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Research Paper

Treatment with oestrogen-receptor agonists or oxytocin in conjunction with exercise protects against myocardial infarction in ovariectomized rats

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New Findings

- **What is the central question of this study?**
  Could the activation of oxytocin or oestrogen receptors be protective against myocardial injury after ovariectomy? If so, would exercising have an additional ameliorating effect?
- **What is the main finding and its importance?**
  The results revealed that when accompanied by exercise, both oestrogen receptor agonists and oxytocin improved cardiac dysfunction, inhibited the generation of pro-inflammatory cytokines and reduced myocardial injury in ovariectomized female rats, suggesting a new approach for protecting postmenopausal women against ischaemia-induced myocardial injury.

To investigate the putative protective effects of oxytocin or oestrogen receptor agonists against myocardial injury of ovariectomized sedentary or exercised rats, female Sprague–Dawley rats assigned to sham-operated control and ovariectomized (OVX) groups were kept sedentary or undertook swimming exercise for 4 weeks and were treated with saline, an oestrogen receptor (ER) β (DPN) or ERα agonist (PPT) or oxytocin. Ovariectomy increased weight gain and anxiety in sedentary rats, whereas exercise prevented weight gain. When accompanied by exercise, both ER agonists and oxytocin inhibited weight gain and anxiety; oxytocin, in the absence or presence of exercise, increased the left ventricular diastolic dimensions and ejection fraction, whereas ER agonists also increased left ventricular diameter when given to exercised rats. Upon the induction of myocardial ischaemia–reperfusion in the OVX rats, plasma creatine kinase-(muscle–brain) was depressed by PPT and oxytocin, whereas DPN, PPT and OT reduced plasminogen activator inhibitor-1 concentrations. The increased tumour necrosis factor-α concentration in OVX rats was also suppressed by exercise or DPN, PPT or oxytocin treatments, whereas the interleukin-6 concentration was diminished by all the treatments when given in conjunction with exercise. Disorganization of cardiac muscle fibres was reduced in all exercised rats. Oestrogen receptor agonists, as well as oxytocin, in conjunction with exercise may be effective new therapeutics to protect against myocardial ischaemia in postmenopausal women.

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Introduction

Despite the advances in healthcare, cardiovascular diseases and, most commonly, coronary heart disease (CHD) remain as the major causes of mortality and morbidity and are predicted to be the leading cause of individual deaths in the near future (Lopez & Murray, 1998). Numerous experimental studies and randomized clinical trials have demonstrated that the incidence of cardiovascular diseases increases in women after menopause, and it was suggested that hormone replacement therapy started at the initiation of menopause could provide cardiovascular benefit (Wenger, 2002). Although the cardioprotective effect of the major oestrogen, 17β-estradiol (E_2), was demonstrated in several models of ischaemia–reperfusion (I/R)-induced myocardial injury (Node *et al.* 1997; Zhai *et al.* 2000*b*), oestrogens in hormone replacement therapy trials have not reduced the overall rate of CHD events and have even facilitated their development (Rossouw *et al.* 2002; Anderson *et al.* 2004). Moreover, use of hormone replacement therapy in postmenopausal women has increased the rate of thromboembolic events, stroke, gallbladder disease and ovarian and breast cancers (Hou *et al.* 2013; Sidaway, 2015). Given that the beneficial effects of non-selective E_2 were thought to be overridden by its potential negative effects, selective oestrogen receptor (ER) ligands that exhibit partial agonist activity have become the focus of interest (Sun *et al.* 1999). Via its receptors in the endothelial and vascular smooth muscle cells, E_2 exerts genomic and non-genomic vascular effects, resulting in vasodilatation, decreased contraction and reduced vascular remodelling (Orshal & Khalil, 2004). Both ERα and ERβ were shown to be involved in mediating E_2-induced rapid cardioprotection against I/R injury in isolated, perfused hearts from adult male mice (Hutchens *et al.* 2012), but their putative protective effects on postmenopausal female hearts have not been studied before.

