Vildagliptin and caloric restriction for cardioprotection in pre-diabetic rats

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Abstract

Long-term high-fat diet (HFD) consumption causes cardiac dysfunction. Although calorie restriction (CR) has been shown to be useful in obesity, we hypothesized that combined CR with dipeptidyl peptidase-4 (DPP-4) inhibitor provides greater efficacy than monotherapy in attenuating cardiac dysfunction and metabolic impairment in HFD-induced obese-insulin resistant rats. Thirty male Wistar rats were divided into 2 groups to be fed on either a normal diet (ND, $n=6$) or a HFD ($n=24$) for 12 weeks. Then, HFD rats were divided into 4 subgroups ($n=6$ /subgroup) to receive just the vehicle, CR diet (60% of mean energy intake and changed to ND), vildagliptin (3 mg/kg/day) or combined CR and vildagliptin for 4 weeks. Metabolic parameters, heart rate variability (HRV), cardiac mitochondrial function, left ventricular (LV) and fibroblast growth factor (FGF) 21 signaling pathway were determined. Rats on a HFD developed insulin and FGF21 resistance, oxidative stress, cardiac mitochondrial dysfunction and impaired LV function. Rats on CR alone showed both decreased body weight and visceral fat accumulation, whereas vildagliptin did not alter these parameters. Rats in CR, vildagliptin and CR plus vildagliptin subgroups had improved insulin sensitivity and oxidative stress. However, vildagliptin improved heart rate variability (HRV), cardiac mitochondrial function and LV function better than the CR. Chronic HFD consumption leads to obese-insulin resistance and FGF21 resistance. Although CR is effective in improving metabolic regulation, vildagliptin provides greater efficacy in preventing cardiac dysfunction by improving anti-apoptosis and FGF21 signaling pathways and attenuating cardiac mitochondrial dysfunction in obese-insulin-resistant rats.

Key Words

- ▶ caloric restriction
- ▶ obesity
- ▶ insulin resistance
- ▶ mitochondria
- ▶ FGF21 sensitivity
- ▶ apoptosis
- ▶ fatty acid β -oxidation
- ▶ cardiac function
- ▶ DPP-4 inhibitor

Journal of Endocrinology
(2017) **232**, 189–204

Introduction

Long-term consumption of high-fat diet (HFD) can lead to obese-insulin resistance (Pratchayasakul *et al.* 2011, Pipatpiboon *et al.* 2012), which is characterized by body

weight gain, hyperinsulinemia and euglycemia and is also associated with metabolic dysregulation and dyslipidemia (Pratchayasakul *et al.* 2011, Pipatpiboon *et al.* 2013,

Apaijai *et al.* 2014). Obese-insulin resistance impairs not only the metabolic parameters but also damages the cardiovascular system including an increased blood pressure and cardiac dysfunction in various models (Fontana *et al.* 2004, Dolinsky *et al.* 2010). In past decades, cardiac mitochondrial dysfunction had been proposed as a crucial mechanism of cardiac dysfunction in high-fat diet-induced obese-insulin-resistant models (Chinda *et al.* 2013, Apaijai *et al.* 2014). Therefore, the therapeutic approaches that can attenuate metabolic complications and cardiac mitochondrial dysfunction caused by obese-insulin resistance may lead to improved cardiac function.

Currently, lifestyle modification, including caloric restriction (CR), is the first therapeutic approach for diabetic management. CR combined with anti-diabetic drugs has been shown to be effective for metabolic regulation and glycemic control in diabetes (Inzucchi *et al.* 2015). Clinical studies showed that CR, with or without exercise, improved glycemic control, metabolic function, increased life spans and also reduced cardiovascular disease (CVD) risk factors in Type 2 diabetes mellitus (T2DM) patients (Wycherley *et al.* 2008). Furthermore, in animal studies, CR also reduced body weight, core temperature, heart rate and motor activity in normal rats (Aydin & Gordon 2013). However, the role of CR on the heart in obese-insulin-resistant rats by switching from HFD to normal diet (ND) form is still unknown.

Fibroblast growth factor 21 (FGF21) is the most recent candidate FGF and plays a major role in metabolic regulation and lipid and energy homeostasis in a pathological state (Kharitonov *et al.* 2009, Chau *et al.* 2010, Itoh & Ornitz 2011). The FGF21 signaling pathways have a protective role in several organs including the liver (Yu *et al.* 2015) and pancreas (Wente *et al.* 2006), and it can also have a direct effect on the heart by activating the receptor complex FGFR1c/ β -Klotho (Planavila *et al.* 2013, 2014, Patel *et al.* 2014, Zhang *et al.* 2015). Previous studies demonstrated that FGF21 plays a cardioprotective role in arteriosclerosis (Lu *et al.* 2010, Wu *et al.* 2014), diabetes cardiomyopathy (DCM) (Yan *et al.* 2015, Zhang *et al.* 2015), cardiac ischemia–reperfusion (I/R) injury (Cong *et al.* 2013, Liu *et al.* 2013), myocardial infarction (MI) (Liu *et al.* 2012, 2013, Joki *et al.* 2015) and cardiac hypertrophy (Planavila *et al.* 2013, 2014). Interestingly, an increase in FGF21 levels is associated with future cardiovascular risk in T2DM patients (Shen *et al.* 2013, Ong *et al.* 2015). The ‘FGF21 resistance’ concept in obesity (Fisher *et al.* 2010, Tan *et al.* 2011) and in the heart (Patel *et al.* 2014) has been proposed. In addition, therapeutic strategies that overcome this condition also need to be investigated, and

these therapies are expected to improve FGF21 sensitivity contributing to the potential cardioprotection role (Tanajak *et al.* 2015). Interestingly, a clinical study in obese children demonstrated that a low-carbohydrate diet and a low-fat diet consumption for 2 months improved the metabolic profile and decreased the serum FGF21 levels, suggesting that this CR could improve FGF21 sensitivity (Wycherley *et al.* 2008).

Vildagliptin is an oral anti-diabetic drug that has been used to treat T2DM patients (Inzucchi *et al.* 2015). Previous studies demonstrated that vildagliptin exerted cardioprotective effects in addition to its glycemic control in obese-insulin-resistant (Apaijai *et al.* 2012, 2013), MI (Inthachai *et al.* 2015) and I/R injury rat models (Apaijai *et al.* 2014). Although both CR and vildagliptin exert cardioprotective effects, the effects of long-term CR, vildagliptin and the combined therapy on metabolic regulation, heart rate variability (HRV), cardiac function, cardiac mitochondrial function and FGF21 signaling pathways in HFD-induced obese-insulin-resistant rats have not been investigated.

