RESEARCH

N-acetylcysteine with low-dose estrogen reduces cardiac ischemia-reperfusion injury

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Abstract

Myocardial damage and mitochondrial dysfunction caused by cardiac ischemia-reperfusion (I/R) injury are intensified by endogenous estrogen deprivation. Although *N*-acetylcysteine (NAC) exerted cardioprotective effects, its benefits when used in combination with hormone therapy are unknown. We tested the hypothesis that a combination of NAC with low-dose estrogen improves cardiometabolic function and protects cardiac mitochondria against I/R injury, to a similar extent to regular-dose estrogen treatment, in estrogen-deprived rats. Female Wistar rats had a bilateral ovariectomy (OVX) or sham operation. Twelve weeks after the operation, OVX rats were treated with regular-dose estrogen (E; 50 µg/kg/day), low-dose estrogen (e; 25 µg/kg/day), NAC (N; 100 mg/kg/day) or combined low-dose estradiol with NAC (eN) for 4 weeks (*n* = 6/group). Metabolic parameters, echocardiography, heart rate variability and then cardiac I/R protocol involving 30-min coronary artery ligation, followed by 120-min reperfusion, were performed. OVX rats had increased body weight, visceral fat, fasting plasma glucose, HOMA-IR index, triglycerides, cholesterol and LDL levels (*P* < 0.05 vs sham). Only OVX-E and OVX-eN had a similarly improved HOMA-IR index. LVEF was increased in all treatment groups, but HRV was restored only by OVX-E and OVX-eN. After I/R, myocardial infarct size was decreased in both OVX-E and OVX-eN groups. OVX-E and OVX-eN rats similarly had a reduced mitochondrial ROS level and increased mitochondrial membrane potential in the ischemic myocardium. In conclusion, combined NAC with low-dose estrogen and regular-dose estrogen therapy similarly improve cardiometabolic function, prevent cardiac mitochondrial dysfunction and reduces the infarct size in estrogen-deprived rats with cardiac I/R injury.

Key Words

- ▶ *N*-acetylcysteine
- ▶ estrogen
- ▶ ischemia-reperfusion injury
- ▶ cardiac mitochondria

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Introduction

Women have been shown to have a lower incidence of cardiovascular disease (CVD) than men at similar age during the active reproductive period. However, the rate is increasing following menopause (Vitale *et al.* 2009).

In women estrogen deprivation by bilateral ovariectomy (OVX) caused an increase in mortality from CVD, while estrogen could reduce the risk, indicating its beneficial effects on the heart (Rivera *et al.* 2009). Also, endogenous

estrogen deficiency resulted in more severe cardiac tissue damage and left ventricular dysfunction after myocardial ischemia and reperfusion (I/R) (Zhai *et al.* 2000). In estrogen-deprived animals undergoing cardiac I/R, cardiac mitochondria were more damaged leading to impaired mitochondrial function (Sivasinprasasn *et al.* 2017). Furthermore, women also have an increased prevalence of metabolic syndrome including dyslipidemia, insulin resistance, visceral fat deposition and central obesity following menopause. Menopause also associates with an increase in total cholesterol, LDL cholesterol, triglycerides and systolic blood pressure which also increase cardiovascular risks which are the leading cause of mortality in women in the western world and many developing countries (Bonithon-Kopp *et al.* 1990).

Estrogen replacement has been widely used in the treatment of menopausal-related symptoms. Estrogen treatments, both in acute and chronic conditions, exhibited cardioprotective effects in ovariectomized animals with cardiac I/R injury by improving cardiac functional recovery, restore mitochondrial function and reducing both myocardial infarct size and reactive oxygen species (ROS) production (Fukumoto *et al.* 2013). Estrogen also helped to improve metabolic parameters in metabolic syndrome, in particular, lipid profiles and insulin sensitivity, in ovariectomized female rats (Xu *et al.* 2012). However, estrogen replacement associated with an increased risk of venous thromboembolism and reproductive cancers (Stuenkel *et al.* 2015). Therefore, alternative remedies are needed to subrogate estrogen in menopausal women who are contraindicated to hormone therapy. Menopausal women are recommended to take estrogen supplements for short duration and at low doses to avoid side effects. Low-dose estrogen supplements are reported as effectively relieving vasomotor and urogenital symptoms (Crandall 2003), but it might be insufficient for preventing other deteriorations such as cardiometabolic impairment and osteoporosis. Evidence shows that antioxidants exert cardioprotective benefits (Bartekova *et al.* 2018). It is therefore of interest to investigate the additional effect of an antioxidant, added to low-dose estrogen treatment, in the management of menopausal-related cardio-metabolism.

N-acetylcysteine (NAC) is a cysteine pro-drug and glutathione precursor, which acts as an anti-oxidant via replenishing cellular cysteine levels to maintain intracellular glutathione and scavenging free radicals (Zhang *et al.* 2011). NAC has been widely used in clinical practice as a mucolytic agent and for the treatment of paracetamol intoxication. Treatment with NAC exhibited

protective effects on cardiomyocytes in hyperglycemia conditions and reduced cardiomyocyte apoptosis (Kumar & Sitasawad 2009). Under cardiac I/R, NAC reduced inflammation and oxidative stress level in the myocardium, thus attenuating the severity of I/R injury in patients undergoing coronary artery bypass grafting surgery (Orhan *et al.* 2006). In rat hearts, treatment with NAC also inhibited sympathetic hyperactivity after coronary artery occlusion, resulting in decreased post-infarcted arrhythmias (Lee *et al.* 2010). Despite these benefits, the effects of NAC on cardiac function under conditions of estrogen-deprivation with or without cardiac I/R injury are still unknown.