Oxytocin (OT), a nonapeptide produced in the paraventricular and supraoptical nuclei of the hypothalamus, is known to exert its effects via its receptors in several organs, including the heart (Gimpl & Fahrenholz, 2001). Oxytocin was shown to have negative inotropic and chronotropic effects (Mukaddam-Daher *et al.* 2001) by controlling blood volume through the release of atrial natriuretic peptide (ANP) from atrial myocytes (Gutkowska *et al.* 1997). In contrast, the cardioprotective effects of regular exercise, verified by several epidemiological studies (Shephard & Balady, 1999; Lavie *et al.* 2009), appear to involve the upregulation of the cardiac OT and ANP systems (Gutkowska *et al.* 2007) and increased hypothalamic OT content and gene expression (Braga *et al.* 2000; Martins *et al.* 2005). Furthermore, in several inflammatory models, including myocardial injury in heart transplant, OT was shown to exert protective effects through the downregulation of inflammatory and oxidative processes (Tuğtepe *et al.* 2007; Çetinel *et al.* 2010; Al-Amran & Shahkolahi, 2014). No data are present regarding the anti-inflammatory effects of OT on menopause-associated myocardial injury.

Regarding the extensive literature relating psychosocial factors to the development of CHD (Everson-Rose & Lewis, 2004), psychological stress and anxiety frequently observed in postmenopausal women (Pedram *et al.* 2010) may be, in part, responsible for the postmenopausal increase in the incidence of CHD. Hormone replacement therapy in postmenopausal women (Stewart *et al.* 1992) or E_2 treatment in ovariectomized female rats significantly decreased anxiety and depression (Wang *et al.* 2010). Likewise, exercise was shown to improve mental health and decrease depressive behaviours in postmenopausal women (Villaverde Gutiérrez *et al.* 2012). However, neither the impact of exercise nor the effect of stimulation of ERs on anxiety and the severity of myocardial injury has been evaluated in the absence of ovarian hormones.

Although the individual cardioprotective and anti-inflammatory effects of exercise, OT and ER agonists have been defined in several models, their impact on menopause-associated myocardial injury has not been elucidated. In relation to this background, the present study was designed to investigate the putative protective effects of OT or ER agonists, and to assess the impact of exercise *per se* or in conjunction with OT or ER agonists on myocardial injury in ovariectomized (OVX) rats by using functional, biochemical and histological parameters.

Methods

Animals

An *in vivo* study was conducted in female Sprague–Dawley rats (200–250 g, 16–20 weeks old) supplied by the Marmara University Animal Center (DEHAMER). The rats were kept in a temperature- (21 ± 2°C) and humidity (65–70%)-controlled room with 12 h–12 h light–dark cycles and fed standard pellet chow and water *ad libitum*. All experimental procedures were approved by the Marmara University Animal Care and Use Committee (11.04.2012–38.2012.mar).

Surgery and experimental design

In order to obtain the basline values, a holeboard anxiety test and transthoracic echocardiography were carried out on all rats. The rats were then randomly assigned to two experimental groups, namely sham operation (control; $n = 16$) or OVX ($n = 64$; Fig. 1). Under general anaesthesia induced with ketamine (100 mg kg^{-1}, I.P.) and chlorpromazine (0.75 mg kg^{-1}, I.P.), a mid-line abdominal

incision was made to remove the ovaries bilaterally, with application of silk sutures, whereas the sham-operated rats underwent the abdominal incision without removal of the ovaries. The rats were injected I.P. with acetaminophen (Perfalgan; Bristol Myers Squibb; 0.1 mg kg^{-1} day^{-1}) for analgesia during the 3 day recovery period.

After the recovery period, half of the rats in the sham-operated and OVX groups started the swimming exercise, whereas the other half were kept sedentary throughout the 4 week follow-up period of the experiment (Fig. 1). Ovariectomized groups, either sedentary or exercising, were injected I.P. with (i) saline, or (ii) 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN; 1 mg kg^{-1} day^{-1}; Tocris Cookson, Ellisville, MO, USA), or (iii) 4,4′,4″-[4-propyl-(1*H*)-pyrazole-1,3,5-triyl]*tris*-phenol (PPT; 1 mg kg^{-1} day^{-1}; Tocris Cookson), or (iv) oxytocin (OT; 1 mg kg^{-1} day^{-1}; Tocris Cookson) during the second half of the 4 week period, whereas the sham-operated sedentary and exercising rats were treated with saline (Fig. 1). Each of the 10 subgroups consisted of eight rats. DPN acts as an agonist on both ER subtypes, but has a 70-fold higher relative binding affinity for ERβ than for ERα, whereas PPT is a selective agonist for the ERα with a 410-fold binding selectivity over ERβ (Stauffer *et al.* 2000). The rationale for the doses of oxytocin and ER treatment was based on our previous studies (Kumral *et al.* 2014).