In this study, we hypothesized that long-term CR and vildagliptin increase insulin sensitivity, attenuate FGF21 resistance, improve cardiac mitochondrial fatty acid oxidation (FAO) and reduce cardiac apoptosis and cardiac mitochondrial dysfunction, leading to improved cardiac function in HFD-induced obese-insulin resistant rats and that combined therapy exerts greater cardioprotection than a single regimen.

Material and methods

Ethical approval

All experiments in this study were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee (Permit No. 31/2557), in compliance with NIH guidelines and in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.* 2010).

Animals and diet

Thirty adult male Wistar rats weighing 180–200g were obtained from the National Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand. All rats were housed in an environment with controlled temperature and humidity and a light–darkness (12:12h) cycle. Rats were fed on either a ND or HFD. The ND is a standard laboratory pelleted diet (Mouse Feed Food

No. 082, C.P. Company, Bangkok, Thailand) containing 19.77% energy from fat and gives a total energy of 4.02 kcal/g. The HFD contains 59.28% energy from fat and consists of standard rat diet (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand, 365 g/kg food), casein (250 g/kg food), lard (310 g/kg food), cholesterol (10 g/kg food), vitamins (60 g/kg food), DL-Methionine (3 g/kg food), yeast powder (1 g/kg food) and sodium chloride (1 g/kg food) giving a total energy of 5.35 kcal/g (Pratchayasakul *et al.* 2011). The CR was achieved in rats by switching from HFD to ND and also by decreasing the energy intake, after the 12-week average food intake, to 60% energy intake in ND form and feeding for 30 days (Wilsey & Scarpace 2004).

Drugs and vehicle

The DDP-4 inhibitor vildagliptin (3 mg/kg/day) (Galvus, Novartis, Bangkok, Thailand) was used in this study. It was dissolved into 0.9% normal saline solution (0.9% NSS) (Burkey *et al.* 2005, Apaijai *et al.* 2012, 2013). The 0.9% NSS in an equal volume was used as a vehicle (Apaijai *et al.* 2012, 2014). Rats were fed vildagliptin or the vehicle via oral gavage feeding once a day for 30 days.

The experiments and study protocol

After the acclimatization week, rats were divided into 2 groups and were given either ND ($n=6$) or HFD ($n=24$) for 12 weeks. At week 12, ND rats were continuously fed with ND and vehicle (NDV) for 30 days. For HFD rats, at week 12, they were divided into 4 subgroups ($n=6$ /group) and each group received one of the following: (1) vehicle (HFV); (2) CR diet and vehicle (HFCR); (3) vildagliptin (HFVil) and (4) CR diet plus vildagliptin (HFCRVil). All treatments were continued for 30 days. On week 12 and at the end of the experiments, rats in all groups were examined. The data collected were body weight, food intake, heart rate variability (HRV), cardiac function by echocardiography and tail blood pressure (BP) measurement using a tail cuff. In addition, the rats were fasted for 6 h, and then blood was collected from the tail vein to determine the plasma metabolic parameters (fasting blood glucose (FPG), insulin, homeostasis model assessment (HOMA), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C)) and plasma FGF21 by ELISA kit (R&D Systems), and serum malondialdehyde (MDA) levels by high-performance

liquid chromatography (HPLC) base assay. In addition, rats were fasted overnight to determine the insulin sensitivity using the oral glucose tolerance test (OGTT) (Apaijai *et al.* 2012, 2013). In addition, rats were fasted overnight to determine the insulin sensitivity using the oral glucose tolerance test (OGTT). An OGTT was carried out consisting of 2 g/kg body weight glucose fed through a gavage (Apaijai *et al.* 2012, 2013). Blood was centrifuged at 3300 g, 4°C for 5 min for plasma collection and 825 g, 4°C for 5 min for serum collection, then plasma and serum were kept at -80°C until required (Thummasorn *et al.* 2011, Apaijai *et al.* 2012, 2013, Pipatpiboon *et al.* 2012, 2013, Surinkaew *et al.* 2013, Chinda *et al.* 2014). At the end of the experiments, rats had their left ventricular (LV) function determined by pressure-volume (P-V) loop measurement, and they were then killed to allow the following to be studied: cardiac mitochondrial function, cardiac mitochondrial morphology by transmission electron microscopy (TEM), cardiac tissue MDA levels by HPLC base assay and cardiac FGF21 signaling cascade protein expression by Western blot analysis. Moreover, the visceral fat was weighed on removal from the rats after killing. The experimental protocol of this study is shown in Fig. 1.

Metabolic parameters assessments

A commercial colorimetric assay kit (Erba Diagnostics Mannheim GmbH, Mannheim, Germany) was purchased for the determination of FPG, TC and TG levels (Pratchayasakul *et al.* 2011, Apaijai *et al.* 2014). A second commercial colorimetric assay kit (Biovision) was purchased for determining the plasma HDL-C level (Apaijai *et al.* 2013). After that the Friedewald equation was used to calculate the plasma LDL-C levels (Friedewald *et al.* 1972, Apaijai *et al.* 2014). The commercial sandwich ELISA kit (LINCO Research) was purchased to determine the plasma insulin levels (Apaijai *et al.* 2013, 2014). The degree of insulin sensitivity was determined by using the HOMA index, an increase in the HOMA index indicates a higher degree of insulin resistance (Pipatpiboon *et al.* 2012).

Plasma FGF21 assessment

Plasma FGF21 levels were determined using a quantitative sandwich enzyme immunoassay technique by using a mouse/Rat FGF21 ELISA kit (R&D Systems) (Yan *et al.* 2015).

Figure 1

The experimental protocol of the study. HFVCR, high-fat diet treated with CR diet; HFVCRVil, high-fat diet treated with CR diet and vildagliptin; HFD, high-fat diet; HFV, high-fat diet treated with vehicle; HFVil, high-fat diet treated with vildagliptin; HPLC, high-performance liquid chromatography; MDA, Malondialdehyde; MMP, mitochondrial membrane potential; ND, normal diet; NDV, normal diet treated with vehicle; OGTT, oral glucose tolerance test; P-V loop, pressure-volume loop; ROS, reactive oxygen species.