In this study, the therapeutic efficacy of estrogen, NAC and combined low-dose estrogen with NAC on cardiometabolic status were investigated under basal and cardiac I/R conditions in female rats with estrogen deprivation. We hypothesized that a combination of low-dose estrogen with NAC exerts cardiometabolic benefits similar to that of regular-dose estrogen treatment in estrogen-deprived female rats.

Materials and methods

Animal preparation and ethical approval

Female Wistar rats (8 weeks of age, weighing 200–220 g) were obtained from Nomura Siam International Co, Ltd. (Bangkok, Thailand). The rats were fed on a standard diet (containing 19.77% energy from fat) with food and drinking water *ad libitum*. Rats were housed in a temperature-controlled room (25°C) with a 12-hour darkness/light cycle setting throughout the study. The experimental protocol was ethically considered and approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University, in compliance with NIH and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Experimental protocol

The rats were randomized into a sham (Sham) or ovariectomized (OVX) group. Twelve weeks after surgery, OVX rats were divided randomly and treated with one of the following interventions ($n=12$ /group) for 4 weeks: regular-dose estrogen replacement (E: estradiol 50 µg/kg/day via subcutaneous injection); low-dose estrogen (e: estradiol 25 µg/kg/day via subcutaneous injection); NAC (N: NAC 100 mg/kg/day via gavage feeding); combined low-dose E2

with NAC (eN: estradiol 25 µg/kg/day combined with NAC 100mg/kg/day) or vehicle (subcutaneous injection with sesame oil, equal volume as in the regular-dose estrogen treatment).

Estrogen treatment was prepared using estradiol powder (Sigma-Aldrich Co.) dissolved in ethanol and then mixed with sesame oil (Sigma-Aldrich Co.). The regular dose of estradiol used in this study was previously demonstrated to improve cardiometabolic status in ovariectomized female rats (Sivasinprasasn *et al.* 2016, 2017). The NAC solution was prepared by mixing NAC powder with normal saline (NSS) using the concentration of NAC 40 mg per 200 µL of NSS. Then, oral NAC treatment was administrated via gavage which contained NAC solution in the total volume at 14–20 µL per day according to the body weight of each rat. The oral gavage was used to assure that each rat received the exact amount of NAC it needed each day. At the end of the 4-week treatment, echocardiography and heart rate variability (HRV) were assessed. Fasted blood samples were collected from the tail vein and were centrifuged for plasma preparation. Then, the *in vivo* cardiac I/R procedure was performed and the heart was investigated for cardiac mitochondrial function, biochemical activities and myocardial infarction.

Ovariectomy

Rats were anesthetized with Isoflurane (Forane, 2–4% delivered with oxygen). Bilateral ovariectomy was done by removing both ovaries, and uterine horns were returned into the abdominal cavity. Tolfenamic acid (Tolfedine, 40 mg/kg) and marbofloxacin (Marbocyl, 2 mg/kg) were used as analgesic and antibiotics agents via subcutaneous injection (Sivasinprasasn *et al.* 2015). After the operation for 1 week, rats were randomized into the experimental groups.

Determination of metabolic parameters and hormone levels

Fasting plasma insulin level was detected using a sandwich ELISA kit (Millipore). Fasting plasma glucose and triglyceride levels were determined by a colorimetric assay from a commercially available kit (Biotech, Bangkok, Thailand). Fasting plasma HDL was defined using commercial kits (ERBA diagnostic, Mannheim, Germany). LDL level was calculated using Friedewald's formula and HOMA-IR index was calculated from fasting

insulin and glucose levels plasma (Matthews *et al.* 1985). Plasma estradiol concentration was measured using a competitive enzyme immunoassay kit (Cayman Chemical Company).

Echocardiography protocol

Rats were lightly anesthetized (2% isoflurane with oxygen, via inhalation). An echocardiograph (SONOS4500, Philips with S12 probe, Hewlett Packard) was used for collecting the data. Signals from pulsed-wave Doppler and M-mode echocardiography at the papillary muscle level were recorded (Sivasinprasasn *et al.* 2017).

Electrocardiography (ECG) and heart rate variability (HRV) protocol

Rats were anesthetized by isoflurane inhalation, and the needle electrodes were subcutaneously inserted into the right arm and left leg of the rat for lead II electrocardiograms (ECG) recording. The ECG recording was operated through a signal transducer using Chart 5.0 program (PowerLab 4/25T transducer, ADInstrument). The rat was then fully conscious in a restrainer during the ECG recording for 20 consecutive minutes. HRV is an assessment of cardiac autonomic innervation activity. The ECG data were analyzed for HRV parameters using the frequency-domain method of MATLAB program. The basal heart rate, high-frequency (HF) component and low-frequency (LF) component were obtained. Increased LF/HF ratio was used as an indication of cardiac sympathovagal function (Incharoen *et al.* 2007).

Cardiac ischemia/reperfusion (I/R) protocol

The rats were anesthetized by intramuscular injection with Zoletil (50 mg/kg, Vibbac Laboratories, Carros, France) and Xylazine (0.15 mg/kg, Laboratories Carlier, SA, Barcelona, Spain) and were ventilated with room air via tracheostomy with a ventilator (Harvard rodent ventilator model 683, Harvard apparatus, MA, USA). Lead II ECG was recorded. After the heart was exposed, left anterior descending coronary artery (LAD) was ligated for 30 min. Myocardial ischemia was indicated by the presence of ST elevation on ECG. Cardiac ischemia was followed by 120 min of reperfusion. The time to 1st VT/VF onset and the mortality rate were recorded. Arrhythmia score was evaluated (Sivasinprasasn *et al.* 2017).

