac mitochondria taken from the nonischemic myocardium. Cardiac mitochondria taken from the ischemic areas of the NDO, HFS and HFO groups had significantly increased mitochondrial ROS (Fig. 5A), increased mitochondrial depolarization indicated by a decrease in the red/green fluorescent intensity ratio (Fig. 5B), and increased mitochondrial swelling indicated by a decrease in absorbance at 540 nm (Fig. 5C), when compared with that from the NDS group.

Testosterone replacement and vildagliptin treatment in both ND and HFD groups attenuated mitochondrial dysfunction in the ischemic myocardium of all ORX groups as indicated by reduced ROS production (Fig. 5A), attenuated mitochondrial depolarization (Fig. 5B) and

Figure 4

Effects of testosterone and vildagliptin on the infarct size, anti-apoptotic and pro-apoptotic proteins after ischemic-reperfusion period in a normal diet-fed, obese-insulin resistant with testosterone deprivation model. The area at risk (AAR) was not different between all groups. (A and B) Testosterone and vildagliptin reduced the infarct size by 35.7 and 44.1%, respectively, compared with those in the NDO group and reduced the infarct size by 55.4 and 49.5%, respectively, compared with those in the HFO group. Testosterone and vildagliptin attenuated apoptotic process (C) by decreasing pro-apoptotic/anti-apoptotic ratio (Bax/Bcl-2 ratio) and (D) increasing pro-caspase3 expression, compared with the NDO and HFO groups (* $P < 0.05$ vs NDS, † $P < 0.05$ vs NDO, ‡ $P < 0.05$ vs HFS, # $P < 0.05$ vs HFO). HFO, high fat + orchiectomy; HFOT, high fat + orchiectomy + testosterone; HFOViI, high fat + orchiectomy + testosterone; HFS, high fat + sham; I, ischemic area; NDO, normal diet + orchiectomy; NDOT, normal diet + orchiectomy + testosterone; NDOViI, normal diet + orchiectomy + vildagliptin; NDS, normal diet + sham; R, remote area.

Figure 5

Effects of testosterone and vildagliptin on cardiac mitochondria after ischemic-reperfusion period in a normal diet-fed, obese insulin-resistant with testosterone deprivation model. Testosterone and vildagliptin improved cardiac mitochondrial function by (A) reducing reactive oxygen species (ROS) production, (B) attenuating mitochondrial membrane depolarization and (C) attenuating mitochondrial swelling in ischemic areas. (D) Testosterone and vildagliptin also attenuated the deterioration of cardiac mitochondrial morphology (* $P < 0.05$ vs NDS, † $P < 0.05$ vs NDO, ‡ $P < 0.05$ vs HFS, # $P < 0.05$ vs HFO). HFO, high fat+orchiectomy; HFOT, high fat+orchiectomy+testosterone; HFOVII, high fat+orchiectomy+testosterone; HFS, high fat+sham; NDO, normal diet+orchiectomy; NDOT, normal diet+orchiectomy+testosterone; NDOVII, normal diet+orchiectomy+vildagliptin; NDS, normal diet+sham; ROS, reactive oxygen species.

reduced mitochondrial swelling (Fig. 5C), compared with the ORX group of the same diet regimen. Representative transmission electron microscope (TEM) pictures of cardiac mitochondria in the ischemic myocardium demonstrated that NDO, HFO and HFS groups had more severe mitochondrial swelling, which was indicated by abnormal morphology together with the loss of cristae in the cardiac mitochondrion, than the NDS and ORX group on testosterone or vildagliptin treatment (Fig. 5D). These findings suggest that testosterone replacement and vildagliptin treatment shared similar efficacy in attenuating the cardiac mitochondrial dysfunction by reducing cardiac mitochondrial ROS production, mitochondrial depolarization and mitochondrial swelling in the I/R hearts of testosterone-deprived rats with obese insulin resistance.