Heart rate variability (HRV) determination

Electrocardiography was performed by immobilizing the limbs of rats in a prone position under 2.5% isoflurane inhalation anesthesia. Needle electrodes were inserted subcutaneously into the positions of lead II electrocardiogram (ECG). Rats were allowed to gain full consciousness prior to ECG recording. During ECG recording, rats were kept restrained and prohibited from movement. HRV was determined from lead II ECG by using PowerLab (AD Instruments, Colorado Springs, CO, USA) equipped with the Chart 5.0 program for 20 min. A stable ECG was used to determine the relationship between the RR interval and the beat numbers (Tachogram) using a MATLAB program (Chattipakorn *et al.* 2007, Prachayasakul *et al.* 2011, Apaijai *et al.* 2013). Power spectra of RR interval variability were obtained using the fast Fourier transform (FFT) algorithm. The high frequency (HF; 0.6–3 Hz) band, low frequency (LF; 0.2–0.6 Hz) band and very low frequency (VLF; below 0.2 Hz) band were detected. Each spectral component was calculated as intervals under the respective part of the power spectral density function and was presented in absolute units (ms^2) (Chattipakorn *et al.* 2007). To minimize the effect of changes in total power on the LF and HF bands, LF and HF were expressed as normalized units by dividing the reading by the total power minus VLF (Chattipakorn *et al.* 2007). The increase of the LF/HF ratio indicates an increase in sympathetic

activity or depressed HRV (Pongchaidecha *et al.* 2009, Apaijai *et al.* 2013).

Tail cuff blood pressure measurement

Blood pressure was measured by using the non-invasive CODA 2 on the volume-pressure recording (VPR) tail-cuff method (Kent Scientific Corporation, Torrington, CT, USA) (Feng *et al.* 2008). Rat tails were attached to an occlusion cuff proximally (O-Cuff) and VPR distally and acclimatized in the holder on the infrared warming platform 32–35°C using levels for this temperature control for at least 5 min. The first five cycles were acclimatization cycles and were not included in the data analysis. The mean blood pressure was calculated from the next 20 consecutive cycles.

LV function study for echocardiography

Rats were anesthetized by inhaling isoflurane and oxygen supplementation at 3 liters per minute for 3–5 min. During anesthesia, the LV function was determined using a Vivid i echocardiograph (GE Medical Systems). The image by echocardiography was produced by M-mode and collected in the parasternal short axis of the heart at the papillary muscle levels and % fractional shortening (%FS) was determined. Increased %FS indicated increased LV contractile function (Apaijai *et al.* 2012, 2013).

LV function using a P–V loop

Rats were anesthetized by intramuscular injection with Zoletil (50 mg/kg, Vibbac Laboratories, Carros, France) plus Xylazine (0.15 mg/kg, Laboratories Calier, S.A., Barcelona, Spain). After that an incision was made on the midline of the anterior cervical area and then a tracheostomy tube was inserted into the trachea. Subsequently, the right common carotid artery (CCA) was identified and the P–V loop catheter (Scisense, Ontario, Canada) was inserted into the right CCA and placed into the ascending aorta allowing the recording of systolic blood pressure (SBP) and diastolic blood pressure (DBP). Then, the catheter was advanced into the LV chamber for determining the LV function including heart rate (HR), end-systolic pressure (ESP), end-diastolic pressure (EDP), $dP/dt_{\max}$, $dP/dt_{\min}$ and stroke volume (SV). Data from the P–V loop measurement were recorded using an analytical software program (Labscribe2, Dover, New Hampshire, USA) (Apaijai *et al.* 2013, Chinda *et al.* 2014). At the end of this protocol, the rats were decapitated, and the hearts were removed rapidly for further cardiac tissue protocols described below.

Determination of cardiac mitochondrial function and morphology

The rats hearts were perfused with 10 mL of 0.9% NSS and removed rapidly. The cardiac mitochondrial isolation protocols were performed as previously described (Thummasorn *et al.* 2011, Apaijai *et al.* 2012, 2013). The cardiac mitochondria reactive oxygen species (ROS) production was determined by incubating the mitochondria with 2 M DCFH-DA dye at 25°C for 20 min, and the fluorescent intensity of the solution was detected by a fluorescent microplate reader with excitation wavelength at 485 nm and emission wavelength at 530 nm (BioTek Instruments). An increase in the fluorescent intensity indicated an increased mitochondrial ROS production (Thummasorn *et al.* 2011, Chinda *et al.* 2013).

The cardiac mitochondrial membrane potential change ($\Delta\Psi$) was determined using 5- μ M JC-1 dye and incubating the mitochondria with JC-1 at 37°C for 30 min (Thummasorn *et al.* 2011, Apaijai *et al.* 2014). The cardiac mitochondrial membrane potential changes were detected using a fluorescent microplate reader. The JC-1 monomer form concentration is represented by the green fluorescence and is excited by a wavelength of 485 nm and the emission is detected at 590 nm. The aggregate form of

JC-1 is represented by the red fluorescence and is excited at a wavelength of 485 nm and the emission is detected at 530 nm. A decrease in the red/green fluorescent intensity ratio indicates an increase in cardiac mitochondrial membrane depolarization (Thummasorn *et al.* 2011, Chinda *et al.* 2013).

The cardiac mitochondrial swelling was determined by incubating cardiac mitochondria with 1.5-mM respiration buffer. The absorbance was determined using a spectrophotometer. A decreased absorbance indicated increased cardiac mitochondrial swelling (Thummasorn *et al.* 2011, Apaijai *et al.* 2013).

The cardiac mitochondria were collected during the cardiac mitochondrial function assessment and fixed by 2.5% glutaraldehyde in a 0.1-M phosphate buffer overnight. After that, they were fixed in a 1% cacodylate-buffer osmium tetroxide for 2 h and a graded series of ethanol were used to dehydrate them. A diamond knife was used to cut the cardiac mitochondria embedded in Epon-Araldite and stained with uranyl acetate. Finally, the cardiac mitochondria's morphology was observed using a transmission electron microscope (TEM) at 15,000 $\times$ by JEM-2200FS field emission electron microscope (Thummasorn *et al.* 2011, Apaijai *et al.* 2014).