Pressure-volume (P-V) loop study for LV function during cardiac I/R

A pressure-volume (P-V) loop catheter (Scisence, Ontario, Canada) was inserted through the right carotid artery at before the cardiac I/R protocol. The catheter tip was placed in the LV chamber to record the LV pressure and LV volume. After allowing 5 min for stabilization, the cardiac I/R protocol was performed. All P-V loop parameters were recorded and analyzed using the Labscribe analytical software (Labscribe, Dover, NH, USA).

Determination of myocardial infarct size

After the cardiac I/R experiment, the heart was removed, and a modified Langendorff apparatus was inserted into the aorta. The heart was dyed with Evan blue dye and triphenyltetrazolium chloride (TTC). The myocardial area without blue-dyed color was defined as the area at risk (AAR). The viable tissue was in red, and the area which exhibited neither blue nor red was identified as the myocardial infarction (Sivasinprasasn *et al.* 2017). Myocardial infarction was determined using the Image tool software version 3.0. The infarct size was calculated using the weight of each myocardium slice according to Reiss *et al.*'s formula and was reported as %infarct/AAR (Riess *et al.* 2009).

Cardiac mitochondrial function study

Cardiac tissues from the non-ischemic (remote area, R) and ischemic areas (I) were separately homogenized and centrifuged to isolate the cardiac mitochondria for investigation of mitochondrial ROS level, mitochondrial membrane potential change and mitochondrial swelling.

Dichlorohydrofluorescein diacetate (DCFDA) dye was used for mitochondrial ROS level measurement using a fluorescent microplate reader (BioTek, Winooski, VT, USA). Membrane potential change ($\Delta\Psi$) of the isolated cardiac mitochondria was detected using JC-1 dye. A decreased ratio of red/green fluorescence intensity indicates mitochondrial membrane depolarization (Thummasorn *et al.* 2011). Mitochondrial absorbance intensity was detected using a spectrophotometer at 540 nm, and the swelling of mitochondria was indicated by a decrease in the absorbance of a mitochondrial suspension. Cardiac mitochondrial morphology was determined at 15,000 $\times$ using a transmission electron microscope (TEM) (JEOL JEM-1200 EX II, Peabody, MA, USA) (Sivasinprasasn *et al.* 2017).

Determination of myocardial oxidative stress

Oxidative stress levels in cardiac issue were indicated by increased malondialdehyde (MDA) level. Proteins from ischemic and remote areas were determined the MDA concentration measured by using a high-performance liquid chromatography (HPLC) system (Thermo Scientific). The cardiac MDA concentration was reported as I/R ratio (Sivasinprasasn *et al.* 2017).

Cardiac expression of Bax, Bcl-2, phospho-Connexin 43 and Connexin 43

Protein expression levels were determined by western blot analysis. The myocardial tissues from I and R areas were separately homogenized in a lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS). The prepared protein was loaded into a 10% SDS-polyacrylamide gel and then transferred to a PVDF membrane in a transfer system (Bio-Rad). The protein-containing membranes were exposed to anti-Bax, anti-Bcl-2, anti-phospho-Cn43, anti-total-Cn43, and anti-GAPDH. Horseradish peroxidase-conjugated with anti-rabbit or anti-mouse IgG was administered to induce a peroxidase reaction, which further developed the signal by enhanced chemiluminescence (ECL) detection reagents. Finally, the protein band density was measured using the ImageJ analysis program (NIH image) (Apaijai *et al.* 2014).

Chemicals and antibodies

Estradiol, NAC and sesame oil (vehicle) were obtained from Sigma-Aldrich. The EIA kit for serum estradiol level detection was from Cayman Chemical Company. Investigation of plasma glucose, cholesterol, and triglycerides levels was performed using colorimetric assay kits from Biotech (Bangkok, Thailand). ELISA kit for the measurement of plasma insulin level was from Millipore. The antibodies used against Bax, Bcl-2, and p-Cx43 were from Cell Signaling Technology, total-Cx43 was from Santa Cruz Biotechnology (Santa Cruz) and the anti-GAPDH was from Sigma-Aldrich. The horseradish peroxidase conjugated with anti-rabbit and anti-mouse IgG was from Santa Cruz Biotechnology (Santa Cruz).

Statistical analysis

All data are presented as mean $\pm$ standard error of mean (S.E.M). A one-way ANOVA followed by *post hoc* Fisher's LSD test was performed using GraphPad Prism software

(GraphPad Prism version 7, GraphPad Software) to determine the differences between the means. A *P* value of less than 0.05 is considered statistically significant.

Results

Effect of interventions on metabolic status

Removal of bilateral ovaries induced a significant decrease in both serum estradiol level and uterus weight in OVX rats ($P < 0.05$ vs sham; Table 1). Only OVX rat had significantly increased visceral fat ($P = 0.04$ vs sham). The uterus weight decreased in all OVX rats when compared with sham ($P < 0.05$). In the estrogen-treated group, uterus weight increased in OVX-E, OVX-e and OVX-eN ($P < 0.05$ vs OVX). However, the uterus weight was lower in OVX-e and OVX-eN, when compared with those of OVX-E ($P < 0.05$ vs OVX-E).