At the end of the 4 weeks, cardiac function was re-assessed by echocardiography, and the holeboard test was repeated (Fig. 1). Following the holeboard test, urethane (Sigma, St Louis, MO, USA; 780 mg kg^{-1}) was injected I.P. for non-recovery anaesthesia. The level of anaesthesia was evaluated by the loss of pedal reflex to toe pinch. After a 10 min baseline recording, all the rats underwent myocardial ischaemia for 30 min followed by 60 min reperfusion. At the end of the reperfusion period, cardiac puncture was performed to obtain blood for the measurements of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), cardiac troponin-I, plasminogen activator inhibitor-1 (PAI-1) and creatine kinase-(muscle–brain) (CK-MB). The rats were exsanguinated and the cardiac tissues removed, dissected and taken for histopathological examination.

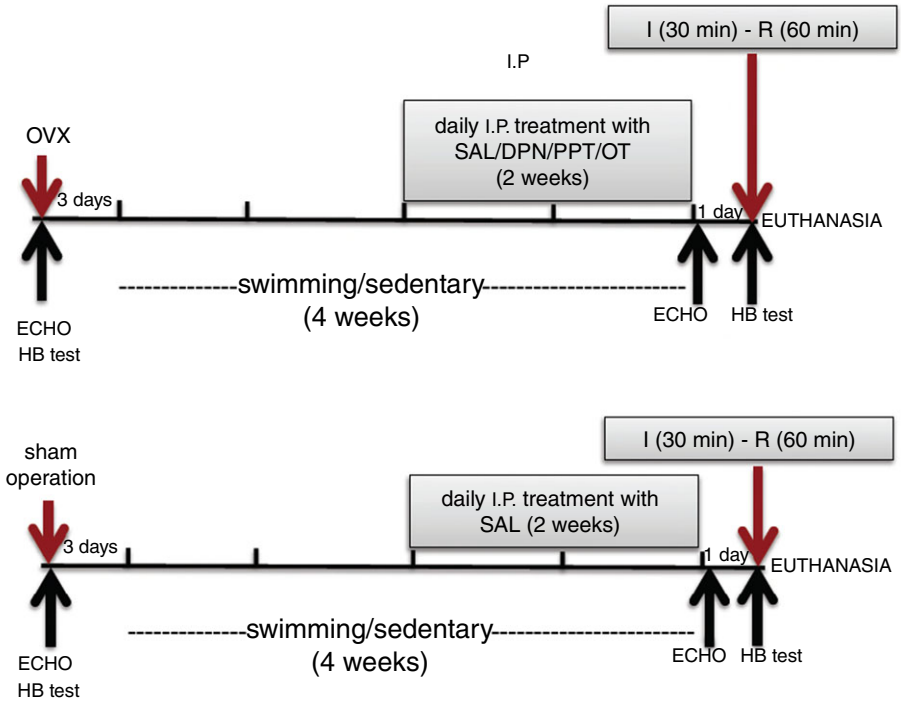

Figure 1. Flowchart illustrating the study design
Rats underwent a sham operation ($n = 16$) or ovariectomy (OVX; $n = 64$), and both groups were further divided into exercised and sedentary groups. Swimming exercise was continued for 4 weeks, and during the last 2 weeks of the exercise or sedentary period, a daily I.P. injection of saline (SAL), oestrogen receptor-α agonist (PPT; 1 mg kg^{-1}), oestrogen receptor-β agonist (DPN; 1 mg kg^{-1}) or oxytocin (OT; 1 mg kg^{-1}) was administered. Myocardial ischaemia–reperfusion, with 30 min ischaemia (I) and 60 min reperfusion (R) periods, was induced in all rats. Echocardiographic (ECHO) evaluation and a holeboard (HB) test were performed before the initial surgeries and were repeated before the terminal anaesthetic.

Exercise protocol

A moderate-load swimming exercise model without workload was selected in the exercise groups (Cakir *et al.* 2010). Exercise training was carried out as 30 min swimming sessions in a cylindrical glass pool (diameter: 100 cm; height: 50 cm) filled to a depth of 35 cm with lukewarm water. Swimming sessions were continued 5 days per week for 4 consecutive weeks. The sedentary rats were taken out from their cages daily and placed in empty glass containers for 30 min at identical time points to the exercised rats.

Evaluation of anxiety

It is well known that increased anxiety reduces the natural exploratory behaviour in rats, and this behaviour can be evaluated by the holeboard test (Marco *et al.* 2005). The holeboard test provides a measure of directed exploration in rats by placing the rat at the centre of a wooden board (40 cm × 40 cm) with 16 equally spaced holes (each 3.8 cm in diameter) and videotaping for 5 min (Çetinel *et al.* 2010). At the end of the recordings, the number of head-dips in a 5 min period was counted from the videotapes by a blinded observer, and the reduction in the number of head dips indicated a reduction in exploratory behaviour and thus increased anxiety. The holeboard test was applied to all rats on two occasions; baseline recordings were made on the first day of the experiment before rats were assigned to experimental groups, and recordings were repeated on the day of the I/R procedure.