Determination of MDA concentration

The cardiac tissue and plasma MDA concentrations were determined using a HPLC-based assay (Thermo Scientific) (Apaijai *et al.* 2012, 2013). Plasma and cardiac MDA were mixed with H_3PO_4 and thiobarbituric acid (TBA) to produce TBA-reactive substances (TBARS). The plasma and cardiac TBARS concentration were determined directly from a standard curve and reported as equivalent to the MDA concentration (Apaijai *et al.* 2012, 2013).

Cardiac protein expression by Western blot analysis

Cardiac tissues for determining the protein expressions were obtained from the fresh hearts at the LV apex. Myocardial protein extracts were prepared by homogenization of nitrogen-frozen myocardial tissues in a 300- μ L extraction buffer containing 20-mM Tris-HCl (pH 6.8), 1-mM sodium orthovanadate, 5-mM sodium fluoride and protease inhibitor. Total protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories). Total protein was mixed with loading buffer (5% beta mercaptoethanol, 0.05% bromophenol blue, 75 mM Tris-HCl (pH 6.8), 2% SDS

Table 1 The effects of calorie restriction, vildagliptin and calorie restriction plus vildagliptin on metabolic parameters in HFD rats after treatment for 4 weeks. Body weight and visceral fat were recorded at the end of experiment. Moreover, the rats were fasted for 6 h then blood was collected from the tail vein to determine the plasma metabolic parameters (fasting plasma glucose FBS, insulin, Homeostasis Model Assessment (HOMA)-index, total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C), serum maladehyde (MDA) levels and cardiac tissue MDA by high liquid chromatography (HPL)C base assay. In addition, rats were fasted overnight to determine the insulin sensitivity using the area under the curve (AUCg) by oral glucose tolerance test (OGTT).

Parameters	Groups				
	NDV	HFV	HFCR	HFVil	HFCRVil
Body weight (g)	519±24	627±21*	523±23 [†]	625±38*. [‡]	518±14 [†] . [#]
Visceral fat (g)	32±3	54±4*	36±4 [†]	58±5*. [‡]	30±2 [†] . [#]
Plasma glucose (mM)	8.1±1.0	8.4±0.2	8.5±0.1	8.5±0.3	8.6±0.3
Plasma TC (mM)	3.9±0.3	5.4±0.2*	4.2±0.1 [†]	4.3±0.5 [†]	4.2±0.3 [†]
Plasma TG (mM)	2.9±0.5	3.1±0.3	2.8±0.3	2.7±0.2	2.8±0.4
Plasma HDL-C (mM)	1.9±0.1	1.4±0.1*	2.1±0.1 [†]	1.9±0.1 [†]	2.0±0.1 [†]
Plasma LDL-C (mM)	1.3±0.1	3.0±0.4*	1.6±0.4 [†]	1.4±0.2 [†]	1.4±0.2 [†]
Plasma insulin (ng/mL)	4.3±0.3	6.7±0.8*	4.1±0.5 [†]	3.8±0.5 [†]	4.6±0.6 [†]
HOMA index	33.2±6.1	61.5±10.0*	33.6±5.1 [†]	29.8±3.8 [†]	33.8±7.2 [†]
Serum MDA (μmol/mL)	1.5±0.1	6.0±1.4*	1.7±0.1 [†]	1.2±0.1 [†]	1.5±0.1 [†]
Tissue MDA (μmol/mg protein)	0.80±0.08	2.91±0.54*	0.71±0.08 [†]	0.64±0.07 [†]	0.72±0.06 [†]
AUCg (mM×min×10 ⁵)	9.23±0.25	12.6±0.70*	8.87±0.57 [†]	9.81±0.27 [†]	9.08±0.32 [†]

**P*<0.05 vs NDV, [†]*P*<0.05 vs HFV, [‡]*P*<0.05 vs HFCR.

HFCR, high-fat diet treated with CR diet; HFCRVil, high-fat diet treated with CR diet and vildagliptin; HFD, high-fat diet; HFV, high-fat diet treated with vehicle; HFVil, high-fat diet treated with vildagliptin; ND, normal diet; NDV, normal diet treated with vehicle.

and 10% glycerol) and boiled at 95°C for 10 min. Forty micrograms of total proteins from each group were loaded in each well (total volume was 20 μL/well). The samples were loaded onto the 10% SDS-polyacrylamide gels (Apaijai *et al.* 2014, Pongkan *et al.* 2016). Then, proteins were transferred to nitrocellulose membranes in a glycine/methanol transfer buffer in a wet/tank blotting system (Bio-Rad Laboratories). Membranes were blocked in either 5% skim milk or 5% bovine serum albumin in Tris-buffered saline and Tween (TBST) buffer. The FGF21 signaling proteins including fibroblast growth factor receptor (FGFR) 1 and phospho-FGFR1 (p-FGFR1) (1:200 dilution, Sigma-Aldrich), β-Klotho (1:200 dilution), extracellular signal-regulated kinase (ERK1/2), phospho-ERK1/2 (p-ERK1/2) (1:1000 dilution, Santa Cruz technologies), Akt and p-Akt (1:500 dilution, Cell Signaling Technology) were determined. Moreover, Bax (1:1000 dilution), cleaved caspase-3 (1:1000 dilution), proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (1:200 dilution), carnitine palmitoyltransferase 1 (CPT1) (1:200 dilution), β-actin (1:2000 dilution, Santa Cruz Biotechnology) and B-cell lymphoma 2 (Bcl2) (1:1000 dilution, Cell Signaling Technology) were determined. Bound antibody for β-actin (Surinkaew *et al.* 2013)

was detected by horse anti-mouse IgG-conjugated horseradish peroxidase (HRP)-linked antibody (1:2000 dilution, Cell Signaling Technology). Bound antibody for β-Klotho was detected using a rabbit anti-goat IgG-conjugated HRP-linked antibody (1:2000 dilution, Santa Cruz Biotechnology). Bound antibodies for FGFR1, p-FGFR1, ERK1/2, p-ERK1/2, Akt, p-Akt, Bax, Bcl-2, cleaved caspase-3, PGC-1α and CPT-1 were detected using a goat anti-rabbit IgG-conjugated HRP-linked antibody (1:2000 dilution, Cell Signaling Technology). Enhanced chemiluminescence (ECL) detection reagents were used to visualize peroxidase reaction products (Clarity ECL Western blotting substrate, Bio-Rad) (Pongkan *et al.* 2016). However, due to our protocol, we used invisible markers BLUeye Prestained Protein Ladder (Cat. No. PM007-0500; GeneDireX, Inc. Miaoli County 350, Taiwan, R.O.C.). The WesternSure Pen (Li-cor biotechnology, Lincoln, Nebraska, USA) was used to annotate visible protein ladders prior to detecting chemiluminescent intensity from Western blot using the ChemiDoc system. The membranes were developed in the ChemiDoc touch imaging system (Bio-Rad Laboratories) by placing a membrane on the chemi/UV/strain-free tray. Then, the image was adjusted to an appropriate size, and the blot

Table 2 The effects of calorie restriction, vildagliptin, and calorie restriction plus vildagliptin on heart rate and blood pressure in HFD rats after treatment for 4 weeks. At the end of experiment, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) were measured by using the non-invasive CODA 2 on the volume–pressure recording (VPR) tail-cuff method.