Plasma lipid levels including cholesterol, triglyceride and LDL were increased, whereas plasma HDL was decreased, in OVX rats. OVX rats also had elevated fasting plasma glucose, fasting plasma insulin and demonstrated the higher HOMA-IR index when compared with sham ($P < 0.05$). In the treatment groups, treatment with regular-dose estrogen (OVX-E) increased serum estradiol level compared to OVX ($P < 0.05$), and the level was higher than that in rats treated with low-dose estrogen ($P < 0.05$ vs OVX-e and OVX-eN). There was no difference in the serum estradiol level between sham and OVX-E. The low-dose estrogen treatment both in the monotherapy group (OVX-e) and in the combined treatment with NAC (OVX-eN) increased the level of serum estradiol when compared with the vehicle group ($P < 0.05$). OVX-OVX-N and OVX-e groups also had higher levels of fasting plasma glucose, insulin, and HOMA index ($P < 0.05$ vs sham), whereas these parameters were significantly reduced in E and eN groups. OVX-E, OVX-N and OVX-eN groups showed significantly reduced levels of fasting plasma cholesterol and LDL ($P < 0.05$ vs OVX); however, OVX-e did not show significant changes in those parameters. Plasma triglyceride levels were reduced in the OVX-N group when compared with OVX ($P < 0.05$). The level of food intake was no different among all groups.

Effect of interventions on cardiac function and heart rate variability in the basal state

Cardiac LV performance was impaired in OVX rats as indicated by the reduced level of %LVEF ($P < 0.05$ vs sham;

Fig. 1B). The level of %LVEF was increased in all treatment groups ($P < 0.001$ vs OVX). The %LVEF level of OVX-E and OVX-eN was not significantly different from those of sham ($P > 0.05$), whereas the %LVEF in OVX-e and OVX-N was lower than that in the sham group (Fig. 1B).

The LV internal diameter in systole (LVIDs) significantly increased in OVX, OVX-e, and OVX-N suggested the increased LV dimension when compared with sham ($P < 0.05$; Fig. 1C). The dilated LVIDs were reduced in OVX-E and OVX-eN ($P < 0.05$ vs OVX). Left ventricular posterior wall thickness in systole (LVPWs) was significantly decreased in OVX when compared with sham ($P < 0.05$; Fig. 1D). However, the LVPWs was increased in all treatment groups ($P < 0.05$ vs OVX).

Mitral valve (MV) E/A ratio was reduced and MV deceleration time was increased in OVX ($P < 0.05$ vs sham). These diastolic function parameters were significantly improved in OVX-E, OVX-e and OVX-eN groups ($P < 0.05$ vs OVX; Fig. 1F and G), and these increased levels of MV E/A ratio and MV Dec time were not different with those of the sham group (Fig. 1F and G). However, these diastolic parameters were not restored in the OVX-N group (Fig. 1F and G).

Regarding cardiac sympathovagal balance, OVX rats exhibited HRV depression as indicated by an increased ratio of LF/HF when compared with sham ($P < 0.0001$; Fig. 2A). Only OVX-E and OVX-eN had significantly restored LF/HF ratios to similar levels of sham ($P > 0.05$ vs sham; Fig. 2A). The resting heart rate showed no significant difference between groups (Fig. 2B).

Effect of interventions on the heart during cardiac I/R injury

Effect of cardiac I/R injury and the efficacy of the treatments on cardiac function

The data from the P-V loop study recorded during cardiac I/R injury are in Fig. 3. At baseline, OVX rats showed a significant decrease in the stroke volume (SV), left ventricular ejection fraction (%LVEF), and an increase in the end-diastolic pressure (LVEDP) when compared with the sham group ($P < 0.05$ vs sham; Fig. 3A, B and D). The SV level was increased only in OVX-E, whereas the %LVEF was increased in both OVX-E and OVX-eN when compared with OVX ($P < 0.05$ vs OVX). These results indicate that at the basal condition the combined treatment of low-dose estrogen with N shared similar efficacy as E in improving LV function.

During the cardiac ischemic period, SV and %LVEF were reduced in all groups ($P < 0.05$ vs baseline; Fig. 3A and

Table 1 Metabolic parameters in experimental groups after 4 weeks of treatments.

Parameters	Sham			OVX			
	Vehicle	E	e	N	eN		
Body weight (g)	342 ± 7*	308 ± 10†	303 ± 11†	335 ± 7*	305 ± 8†		
Visceral fat/body weight	0.037 ± 0.002*	0.028 ± 0.002	0.031 ± 0.003	0.034 ± 0.003	0.033 ± 0.001		
Uterus weight/body weight (×10 ⁻⁴)	2.6 ± 0.4*	12 ± 1*†	6.2 ± 0.4*†,‡	2.3 ± 0.1*†	8.3 ± 0.4*†,‡		
Fasting plasma glucose (mg/dL)	172 ± 6*	137 ± 6†	153 ± 14*	156 ± 10*	131 ± 9†		
Fasting plasma insulin (ng/mL)	3.8 ± 0.5*	2.0 ± 0.4†	4.0 ± 0.8*	3.7 ± 0.7*	2.1 ± 0.2†		
HOMA index	15.5 ± 2	18.3 ± 4†	41.1 ± 7*	34.6 ± 7*	26.9 ± 7		
Fasting plasma cholesterol (mg/dL)	71 ± 5	71 ± 3†	75 ± 2	64 ± 5†	65 ± 2†		
Fasting plasma HDL (mg/dL)	26 ± 1	25 ± 1	24 ± 1	24 ± 1	24 ± 1		
Fasting plasma LDL (mg/dL)	50 ± 3	52 ± 2†	58 ± 3	48 ± 4†	48 ± 1†		
Fasting plasma triglyceride (mg/dL)	42 ± 2	50 ± 4	55 ± 1	43 ± 3†	52 ± 6		
Serum estradiol level (pg/mL)	76 ± 13	94 ± 21†	49 ± 10†,‡	12 ± 3*†	51 ± 14†,‡		
Food intake per day (g/BW (g)	0.047 ± 0.003	0.044 ± 0.002	0.045 ± 0.003	0.040 ± 0.001	0.049 ± 0.005		

Values are mean ± S.E.M.