Discussion and conclusions

This study has demonstrated the potential cardioprotective effects of testosterone replacement and DPP-4 inhibitor

on the I/R hearts of testosterone-deprived rats with obese insulin resistance. The major findings of this study are as follows. First, high-fat diet, but not testosterone deprivation, induced obese insulin resistance, whereas testosterone replacement and DPP-4 inhibitor (vildagliptin) treatment improved insulin sensitivity and metabolic parameters in testosterone-deprived rats with obese insulin resistance. Secondly, LV function and the HRV were impaired with testosterone deprivation in both obese and nonobese insulin resistance models. Thirdly, testosterone and vildagliptin provided cardioprotective effects in cases of testosterone deprivation alone and in a testosterone-deprived rat with obese insulin resistance model. Fourthly, during the I/R period, testosterone and vildagliptin exerted cardioprotective effects in a testosterone deprivation model and in a testosterone deprivation with obese insulin resistance model, identified by improving LV pressure and stroke volume, reducing cardiac arrhythmias, decreasing the infarct size and attenuating cardiac mitochondrial dysfunction caused by I/R injury.

This study demonstrated that testosterone deprivation alone (NDO group) neither induced insulin resistance nor had adverse effects on metabolic parameters. On the other hand, HFS and HFO groups developed insulin resistance, which is consistent with previous studies which reported that long-term consumption of a HFD caused obese insulin resistance, which is indicated both by increased plasma insulin levels and HOMA index (Pratchayasakul *et al.* 2011, Pintana *et al.* 2015, Pongkan *et al.* 2016). As the severity of insulin resistance at 16 weeks was not significantly different between the HFS and HFO group, this finding suggests that these two conditions were not additive in terms of insulin resistance. Therefore, this finding suggests that these two conditions were not additive in terms of insulin resistance. In this study, our results further demonstrated that testosterone deprivation also did not aggravate the severity of the cardiac impairments such as LV dysfunction and cardiac sympathovagal imbalance in the obese insulin resistant model.

In addition, low testosterone levels and obese insulin resistance are known as risk factors of cardiac dysfunction (Culic & Busic 2015) due mainly to increased oxidative stress (Hwang *et al.* 2011) in the heart, and leading to depressed HRV (Brook *et al.* 2010). Moreover, previous studies found the association of low testosterone and the increase in the reactive oxygen species (ROS) level (Pongkan *et al.* 2015, 2016). Lateef and coworkers demonstrated that low testosterone increased the

synthesis of nitric oxide (NO), which could upregulate urokinase plasminogen activation receptor (uPAR) expression, resulting in plasmin formation, which activates matrix metalloproteinase 9 (MMP-9), leading to extracellular matrix degradation resulting in increased ROS, especially in the mitochondria (Lateef *et al.* 2013). Moreover, they found that ROS production from extracellular matrix disruption also induced lipid peroxidation and caused the reduction of antioxidant levels such as glutathione reductase, catalase and glutathione-dependent enzyme (Lateef *et al.* 2013). When the accumulation of ROS in mitochondria reach a critical threshold level, it triggers the opening of the mitochondrial permeability transition pores (mPTP), resulting in induced mitochondrial membrane depolarization (Hausenloy *et al.* 2003). Moreover, the prolonged opening of mPTP could lead to an increase in the matrix osmotic pressure, resulting in mitochondrial swelling and/or mitochondrial membrane rupture. This will cause the release of pro-apoptotic cytochrome C and other pro-apoptotic proteins, leading to apoptotic cell death (Halestrap & Pasdois 2009). The increase in apoptotic cells induces the enlargement of myocardial infarct size and, as myocardial infarction plays an essential role in cardiac dysfunction, reduction in the infarct size would be of great benefit regarding contractility. This study is consistent with previous studies, demonstrating that NDO, HFS and HFO groups exhibit cardiac dysfunction and depressed HRV in both 12 and 16 weeks.

Regarding therapeutic outcomes, after treatment with a physiological dose of testosterone for 4 weeks, the level of plasma testosterone in the NDOT and HFOT groups was restored back to the same level as the NDS group, whereas plasma testosterone levels in the HFS was significantly lower than those in the NDS and testosterone-treated groups. In NDOVil and HFOVil groups, the level of testosterone was not altered. Interestingly, our study demonstrated that testosterone replacement and vildagliptin treatment in ORX rats with obese insulin resistance (HFO) improved insulin resistance by decreasing plasma insulin levels, HOMA index, total cholesterol levels and plasma LDL cholesterol levels. This is consistent with our previous studies (Apaijai *et al.* 2012, Pintana *et al.* 2015) as well as others (Traish *et al.* 2014, Bayram *et al.* 2016), which demonstrated that testosterone replacement and vildagliptin treatment could attenuate insulin resistance with testosterone deprivation with obese insulin resistance model. These findings suggest that vildagliptin gives beneficial effects similar to testosterone replacement by improving insulin resistance and other metabolic parameters in testosterone-deprived

rats with obese insulin resistance. Moreover, decreasing insulin resistance in several anti-diabetic drugs of different classes such as metformin, liraglutide and vildagliptin demonstrated the cardioprotective effect (Apaijai *et al.* 2012, 2014, Barreto-Vianna *et al.* 2016). However, DPP-4 inhibitor itself might exert direct beneficial effects on the cardiomyocyte, for example, promote the endothelial cell function and upregulate protein expression resulting in cardioprotection (Giannocco *et al.* 2013, Ishii *et al.* 2014).