Parameters	Groups				
	NDV	HFV	HFCR	HFVil	HFCRVil
SBP (mmHg)	132 ± 5	156 ± 3*	152 ± 8*	134 ± 4 ^{†,‡}	129 ± 5 ^{†,‡}
DBP (mmHg)	87 ± 7	88 ± 3	87 ± 6	82 ± 5	87 ± 6
MAP (mmHg)	104 ± 4	117 ± 2*	118 ± 3*	103 ± 3 ^{†,‡}	100 ± 3 ^{†,‡}
HR (bpm)	401 ± 6	436 ± 10*	429 ± 10*	383 ± 21 ^{†,‡}	392 ± 12 ^{†,‡}

* $P < 0.05$ vs NDV, [†] $P < 0.05$ vs HFV, [‡] $P < 0.05$ vs HFCR.

HFCR, high-fat diet treated with CR diet; HFCRVil, high-fat diet treated with CR diet and vildagliptin; HFV, high-fat diet treated with vehicle; HFVil, high-fat diet treated with vildagliptin; ND, normal diet; NDV, normal diet treated with vehicle.

chemiluminescence category was chosen for exposure and to take a Western blot image. The densitometric analysis was done by the ImageJ program (Chinda *et al.* 2014). Each protein expression was normalized with β -actin expression.

Statistical analysis

Data were expressed as mean $\pm$ S.E.M. One-way analysis of variance (ANOVA) by least significant difference (LSD) *post hoc* test was used to test the difference between the groups. $P < 0.05$ was considered statistically significant.

Results

CR, vildagliptin and combined therapy restored metabolic disturbance in obese-insulin-resistant rats

At 16 weeks after HFD feeding, HFV rats had significantly increased BW and visceral fat, compared with the NDV group (Table 1). The dietary intervention HFCR group had significantly decreased BW and visceral fat, compared with the HFV group. However, no differences in the BW and visceral fat were found in the HFVil rats, compared with the HFV group. Nevertheless, the combined therapy HFCRVil rats had significantly decreased BW and visceral fat, compared with the HFV and HFVil groups (Table 1).

The plasma TC, plasma LDL-C, plasma insulin, HOMA index, serum MDA, cardiac MDA, AUCg and the HOMA index were increased, whereas the plasma HDL-C was decreased in the HFV rats, compared with the NDV group (Table 1). These parameters were improved in the HFCR, HFVil and HFCRVil rats, when compared with the HFV

group. However, plasma glucose and plasma TG showed no difference between the groups ($P > 0.05$, Table 1). Moreover, the HFV rats had significantly increased HR, SBP and MAP, compared with the NDV group. Only rats in the vildagliptin-treated groups (HFVil and HFCRVil) had significantly decreased HR, SBP and MAP, compared with the HFV group (Table 2).

Vildagliptin increased cardiac autonomic regulation and LV function in obese-insulin-resistant rats more extensively than CR

Regarding HRV, the results showed that HFD results showed a significant increase in the LF/HF ratio since week 12 in the HFD rats, compared with the ND group (Fig. 2A). In the same way, it was found that the %FS was significantly decreased at week 12 in the HFD group, compared with the ND group (Fig. 2B). After 16 weeks of HFD feeding, there was still a significant increase in the LF/HF ratio and decreased %FS in the HFV rats, compared with the NDV group. In addition, the LF/HF ratio was significantly decreased in the HFCR group (Fig. 2C), when compared with the HFV group. Moreover, the LF/HF ratio was also significantly decreased in the HFVil and HFCRVil groups (Fig. 2C), when compared with the HFV and HFCR groups. Furthermore, the %FS was significantly increased in the HFCR group (Fig. 2D), when compared with the HFV group. Moreover, the %FS was also significantly increased in the HFVil and HFCRVil groups (Fig. 2D), when compared with the HFV and HFCR groups.

At 16 weeks after HFD feeding, the P–V loop showed that the HFV rats had significantly increased SBP and MAP and also had impaired cardiac function identified by increased HR and EDP, and decreased ESP, $\pm dP/dt$

The levels of plasma FGF21 were significantly higher in the HFV group, compared to the ND rats at week 12 of HFD feeding (Fig. 3A). Plasma FGF21 levels at week 16 were also

HFCR, high-fat diet treated with CR diet; HFCRVil, high-fat diet treated with CR diet and vildagliptin; HFD, high-fat diet; HFV, high-fat diet treated with vehicle; HFVil, high-fat diet treated with vildagliptin; ND, normal diet; NDV, normal diet treated with vehicle.

Figure 3

The effects of HFCR, vildagliptin and combined HFCR and vildagliptin on plasma FGF21 levels, FGFR1/β-Klotho receptors complex and intracellular Erk1/2 expression and phosphorylation. (A) Plasma FGF21 levels at weeks 12, (B) plasma FGF21 levels post treatment, (C) p-FGFR1, (D) t-FGFR1, (E) p-FGFR1/t-FGFR1, (F) β-Klotho, (G) p-Erk1/2, (H) t-Erk1/2, and (I) p-Erk1/2/t-Erk1/2 ratio in ND and HFD rats treated with vehicle, Calorie restriction (CR), vildagliptin, and CR combined with vildagliptin. * $P < 0.05$ vs NDV, † $P < 0.05$ vs HFV, ‡ $P < 0.05$ vs HFCR (1-WAY ANOVA, LSD *post hoc* test, $n = 4-6$ per subgroups). Erk1/2, extracellular signal-regulated protein kinases 1 and 2; FGFR1, fibroblast growth factor receptors 1; HFCR, high-fat diet treated with CR diet; HFCRVil, high-fat diet treated with CR diet and vildagliptin; HFD, high-fat diet; HFV, high-fat diet treated with vehicle; HFVII, high-fat diet treated with vildagliptin; ND, normal diet; NDV, normal diet treated with vehicle; p-FGFR1, phospho FGFR1; p-ERK1/2, phospho ERK1/2; t-ERK1/2, total ERK1/2.