Echocardiographic evaluations

Echocardiographic measurements were made on two occasions, i.e. on the first day of the experiment and 1 day before the I/R procedure. Echocardiographic imaging and calculations were carried out using a 12 MHz linear transducer and 5–8 MHz sector transducer (Vivid 3; General Electric Medical Systems Ultrasound, Tirat Carmel, Israel) according to the guidelines published by the American Society of Echocardiography (Schiller *et al.* 1989). Under general anaesthesia induced with ketamine (50 mg kg^{-1}, I.P.), transthoracic echocardiography was carried out in M-mode, and after observing at least six cardiac cycles, two-dimensional images were obtained in the parasternal long and short axes at the level of the papillary muscles. Interventricular septal thickness (IVS) and left ventricular diameter (LVD) were measured during systole (s) and diastole (d). The ejection fraction (EF) and fractional shortening (FS) were calculated from the M-mode images using the following formulas: percentage EF $= (\mathrm{LVDd})^3 - (\mathrm{LVDs})^3/(\mathrm{LVDd})^3 \times 100$; and percentage FS $=$ LVDd $-$ LVDs/LVDd $\times$ 100 (Schiller *et al.* 1989).

Electrocardiographic monitoring, myocardial I/R procedure and analysis of ST segment resolution

The rats were ventilated (60–70 cycles min^{-1}) through a tracheotomy using a rodent ventilator (Harvard Apparatus, Holliston, MA, USA). Standard limb lead II was continuously monitored on the ECG using a computerized data acquisition system (Power Lab; ADInstruments, Radon Medical Ltd, Ankara, Turkey). A baseline ECG was recorded before the ischaemia, and follow-up ECGs were recorded during the reperfusion period.

The fourth rib was cut 3 mm below the left lateral sternal border. The pericardium was incised, and a suture (6–0 silk; Ethicon) was placed around the left anterior descending coronary artery close to its origin, immediately below the left atrial appendage. Both ends of the ligature were passed through a small plastic tube. To induce transient myocardial ischaemia, the artery was occluded by applying tension to the plastic tube–silk string. At the end of the period of ischaemia, the ligature was released to remove the tension, and reperfusion was initiated. Coronary artery occlusion and reperfusion were confirmed by epicardial cyanosis and hyperaemia, respectively.

As defined previously (de Lemos & Braunwald, 2001), ST segments were measured retrospectively by an observer (M.A.) blinded to the experimental groups, using callipers at 60 ms beyond the J-point. Maximal ST segment elevation in the initial record was used for comparison. Successful reperfusion was taken as >50% resolution of the greatest ST segment elevation recorded at 30 min. The ST resolution index was calculated as the ST segment elevation (in millimetres) during left anterior descending coronary artery occlusion divided by the ST segment elevation (in millimetres) at 60 min of reperfusion.

Blood assays

Blood samples were collected, immediately centrifuged at 4000 g for 10 min, and stored at −80°C for the subsequent determination of plasma TNF-α, IL-6, IL-8, cardiac troponin I (cTnI), PAI-1 and CK-MB concentrations. Plasma concentrations were quantified according to the manufacturer's instructions and guidelines using the enzyme-linked immunosorbent assay method. All samples were assayed in triplicate using commercial kits (Cusabio Life Science, Hubei, China), and the values were expressed in picograms per millilitre. These assay kits were selected because of their high degree of sensitivity, specificity, inter- and intra-assay precision, and the small volume of plasma required to conduct the assay.

Table 1. Transthoracic echocardiography measurements obtained at the end of 4 weeks, during which the rats were either kept sedentary or undertook swimming exercise, indicating the cardiac functional states of the experimental groups before they underwent myocardial ischaemia–reperfusion