In addition to the benefits on metabolic parameters, testosterone replacement and vildagliptin treatment also exhibited similar beneficial effects on cardiac function and HRV in ORX rats with/without obese insulin resistance (HFO and NDO groups) by increasing %fractional shortening and decreasing the LF/HF ratios, respectively, when compared with the ORX group. Although previous studies demonstrated that either testosterone or vildagliptin exerted beneficial effects on cardiac function in HFO and NDO groups in a testosterone-deprived model (Pongkan *et al.* 2015), obese-insulin resistance model (Apaijai *et al.* 2012, 2014) and testosterone-deprived with obese-insulin resistance model (Francomano *et al.* 2014), our study is the first to compare the efficacy of testosterone replacement and vildagliptin for their cardioprotection. Regarding the effects of testosterone replacement and vildagliptin on the suppression of mitochondrial ROS production and apoptosis, previous studies demonstrated that either testosterone (Hwang *et al.* 2011, Zhang *et al.* 2013, Pintana *et al.* 2015) or vildagliptin (Avila Dde *et al.* 2013, Apaijai *et al.* 2014, Chinda *et al.* 2014) could effectively reduce ROS generation and suppressed oxidative stress. In addition, Alessandro and coworkers reported that testosterone decreased ROS production by binding to cytoplasmic androgen receptor (AR) and promoting its translocation to the nucleus, resulting in enhanced extracellular matrix (ECM) production via the transcription-inducing complex c-FOS/c-JUN (Bertolo *et al.* 2014). Moreover, previous studies demonstrated that both testosterone (Mancini *et al.* 2008, Zhang *et al.* 2013) and vildagliptin (Avila Dde *et al.* 2013, El-Sherbeeney & Nader 2016) could decrease oxidative stress by increasing the antioxidative substance such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). This hypothesis is supported by the findings in this study that HFO and NDO groups had increased cardiac mitochondrial ROS production and that both testosterone and vildagliptin effectively attenuated cardiac mitochondrial ROS levels and reduced mitochondrial dysfunction (Chinda *et al.* 2014, Pongkan *et al.* 2015). Therefore, it is possible

that the enhancement of the antioxidant defense system and reduction in the levels of oxidative stress of testosterone and vildagliptin could be responsible for their cardioprotection. As both testosterone replacement and vildagliptin share similar efficacy in cardioprotection, these findings could provide an implication for future clinical treatment options in those patients who cannot be treated with testosterone.

During the I/R injury, the results clearly demonstrated that the HFS, HFO and NDO groups demonstrated an impairment of LV function, whereas either testosterone or vildagliptin exerted similar efficacy in cardioprotection in this case. This could be due to their similar efficacy in reducing the infarct size and improving cardiac mitochondrial function, which was impaired during I/R injury. Moreover, as myocardial infarct size has been shown to be associated with the severity of left ventricular dysfunction (Chinda *et al.* 2014), reduction in the infarct size would be of great benefit regarding improved LV function. In this study, HFS, HFO and NDO groups demonstrated a larger infarct size/area at risk than NDS group. Interestingly, either testosterone or vildagliptin exerted similar efficacy in reducing the infarct size in this case. Previous studies on myocardial apoptosis attenuation demonstrated that testosterone replacement could reduce the myocardial infarct size and the potential mechanism could be due to reduced apoptosis and the reduction of cardiac mitochondrial dysfunction, which was demonstrated that testosterone attenuated myocardial apoptosis by increasing anti-apoptotic (Bcl-2) proteins and reducing pro-apoptotic (Bax) proteins (Pongkan *et al.* 2015). Moreover, Huang and coworkers demonstrated that chronic exogenous testosterone replacement improved cardiac function via Akt/PKB signaling pathway resulting in decreased pro-apoptotic (Bax, Fas-L protein) and increased anti-apoptotic proteins (Bcl-2, p-Bad, p-FOXO3a) in isolated heart investigation (Huang *et al.* 2010). For vildagliptin, it has been shown that this drug reduced the infarct size by attenuating myocardial apoptosis via increased anti-apoptotic (Bcl-2) proteins (Apaijai *et al.* 2014, Chinda *et al.* 2014), increased pro-caspase 3 (Chinda *et al.* 2014) and decreased pro-apoptotic (Bax) proteins (Apaijai *et al.* 2014) in I/R injury model as well as renal ischemia-reperfusion injury model (Glorie *et al.* 2012). Moreover, treatment with DPP4 inhibitor in obese, prediabetic (DIO) rats for 4 weeks has been shown to reduce myocardial infarct size after I/R via an increase of the phosphorylation of Akt/PKB signaling proteins, which involved in the RISK (reperfusion-induced salvage kinase) pathway