significantly increased in the HFV group, compared with the NDV group (Fig. 3B). However, the plasma FGF21 levels were significantly decreased in the HFCR, HFVII and HFCRVil groups (Fig. 3B). The FGF21 receptor complex and signaling cascade protein expression and phosphorylation by Western blot analysis demonstrated that the HFV group had decreased p-FGFR1 (Fig. 3C) and also a p-FGFR1/t-FGFR1 ratio (Fig. 3E) when compared with the NDV group. HFCR, HFVII and HFCRVil groups had restored p-FGFR1 (Fig. 3C) and p-FGFR1/t-FGFR1 ratios when compared with the HFV group (Fig. 3E). However, no alteration of t-FGFR1 (Fig. 3D) and β-Klotho (Fig. 3F) expression were found in

all groups. Regarding downstream signaling, the HFV rats had decreased p-ERK1/2 (Fig. 3G) and also p-ERK1/2/t-ERK1/2 ratio (Fig. 3I), whereas all treatments increased p-ERK1/2 (Fig. 3G) and p-ERK1/2/t-ERK1/2 ratios (Fig. 3I). However, no alteration of t-ERK1/2 (Fig. 3H) expression was found in all groups. In addition, the p-Akt/t-Akt ratio was not significantly different between groups (0.57 ± 0.05 , 0.49 ± 0.06 , 0.55 ± 0.12 , 0.47 ± 0.07 and 0.51 ± 0.10 unit for NDV, HFV, HFCR, HFVII and HFCRVil, respectively).

In regard to anti-apoptotic signaling pathways, rats in the HFV group had significantly increased apoptotic protein expression including Bax (Fig. 4A), Bax/BCL-2

Figure 4

Effects of HFCR, vildagliptin and combined HFCR and vildagliptin on intracellular FGF21 signaling pathways including anti-apoptosis and fatty acid oxidation pathways. (A) Bax, (B) BCL-2, (C) Bax/BCL-2 ratio, (D) Cleaved Caspase-3/t-Caspase-3 ratio, (E) PGC-1α, and (F) CPT-1 in ND and HFD rats treated with vehicle, Caloric restriction (CR), vildagliptin, and CR combined vildagliptin. * $P < 0.05$ vs NDV, † $P < 0.05$ vs HFV, ‡ $P < 0.05$ vs HFCR (1-WAY ANOVA, LSD *post hoc* test, $n = 4-6$ per subgroups). CPT1, carnitine palmitoyltransferase 1; HFCR, high-fat diet treated with CR diet; HFCRVil, high-fat diet treated with CR diet and vildagliptin; HFD, high-fat diet; HFV, high-fat diet treated with vehicle; HFVil, high-fat diet treated with vildagliptin; ND, normal diet; NDV, normal diet treated with vehicle; PGC-1α, proliferator-activated receptor gamma coactivator 1-alpha.

ratio (Fig. 4C) and cleaved caspase-3 (Fig. 4D), when compared with the NDV group. Moreover, the HFV group also had significantly decreased anti-apoptotic signaling molecules as shown by decreased BCL-2 (Fig. 4B), when compared with the NDV group. Our results showed that HFCR rats had significantly decreased Bax (Fig. 4A), Bax/BCL-2 ratio (Fig. 4C) and cleaved caspase-3 (Fig. 4D), when compared with the HFV group. Moreover, the HFCR group had significantly increased levels of BCL-2 (Fig. 4B), when compared with the HFV group. Interestingly, we found that vildagliptin-treated groups (HFVil and HFCRVil) had a greater reduction in Bax (Fig. 4A), Bax/BCL-2 ratio (Fig. 4C) and cleaved caspase-3 (Fig. 4D), when compared with the HFV and HFCR groups. Vildagliptin-treated groups (HFVil and HFCRVil) also had greater increased levels of BCL-2 (Fig. 4B), when compared with HFV and HFCR groups.

CR and vildagliptin restored fatty acid β-oxidation (FAO) pathway protein expression in the heart

At the end of 16 weeks of feeding on a HFD, the results in mitochondrial FAO pathways protein expression demonstrated that HFV rats had significantly decreased PGC-1α (Fig. 4E) and CPT-1 (Fig. 4F) protein expression

when compared with the NDV group. All treatments in HFCR, HFVil, and HFCRVil groups had led to significantly increased PGC-1α (Fig. 4E) and CPT-1 (Fig. 4F) protein expression when compared with the HFV group.

Vildagliptin exerted more effective cardiac mitochondrial protection than CR in obese-insulin-resistant rats

At the end of 16 weeks of feeding on a HFD, the results regarding cardiac mitochondrial function demonstrated that HFD consumption significantly increased cardiac mitochondrial ROS production (Fig. 5A), decreased cardiac mitochondrial membrane potential changes verified by an attenuated red/green fluorescent intensity ratio ($\Delta\Psi$; Fig. 5B) and also increased cardiac mitochondrial swelling (Fig. 5C), when compared with the NDV group. HFCR, HFVil and HFCRVil groups had significantly decreased cardiac mitochondrial ROS production when compared with the HFV group (Fig. 5A). Moreover, the HFCR group had improved cardiac mitochondrial function identified by increased cardiac mitochondrial membrane potential changes ($\Delta\Psi$; Fig. 5B) and mitochondrial absorbance (Fig. 5C), when compared with the HFV groups.

Figure 5

Effects of HFCR, vildagliptin and combined HFCR and vildagliptin on cardiac mitochondrial function and cardiac mitochondria morphology. (A) Cardiac mitochondrial ROS production, (B) red/green fluorescent intensity ratio, (C) Cardiac mitochondrial swelling, and (D) Cardiac mitochondria morphology by transmission electron microscope (TEM) in ND and HFD treated with vehicle, CR, vildagliptin, and CR combined with vildagliptin. * $P < 0.05$ vs NDV, † $P < 0.05$ vs HFV, ‡ $P < 0.05$ vs HFCR (1-WAY ANOVA, LSD *post hoc* test, $n = 6$ per subgroups). HFCR, high-fat diet treated with CR diet; HFCRVil, high-fat diet treated with CR diet and vildagliptin; HFD, high-fat diet; HFV, high-fat diet treated with vehicle; HFVil, high-fat diet treated with vildagliptin; ND, normal diet; NDV, normal diet treated with vehicle; ROS, reactive oxygen species.