**P* < 0.05 vs sham; †*P* < 0.05 vs OVX; ‡*P* < 0.05 vs OVX-E.

BW, body weight; e, low-dose estrogen; E, regular-dose estrogen; eN, combined low-dose estrogen with NAC; HOMA, Homeostasis Model Assessment; N, NAC; OVX, ovariectomized rats; Sham, sham-operated rats.

B). Furthermore, OVX rats showed a significant reduction in LVESP (Fig. 3C) and had significantly increased the level of LVEDP (Fig. 3D), when compared with those at their baseline level. In comparison with OVX, only OVX-E had a significantly increased SV during ischemia (Fig. 3A). Both OVX-E and OVX-eN demonstrated an increased level of %LVEF (*P* < 0.05 vs OVX; pink line and dashed line, respectively, in Fig. 3B). All treatments significantly decreased LVEDP and only OVX-E further increased dP/dtMax, when compared with OVX (Fig. 3D and E). At the end of reperfusion, only the E treatment had significantly increased levels of SV and LVESP, together with a significant reduction in LVEDP, when compared with OVX.

Effect of cardiac I/R and efficacy of the treatments on cardiac arrhythmias and mortality

A significantly increased arrhythmia score was observed in the OVX group (*P* = 0.001 vs sham and all treatment groups; Fig. 4A), which was consistent with the shortened time to 1st VT/VF (*P* = 0.003 vs sham; Fig. 4B), and decreased level of p-Cx43 expression in the ischemic area of myocardium (*P* = 0.01 vs sham; Fig. 4C). The changes in these parameters regarding increased susceptibility to arrhythmia during I/R was not found in OVX-E and OVX-eN rats. The time to 1st VT/VF was significantly prolonged in OVX-E and OVX-eN when compared with OVX rats (*P* < 0.05 vs OVX; Fig. 4B). The levels of p-Cx43 were not significantly different when compared to sham, OVX-E and OVX-eN, whereas they were decreased in OVX-e and OVX-N groups (*P* < 0.05 vs sham; Fig. 4C). The percentage of mortality during cardiac I/R in each treatment group is in Fig. 4D, which indicates a significantly increased mortality rate in the OVX group (*P* = 0.009 vs sham). This percentage of mortality was reduced by the E-, e- and eN treatments (*P* < 0.05 vs OVX).

Level of myocardial infarction and the expression of apoptotic and anti-apoptotic proteins after cardiac I/R

After cardiac I/R, the level of AAR was no different in all experimental groups (data not shown). The hearts from the OVX group had an increased percentage of myocardial infarct size per AAR when compared with sham (*P* = 0.001; Fig. 4A). OVX hearts had a significantly elevated level of MDA in ischemic tissues (*P* = 0.01 vs sham; Fig. 5B), together with a significant reduction of the protein Bcl-2 and an increase in protein Bax in ischemic tissues (*P* = 0.01 vs sham; Fig. 5C and D).

Both E and eN treatments similarly decreased the level of myocardial infarct size (*P* < 0.05 vs OVX; Fig. 5A),

Figure 1

Effect of estrogen deprivation and the treatments on cardiac systolic and diastolic function. (A) M-mode of echocardiography in parasternal short axis view; (B) left ventricular ejection fraction (%LVEF); (C) left ventricular internal diameter in systole (LVIDs); (D) left ventricular posterior wall thickness in systole (LVPWs); (E) pulsed-wave Doppler shows mitral inflow in apical four-chamber view; (F) mitral valve E/A ratio and (G) mitral valve deceleration time in sham rats (sham), ovariectomized (OVX) rats and OVX rats treated with normal-dose estrogen (OVX-E), low-dose estrogen (OVX-e), NAC (OVX-N) or combined low-dose estrogen with NAC (OVX-eN). Values are mean $\pm$ s.e.m. for six rats per group. * $P < 0.05$ vs sham and † $P < 0.05$ vs OVX. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0108>.

lowered the MDA level ($P < 0.05$ vs OVX; Fig. 5B) and had preserved levels of Bcl-2 expression in the ischemic areas of the hearts (Fig. 5C). Treatment with E and eN also decreased the level of apoptotic protein Bax when compared with vehicle-treated OVX ($P < 0.05$ vs OVX; Fig. 5D).

Effect of cardiac I/R and efficacy of the treatments on the cardiac mitochondrial status

Data from the cardiac mitochondrial study is in Fig. 6. Cardiac mitochondria from OVX hearts showed a

significant increase in ROS level (Fig. 6A), and a decrease in both $\Delta\Psi$ and absorbance intensity when compared with those from sham (Fig. 6B and C). Only OVX-E and OVX-eN had significant reduced cardiac mitochondrial ROS levels, which is consistent with the significant improvement in mitochondrial membrane potential change ($\Delta\Psi$; Fig. 6B). There was only a significant increase in mitochondrial absorbance intensity in the OVX-E group when compared with vehicle-treated rats (Fig. 6C). The morphology of cardiac mitochondria from the ischemic area of all treatment groups is shown in Fig. 6D.