(Huisamen *et al.* 2011). Consistent with previous studies, our results demonstrated that testosterone and vildagliptin attenuated cardiac mitochondrial dysfunction, which is indicated by decreased mitochondrial ROS production, mitochondrial depolarization and mitochondrial swelling as well as attenuated myocardial apoptosis by decreased Bax/Bcl-2 ratio and increased pro-caspase3 expression. This study also showed that I/R injury induced apoptosis by decreasing pro-caspase3, which is consistent with previous reports in models of I/R injury and obesity (Lu *et al.* 2007, 2016). Increased pro-caspase3 has been shown to play a beneficial role to the cell and acts as the critical point of apoptotic process because pro-caspase3 is an inactive form until it is converted to cleaved caspase3, which is an active form that induced apoptosis (Earnshaw *et al.* 1999, Peterson *et al.* 2009, Walters *et al.* 2009). Mitochondria are the organelles, which play an essential role in cell survival as well as regulation of cell death, especially in cardiomyocytes and increasing intracellular oxidative stress during I/R injury could lead to cardiomyocyte swelling and death (Pongkan *et al.* 2015). In this study, we are reporting for the first time similar beneficial effects of testosterone replacement and vildagliptin during I/R injury in an HFO model, and our findings demonstrate that vildagliptin exhibited similar beneficial effects on the infarct size reduction and improved cardiac mitochondrial function during I/R injury as did testosterone replacement in the HFO and NDO models.

Regarding cardiac arrhythmias, as cardiac mitochondrial depolarization and decreased phosphorylation of Cx43 have been shown to facilitate cardiac arrhythmias (Aon *et al.* 2009, Chinda *et al.* 2014), interventions that prevent these two events could attenuate the arrhythmia incidence (Maruyama *et al.* 2016). Our previous studies (Apaijai *et al.* 2014, Pongkan *et al.* 2015) also demonstrated that both testosterone and vildagliptin could attenuate mitochondrial depolarization and increased phosphorylation of Cx43. Our results in this study demonstrated that the ORX groups were more susceptible to arrhythmias as indicated by decreased time to 1st VT/VF onset and increased arrhythmia scores compared with the sham group of its own diet assignment. As treated groups (NDOT, NDOVil, HFOT and HFOVil) had longer times to 1st VT/VF onset and lower arrhythmia scores, compared with the ORX group, these findings indicated that testosterone and vildagliptin exerted similar antiarrhythmic effects in NDO and HFO groups during I/R injury.

In conclusion, before I/R injury, vildagliptin treatment exhibits similar cardioprotective efficacy as testosterone replacement in testosterone-deprived rats with obese insulin resistance caused by long-term consumption of a HFD, by improving cardiac sympathovagal balance and LV function. During I/R injury, vildagliptin treatment and testosterone replacement also exerted similar cardioprotective effects by ameliorating left ventricular dysfunction and reducing the infarct size and cardiac arrhythmias caused by I/R injury under testosterone deprivation with obese insulin-resistant conditions. These beneficial effects of both testosterone and vildagliptin were due to their ability to increase Cx43 phosphorylation and attenuate cardiac mitochondrial dysfunction and cardiomyocyte apoptosis. These findings suggest that vildagliptin may be used as an alternative to testosterone in testosterone-deprived subjects with or without obese insulin resistance. Whether the combined treatment of vildagliptin and testosterone replacement would provide the synergistic effects needs to be investigated in the future.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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