However, vildagliptin-treated groups (HFVil and HFCRVil) provided greater efficacy in improving cardiac mitochondrial function by restoring the cardiac mitochondrial membrane potential changes ($\Delta\Psi$; Fig. 5B) and mitochondrial absorbance (Fig. 5C), when compared with the HFV and HFCR groups. Moreover, the cardiac mitochondrial morphology viewed by transmission electron microscope (TEM) demonstrated unfolded cristae in the mitochondria in the HFV group, indicating cardiac mitochondrial swelling in the HFV group. Although the cardiac mitochondrial morphology had improved in the HFCR group, when compared with the HFV group, this parameter had been restored to normal levels in vildagliptin-treated groups (HFVil and HFCRVil), when compared with HFV and HFCR groups (Fig. 5D).

Discussion

The major findings of this study are as follows: long-term HFD consumption led to obesity, insulin resistance, FGF21 resistance and increased oxidative stress levels. Moreover, high systolic blood pressure, cardiac mitochondrial dysfunction and cardiac sympathovagal imbalance were observed, and all of these factors contributed to LV dysfunction in obese-insulin-resistant rats. The three interventions in this study, including caloric restriction (CR), vildagliptin and combined CR and vildagliptin, had similar efficacy in improving insulin resistance, FGF21 resistance, metabolic disturbance, cardiac and serum MDA

levels and mitochondrial ROS production. Interestingly, vildagliptin exerted greater cardioprotection than CR as vildagliptin appeared to reduce cardiac apoptosis, preserve cardiac mitochondrial membrane potential and attenuate cardiac mitochondrial swelling more effectively than CR. From all of these beneficial effects, vildagliptin effectively reduced blood pressure, improved cardiac sympathovagal imbalance and cardiac dysfunction in obese-insulin-resistant rats.

In previous reports, 12–16 weeks of HFD consumption did not affect plasma triglyceride levels (Pratchayasakul *et al.* 2011, 2014). However, the excessive fat intake usually does lead to an accumulation of triglyceride (TG) in the liver and adipose tissues. This is due to the fact that it might take time to release (TG) from the liver and adipose tissue into the blood stream. Therefore, a longer duration of HFD feeding may be needed to increase plasma triglyceride levels as shown in previous reports (Manco *et al.* 2004, Gauthier *et al.* 2006, Hwang *et al.* 2010, Pratchayasakul *et al.* 2011, 2014).

In our obese-insulin-resistant rats, plasma glucose and triglyceride levels were not altered, compared to their baseline levels. This suggested that our obese-insulin-resistant rats had hyperinsulinemia with euglycemia and normotriglyceridemia. Treatment with vildagliptin and caloric restriction did not affect plasma glucose and triglyceride levels. However, these interventions reduced plasma cholesterol and low-density lipoprotein (LDL) levels in obese-insulin-resistant rats. As the plasma levels

of glucose and TG were not increased, both interventions did not interrupt the metabolism of glucose and TG at their physiological levels. Consistent with our previous reports in the obese-insulin-resistant model, our high-fat fed rats were considered as pre-diabetic state rats (Pratchayasakul *et al.* 2011, Pongkan *et al.* 2016).

Regarding the effect of DDP4-inhibitor vildagliptin on FBG, reports from our group and others demonstrated that vildagliptin acted as a glycemic control agent without having a hypoglycemic effect (Yin *et al.* 2011, Maeda *et al.* 2012, Apaijai *et al.* 2014). This is due to the fact that vildagliptin reduced glucagon levels during hyperglycemia and at the same time did not inhibit any counter-regulatory glucagon response during hypoglycemia (Farngren *et al.* 2012). Moreover, vildagliptin also enhanced glucose sensitivity in α -cells by promoting the direct effect of low blood glucose levels to stimulate glucagon secretion (Ahren *et al.* 2011, Farngren *et al.* 2012). In addition, the increase of glucose-dependent insulinotropic polypeptide (GIP) was observed by vildagliptin therapy (Ahren *et al.* 2004, Mari *et al.* 2005, Farngren *et al.* 2012), thus stimulating glucagon secretion during low blood glucose levels (Meier *et al.* 2003, Christensen *et al.* 2011). Consistent with previous studies, our results showed that vildagliptin improved insulin sensitivity leading to reduced plasma insulin without affecting plasma glucose levels in obese-insulin-resistant rats (Apaijai *et al.* 2014, Pongkan *et al.* 2016).

Our previous studies have shown that long-term HFD consumption led to obese-insulin resistance with impaired cardiac sympathovagal balance and cardiac dysfunction (Apaijai *et al.* 2013, 2014). Previously, we proposed various mechanisms as being responsible for these impairments, such as metabolic disturbance, oxidative stress and cardiac mitochondrial dysfunction. In this study, we demonstrated that the HFD-fed rats had FGF21 resistance and impaired cardiac metabolism. FGF21 resistance was observed in our obese-insulin-resistant rats and is associated with a reduction in FGF receptor function, as FGF receptor expression and β -Klotho expression were unaffected in our model. However, there are several discrepancies regarding the mechanism responsible for FGF21 resistance. Fisher and coworkers reported that mice fed with a high-fat/high-sucrose diet for 22 weeks had FGF21 resistance and found that FGF21 receptor mRNA expression was reduced, whereas β -Klotho mRNA expression was unchanged in the liver of obese mice, compared to lean mice. However

unlike in the liver, both FGF21 receptor and β -Klotho mRNA expression were reduced in white adipose tissue of obese mice, compared to lean mice (Fisher *et al.* 2010). In addition, our study on the HFD-induced obese-insulin-resistant rat model demonstrated that HFD consumption did not alter FGF21 receptor protein expression in the heart (Tanajak *et al.* 2016). These findings suggest that the mechanism of FGF21 resistance is specific to the organ. However, Patel and coworkers reported that 12 weeks of HFD consumption reduced β -Klotho protein expression in the heart (Patel *et al.* 2014). Although they found that β -Klotho protein expression in the heart was decreased, the p-FGFR1 protein expression had not been investigated. Therefore, the role of HFD consumption of FGF21 receptors complex expression and function in the heart are still controversial. Future studies are needed to investigate the role of HFD consumption in FGF21 resistance, particularly with the changes of FGF21 receptor complex expression (β -Klotho, FGFR1 and p-FGFR1) on the heart at different time points. In this model, however, the expression of β -klotho, the FGFR1 co-receptor, was unchanged. The decrease in FGF21 receptor complex function could lead to the impairment of the downstream signaling molecules, such as reduced ERK1/2 phosphorylation. Furthermore, we also found that the utilization of fatty acids was reduced in the HFD-fed rats as indicated by a reduction in PGC-1 α and CPT1 expression. Therefore, the FGF21 resistance and the alteration of cardiac metabolism could also contribute to cardiac dysfunction in our obese-insulin-resistant rats.