Figure 2

Effect of estrogen deprivation and the treatments on heart rate variability. (A) LF/HF ratio and (B) resting heart rate in sham rats (sham), ovariectomized (OVX) rats and OVX rats treated with normal-dose estrogen (OVX-E), low-dose estrogen (OVX-e), NAC (OVX-N) or combined low-dose estrogen with NAC (OVX-eN). LF is low-frequency power (LF; 0.15–1.5 Hz, in ms²), and HF is high-frequency power (HF; 1.5–5 Hz, in ms²). Values are mean $\pm$ s.e.m. for six rats per group. * $P < 0.05$ vs sham, and † $P < 0.05$ vs OVX.

Discussion

The adverse effect of endogenous estrogen deprivation and efficacy of the treatments on cardiometabolic function in the basal state and in the cardiac I/R condition have been demonstrated in this study. The major findings are summarized as (1) endogenous estrogen deprivation induced metabolic disturbance, cardiac autonomic imbalance and impaired cardiac systolic and diastolic function in the basal state; (2) under conditions of cardiac I/R estrogen deprivation aggravated impairment of the LV performance, increased arrhythmia, increased infarct size, and impaired cardiac mitochondrial function; (3) treatment with low-dose estrogen alone or oral NAC alone was insufficient to improve cardiometabolic function and was inadequate in protecting the heart against cardiac I/R injury and (4) treatment with combined low-dose estrogen with NAC exhibited the therapeutic potentials similar to those of regular-dose estrogen, however, regular-dose estrogen still showed superior potential in improving metabolic status, preserving cardiac performance

during ischemia, and preventing cardiac mitochondrial swelling after I/R injury.

Endogenous estrogen deprivation, from both ovariectomy and natural regression, has been reported as causing metabolic function impairment and increasing the risk of developing metabolic syndrome (Ozdemir *et al.* 2009). Our findings also indicate a loss of estrogen-induced metabolic disturbance without a significant change in the amount of food intake. A previous study similarly demonstrated that the estrogen loss did not alter the level of energy intake; however, it was associated with a decrease in ambulatory activity and energy expenditure, resulting in adipocyte hypertrophy and visceral fat accumulation. Adipose tissue inflammation and hepatic steatosis were also aggravated by estrogen deprivation. Moreover, tissue insulin sensitivity could be diminished by estrogen deprivation since the level of glucose tolerance, and hepatic beta cell function was lowered following OVX treatment (Vogel *et al.* 2013).

A previous study demonstrated that 11-week treatment with NAC (2 g/L of drinking water) in high-fat-diet-induced obese male mice effectively suppressed the metabolic disorders, as indicated by reduction of adipose tissue accumulation, lowered plasma lipid levels, and also inhibition of hyperglycemia and hyperinsulinemia (Ma *et al.* 2016). However, in this study, treatment with either oral NAC alone or low-dose estrogen alone for 4 weeks did not restore the upregulated fasting glucose level, insulin level and HOMA-IR index, indicating that both treatments were insufficient for regulating glucose homeostasis in OVX-induced metabolic impairment. The discrepancy of the findings could be possibly due to the differences between animal model (obese male mice vs estrogen-deprived female rats), NAC dosage (2 g/L of drinking water vs 100 mg/kg/day) and the duration of NAC treatment (11 weeks vs 4 weeks). The present finding was consistent with the previous report that treatment with NAC alone for four weeks did not lead to a significant improvement in glucose tolerance, insulin release and oxidative markers in type 2 DM patients (Szkudlinska *et al.* 2016). However, the combination of low-dose estrogen and NAC administration effectively improved glucose metabolism at a similar rate to the effect of regular-dose estrogen treatment. This finding suggested that the combined therapy provided greater efficacy than the single remedies for insulin resistance regulation under the OVX condition. Interestingly, our results demonstrated that dyslipidemia in the estrogen-deprived condition was sufficiently improved by NAC alone. This finding is consistent with a previous report that showed that, after treatment for

Figure 3

The changes of cardiac performance during cardiac I/R protocol. Data from P-V loop study demonstrate the changes of cardiac parameters during baseline (BL), ischemia (I) and reperfusion (R) of cardiac I/R procedure. (A) Stroke volume per body weight (SV/BW); (B) left ventricular ejection fraction (%LVEF); (C) left ventricular end-systolic pressure (LVESP); (D) left ventricular end-diastolic pressure (LVEDP); (E and F) maximal and minimal slope of the diastolic pressure decrement (dP/dt_{Max} and dP/dt_{Min}). Values are mean $\pm$ S.E.M. for 5–6 rats per group. * $P < 0.05$ vs BL, † $P < 0.05$ vs sham and ‡ $P < 0.05$ vs OVX. Sham, sham rats; OVX, ovariectomized rats; OVX-E, OVX rats treated with normal-dose estrogen; OVX-e, OVX rats treated with low-dose estrogen; OVX-N, OVX rats treated with NAC; OVX-eN, OVX rats treated with combined low-dose estrogen with NAC. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0108>.