Besides ERK1/2, FGF21 receptor activation also regulates Akt, AMPK and adiponectin, which are known to determine cardiac metabolism. However, the definite mechanisms are still controversy. In cardiac ischemia-reperfusion injury model, it has been shown that FGF21 administration exerted cardioprotection via the activation of Akt and AMPK (Patel *et al.* 2014). However, Xu and coworkers demonstrated that FGF21 therapy did not alter Akt in the insulin-resistant C57BL6 mice heart (Xu *et al.* 2009). However, our results showed that there was no difference in the p-Akt/t-Akt ratio between all groups. This could be due to the use of different models and drugs in these studies. Regarding adiponectin, Joki and coworkers demonstrated that activation of FGF21 receptors increased plasma adiponectin, which contributed to reduced cardiac inflammation and exerted cardioprotection against cardiac remodeling in the myocardial infarction mice model (Joki *et al.* 2015).

Future studies are needed to investigate the roles of FGF21 receptor activation on AMPK and adiponectin in obese-insulin-resistant condition.

In this study, although all interventions effectively reduced insulin resistance, only CR led to reduced body weight gain in obese-insulin-resistant rats. This is due to a reduction of caloric intake, which led to reduced visceral fat deposition. Our findings are consistent with previous studies, which also showed that CR reduced body weight gain in various animal models (Wilsey & Scarpace 2004, Park *et al.* 2006, Niemann *et al.* 2010, Takatsu *et al.* 2013), whereas vildagliptin improved insulin resistance without any changes in body weight (Pipatpiboon *et al.* 2013, Apaijai *et al.* 2014). In addition, there is growing evidence that cardiac mitochondrial dysfunction impairs cardiac function and cardiac autonomic function. It has been reported that there is a relationship between cardiac mitochondrial dysfunction, cardiac dysfunction and cardiac autonomic imbalance in diabetic patients (Momiya *et al.* 2002). In this study, vildagliptin led to greater improvement in cardiac mitochondrial function than CR in obese-insulin-resistant rats. Although both vildagliptin and CR have similar effects on oxidative stress, including on levels of MDA and mitochondrial ROS, vildagliptin attenuated cardiac mitochondrial membrane depolarization and mitochondrial swelling more effectively than CR. These findings indicate that vildagliptin exerted greater cardioprotection by protecting mitochondrial dysfunction more effectively than CR in obese-insulin-resistant rats.

Moreover, it has been shown that mitochondria could regulate the apoptotic process. In this study, we found that vildagliptin appeared to reduce cardiac apoptosis more effectively than CR in the obese-insulin-resistant model as shown by greater reduction of Bax and cleaved caspase-3 expression and higher BCL-2 expression. This in turn would have then caused reduced Bax translocation to the mitochondrial outer membrane. Previous reports have shown that Bax translocation after stress stimuli caused mitochondrial membrane depolarization (Smaili *et al.* 2001). Furthermore, Bax binds with voltage-dependent anion channels (VDAC), which is followed by conformational change of the mitochondrial permeability transition pores (mPTP). This process allows proton and water influx across the mitochondrial membrane into the mitochondrial matrix, leading to mitochondrial swelling. These mechanisms could be responsible for the superior effects of vildagliptin to CR on cardiac function and cardiac autonomic function in obese-insulin-resistant rats.

In this study, we also demonstrated that chronic HFD consumption is related to FGF21 resistance. FGF21 has been shown to regulate cardiac function in various models (Cong *et al.* 2013, Planavila *et al.* 2013, 2014, Tanajak *et al.* 2015, Yan *et al.* 2015). Our results showed that both CR and vildagliptin improved FGF21 resistance by improving FGF21 receptor function and its downstream signaling. These beneficial effects could also contribute to improved LV function in obese-insulin-resistant rats observed in this study. Although the vildagliptin-treated group demonstrated greater efficacy than caloric restriction in improving anti-apoptotic pathways and attenuating cardiac mitochondrial dysfunction, the combination of vildagliptin and caloric restriction did not provide an additional effect. It is possible that vildagliptin therapy already had a superior effect to caloric restriction as our results showed that vildagliptin could reverse the metabolic and cardiac adverse effects caused by long-term HFD consumption back to the normal physiological levels, whereas this restoration was not observed in the caloric restriction group.

In conclusion, chronic HFD consumption leads to obese-insulin resistance and FGF21 resistance. Although CR is effective in improving metabolic regulation and FGF21 sensitivity, vildagliptin provides greater efficacy in preventing cardiac dysfunction by improving anti-apoptotic pathways and attenuating cardiac mitochondrial dysfunction in obese-insulin-resistant rats.

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.

Funding

This work was supported by a NSTDA Research Chair Grant from the National Science and Technology Development Agency Thailand (NC), the Thailand Research Fund Royal Golden Jubilee Program (PHD/0078/2557 PT&SC), BRG5780016 (SC) and Chiang Mai University Center of Excellence Award (NC).

Author contribution statement

S C and N C conceived and designed the experiments. P T and H P conducted the experiments. P T, H P, N S, J K, N A, S C and N C analyzed the data. P T, N A, S C and N C wrote the manuscript.

Acknowledgements

The authors would like to thank Piangkwan Sa-nguanmoo for her technical assistance on metabolic parameter assessment and Cicely Proctor for her editorial assistance of this paper.

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Received in final form 19 October 2016

Accepted 13 November 2016

Accepted Preprint published online 14 November 2016