8 weeks, NAC reduced plasma cholesterol and LDL in cholesterol-fed mice through maintaining the level of lipid uptake into cells and thus consequently improved plasma lipid profiles (Korou et al. 2010). The underlying mechanism might also relate to the hepatoprotective

effects of NAC which reduced hepatic lipogenic enzyme activity (Lin & Yin 2008). Moreover, chronic treatment with NAC was reported to decrease plasma triglycerides through the reduction of inflammatory marker levels (TNF and COX-2) and improve oxidative stress in the liver

Figure 4

Cardiac arrhythmia score, time to 1st VT/VF, mortality rate and cardiac p-Cx43 expression, after treatment with E, e, N or eN for 4 weeks. (A) Arrhythmia score during ischemia and reperfusion periods; (B) time to 1st VT/VF following ischemia induction; (C) representative western blot bands and the level of p-Cx43 Ser368 per total Cx43 presented in level of ischemic/remote area; (D) mortality rate during cardiac I/R condition presented in percentage from total rats in the group. Values are mean $\pm$ S.E.M. for six rats per group. * $P < 0.05$ vs sham and † $P < 0.05$ vs OVX. Sham, Sham rats; OVX, ovariectomized rats; OVX-E, OVX rats treated with normal-dose estrogen; OVX-e, OVX rats treated with low-dose estrogen; OVX-N, OVX rats treated with NAC; OVX-eN, OVX rats treated with combined low-dose estrogen with NAC.

Figure 5

Levels of infarct size and cardiac expression of apoptotic and anti-apoptotic proteins, after treatment with E, e, N or eN for 4 weeks. (A) Percentage of myocardial infarct size per area at risk (AAR) and the representatives of the infarcted myocardium. (B) Level of malondialdehyde (MDA) in ischemic tissue (I) normalized by the MDA level of remote (R) tissues. (C) Expression levels of the anti-apoptotic protein Bcl-2 and (D) the apoptotic protein Bax from ischemic area normalized by the level of those in remote area (I/R ratio). Values are mean $\pm$ S.E.M. for six rats per group. * $P < 0.05$ vs sham and † $P < 0.05$ vs OVX. Sham, Sham rats; OVX, ovariectomized rats; OVX-E, OVX rats treated with normal-dose estrogen; OVX-e, OVX rats treated with low-dose estrogen; OVX-N, OVX rats treated with NAC; OVX-eN, OVX rats treated with combined low-dose estrogen with NAC. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0108>.

(Villagarcía *et al.* 2018). Those findings, together with results from our study, indicated that NAC could improve dyslipidemia in obese and estrogen-deprived conditions.

Increased oxidative stress is an important factor in the disruption of tissue insulin sensitivity and the development of insulin resistance and increase cardiovascular disorders. Our study previously demonstrated that oxidative stress markers were increased in both serum and cardiac tissue of estrogen-deprived rats which resulted in impairment of metabolic function, cardiac autonomic balance

and cardiac performance in both basal and cardiac I/R conditions. Hyperactivity of cardiac sympathetic signaling through the activation of β -adrenergic receptors resulted in the increased level of mitochondrial ROS production in cardiomyocytes (Andersson *et al.* 2011). The prolonged cardiac autonomic imbalance caused an excessive energy demand, and together with chronic ROS elevation, could diminish cardiac performance and increase the risks of cardiovascular disorders, cardiac arrhythmia and mortality (Krul *et al.* 2015). Estrogen deprivation induced

Figure 6

Cardiac mitochondrial function in estrogen-deprived rats after treatment with E, e, N or eN for 4 weeks. (A) Mitochondrial ROS level; (B) mitochondrial membrane potential change ($\Delta\Psi$) and (C) absorbance intensity of mitochondria from ischemic myocardium normalized by those from the remote area. (D) Transmission electron microscopy illustrates cardiac mitochondria morphology from ischemic myocardium of rats in each group. Values are mean $\pm$ S.E.M. for six rats per group. * $P < 0.05$ vs sham and † $P < 0.05$ vs OVX. Sham, Sham rats; OVX, ovariectomized rats; OVX-E, OVX rats treated with normal-dose estrogen; OVX-e, OVX rats treated with low-dose estrogen; OVX-N, OVX rats treated with NAC; OVX-eN, OVX rats treated with combined low-dose estrogen with NAC.

HRV depression and impaired cardiac function. These conditions could be abrogated by estrogen replacement through oxidative stress reduction (Sivasinprasasn *et al.* 2016). This study found that either low-dose estrogen alone or NAC alone were insufficient to correct cardiac dysfunction and cardiac autonomic disturbance. This finding was similar to previously reported data which showed that the level of LF/HF ratio was not changed by low-dose estrogen treatment (Campos *et al.* 2014). However, our study first revealed that the combination of NAC with low-dose estrogen significantly improved HRV and LV performance suggesting that the combined therapy could intensify cardioprotective efficacy to a similar level to the regular-dose estrogen in the estrogen-deprived condition.

After cardiac I/R, an increase in MDA concentration, an elevated level of cardiac mitochondrial ROS level, altered mitochondrial membrane potential and mitochondrial swelling was demonstrated in the infarcted myocardia of the estrogen-deprived hearts. Lipid peroxidation of the plasma membrane produced MDA used as an indicator of oxidative stress. Increased level of cardiac MDA negatively affect the calcium-induced calcium release mechanism of cardiocyte in a concentration-dependent manner, therefore, diminished the cardiac contractile function. In the heart, the higher level of mitochondrial ROS associated with the decreased mitochondrial membrane potential led to an impairment of mitochondrial respiratory chain function and lowered ATP production. This interrupted ATP generation negatively affects SERCA activity leading to cardiomyocyte calcium uncoupling. Cardiocyte calcium overload and the increased mitochondrial ROS level caused the opening of mitochondrial permeability transition pores (mPTP) resulting in the swelling mitochondria (Riojas-Hernandez *et al.* 2015). The normal dose estrogen treatment and the combined low-dose estrogen with NAC led to the similar reduction in cardiac MDA levels, lowered ROS concentrations and preserved mitochondrial function in the myocardia of the estrogen-deprived heart that underwent cardiac I/R injury. However, our findings suggested that treatments with oral NAC alone or low-dose estrogen alone were impracticable when it came to the maintenance of mitochondrial membrane potential and suppression of mitochondrial swelling, therefore, failed to protect the hearts against myocardial dysfunction under similar conditions.

This study first demonstrated that E and eN could similarly maintain myocardial Cx43 activity following I/R injury. This finding suggested that cardiomyocyte communication was preserved in addition to the

improvement of cardiac autonomic function. Thus, the susceptibility of I/R-induced cardiac arrhythmia was reduced in these groups. Cardiac Cx43 is an important protein that plays a role in the cell-to-cell traffic in the myocardium. Prolonged myocardial hypoxia/ischemia reduced Cx43 expression, which caused an abrupt communication between the ischemic cardiomyocytes and alteration of cardiocyte calcium signaling, and thus, increased the vulnerability to arrhythmia. The increased level of mitochondrial ROS production was proposed as being a deactivator of Cx43 expression which triggered ventricular arrhythmia and sudden arrhythmogenic death (Sovari *et al.* 2013). In the case of the estrogen-deprived heart, we previously reported that the level of p-Cx43 expression was preserved and the vulnerability of cardiac arrhythmia was reduced by estrogen replacement. It was also previously reported that NAC attenuated the reduction of total cardiac Cx43 in the border zone of infarcted hearts, and thus reduced the susceptibility of post-ischemic arrhythmia (Lee *et al.* 2013). However, the change of p-Cx43 level had never been verified in NAC-treated hearts. Our study investigated the p-Cx43 level in the ischemic zone of the myocardium, and our results further demonstrated that p-Cx43 level was not preserved by using NAC alone, but it was maintained in the heart treated with the combined NAC with low-dose estrogen. As described in the results the time to 1st VT/VF was prolonged and cardiac arrhythmia risk during I/R was reduced by the combined therapy, but not the NAC monotherapy.

Apoptosis of cardiomyocytes could occur as a consequence of mitochondrial swelling and mitochondrial membrane rupture leading to the impairment of cardiac performance (Riojas-Hernandez *et al.* 2015). After cardiac I/R, the level of the apoptotic protein Bax was increased in accordance with the upregulated level of mitochondrial ROS level and mitochondrial swelling in the estrogen-deprived hearts. It has been reported that increased Bax expression and decreased Bcl-2 expression were associated with increased infarct size (Konstantinidis *et al.* 2012). Both the regular-dose estrogen and the combined low-dose estrogen with NAC effectively lowered the level of apoptotic protein Bax expression and preserved the level of anti-apoptotic protein Bcl-2 expression in the infarcted myocardium. The lowered level of myocardial infarction in E and eN treatment groups was consistent with their LV performance during cardiac I/R. Treatment with NAC has been shown to reduce cardiac oxidative stress level and attenuate diastolic dysfunction as it restored LVEDP to normal levels in diabetic rat hearts

(Tuncay *et al.* 2014). Although the present study similarly found that treatment with NAC alone could partially preserve LV systolic and diastolic function in the basal state, however, it did not verify the preservation of the level of SV and %LVEF during cardiac I/R. Our findings demonstrated that the treatment with NAC alone or low-dose estrogen alone could not inhibit mitochondrial dysfunction and could not modulate Bcl-2 and Bax level in the ischemic myocardium, resulting in an aggravated infarction and diminished LV performance during cardiac I/R. This result suggested that both treatments were insufficient to protect cardiac function in the cardiac I/R condition. In contrast, both the regular-dose estrogen and the combined NAC with low-dose estrogen exerted a preferable cardioprotective effect since the %LVEF levels of these groups were restored during cardiac I/R. The cardioprotection afforded by the two remedies may be because of the effect of lowering mitochondrial oxidative stress and mitochondrial swelling leading to a more maintained level of mitochondrial function and viable myocardium. As a result, LV performance was less damaged.

Conclusion

A combination of NAC with low-dose estrogen and the regular-dose estrogen therapy led to similar improvements in cardiometabolic function, lower cardiac arrhythmia, prevention of mitochondrial dysfunction and reduction in myocardial infarction in estrogen-deprived rats with cardiac I/R injury. These findings have important implications for the future application of interventions in menopausal women, particularly under conditions of cardiac I/R. This study may contribute to improving the quality of life of menopausal women where there are contraindications for the use of estrogen.

Study limitation and clinical insights

This study aimed to investigate the efficacy of the treatments on cardiometabolic function and cardio-protection during I/R injury in estrogen-deprived female rats. The findings provide information regarding the therapeutic potential of the treatments which could then contribute to improvement of the quality of life for menopausal women. However, the abrupt estrogen-deprived model in this study was obtained by bilateral removal of the ovaries, which is dissimilar to postmenopausal women,

who have a progressive reduction of endogenous estrogen level with intact ovaries.

The treatments in this study were commenced at 1 week following surgical estrogen deprivation since it is recommended that hormone therapy be initiated early, around the onset of the menopausal period (NAMS 2017). The clinical outcome of the treatments may be different according to the delay in timing of treatment initiation and older age of the postmenopausal women. The different routes of treatments among the experimental groups (i.e., subcutaneous injection and gavage feeding) were considered according to the clinical application of the remedy. However, these different routes of administration may be the cause of different stress conditions and pharmacokinetics.

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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Author contribution statement

N C, S C and S S designed the study. S S, S P, K C, T J, N A and W P performed the experiments. S S, N C and S C performed data analysis and drafted the manuscript. N C, S C and S S revised the manuscript. All authors reviewed the final manuscript.

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