Group (before the induction of myocardial I/R)		IVS (mm)	LVDs (mm)	LVDd (mm)	EF (%)	FS (%)
Sham operated	Sedentary	2.37 ± 0.09	3.24 ± 0.41	5.08 ± 0.37	75.52 ± 2.08	37.44 ± 2.00
	Exercised	2.47 ± 0.11	2.82 ± 0.37	4.71 ± 0.17	77.60 ± 5.25	39.44 ± 4.92
Saline treated, OVX	Sedentary	2.49 ± 0.09	3.340 ± 0.01	5.22 ± 0.35	79.33 ± 1.06	36.71 ± 4.79
	Exercised	2.58 ± 0.09	3.34 ± 0.03	5.57 ± 0.41**	80.39 ± 4.12	41.51 ± 6.21
PPT treated, OVX	Sedentary	2.26 ± 0.14	3.26 ± 0.40	5.22 ± 0.62	79.77 ± 0.56	35.40 ± 3.89
	Exercised	2.41 ± 0.17	3.66 ± 0.57***	5.85 ± 0.58**†	80.34 ± 1.01	41.31 ± 5.91
DPN treated, OVX	Sedentary	2.33 ± 0.22	3.10 ± 0.17	5.12 ± 0.77	77.82 ± 3.41	39.54 ± 3.23
	Exercised	2.55 ± 0.15	3.27 ± 0.03	5.89 ± 0.24**	81.23 ± 2.82	42.78 ± 2.72
OT treated, OVX	Sedentary	2.23 ± 0.21	2.85 ± 0.02	5.40 ± 0.10**	82.47 ± 1.20**‡‡	44.04 ± 1.30**‡‡
	Exercised	2.44 ± 0.21‡‡	2.88 ± 0.03	5.29 ± 0.01**	83.67 ± 0.28**‡‡	45.35 ± 0.28**‡‡

Abbreviations: DPN, oestrogen receptor-β agonist; EF, ejection fraction; FS, fractional shortening; I/R, ischaemia–reperfusion; IVS, interventricular septal thickness; LVDd, left ventricular diameter in diastole; LVDs, left ventricular diameter in systole; OT oxytocin; OVX, ovariectomized; and PPT, oestrogen receptor-α agonist. Each subgroup consisted of eight rats. $^{**}P < 0.01$ compared with sedentary sham-operated group. $^{\dagger}P < 0.05$ compared with respective sedentary group. $^{\ddagger\ddagger}P < 0.01$ compared with respective saline-treated group.

Light microscopy and semi-quantitative analysis

The hearts were fixed in 10% buffered formalin, processed for embedding in paraffin wax by routine protocols and cut into 5-μm-thick sections by a microtome. The sections were stained using Haematoxylin and Eosin and examined using a photomicroscope (Olympus BX51, Japan). In each experimental group, six to eight slides were evaluated and five similar areas randomly selected for the histopathological examination. Cardiac muscle fibres were evaluated microscopically. The scores of the infarction area, haemorrhage and inflammatory cell (neutrophil) infiltration were assessed using a semi-quantitative lesion scoring system modified from Guven Bagla *et al.* (2013). Infarct size and haemorrhage were scored as follows: none, 0; weak, 1; moderate, 2; strong, 3; and very strong, 4. Inflammatory cell infiltration was scored as follows: none, 0; weak, 1; moderate, 2; and strong, 3. The maximal total score for each section was 11.

Statistical analysis

The data were expressed as means ± SEM. The Mann–Whitney *U* test was used for the analysis of microscopic evaluation, whereas Student's unpaired *t* test or one-way ANOVA and Tukey–Kramer multiple comparison tests were used to evaluate the level of statistical significance of other parameters. Values of $P < 0.05$ were regarded as significant. Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Echocardiographic measurements repeated at the end of the 4 weeks, during which the rats were either kept sedentary or undertook swimming exercise, indicated the cardiac functional states of the experimental groups before they underwent myocardial I/R (Table 1). Left ventricular end-diastolic dimensions were increased in all the exercised rats with OVX in comparison to sedentary sham-operated rats ($P < 0.001$). Accordingly, the percentage fractional shortening and ejection fraction were also increased in the OVX exercised rats, but statistical significance was reached only in the OT-treated rats ($P < 0.01$). In addition, end-diastolic LV dimension, ejection fraction and fractional shortening were also found to be higher in the sedentary OVX rats treated with OT.

In sham-operated rats, swimming exercise for 4 weeks resulted in significant weight loss ($P < 0.001$; Fig. 2). In contrast, OVX caused significant weight gain in the sedentary rats compared with sham-operated rats ($P < 0.05$ and $P < 0.001$), but having exercised throughout the 4 week period reduced the weight gains significantly in all the treatment groups ($P < 0.05$ and $P < 0.001$), except for the saline-treated rats.

The test performed before I/R revealed that the level of anxiety, as assessed by reduced numbers of head-dipping on the holeboard test, was increased in sedentary OVX rats treated with saline, PPT or DPN in comparison to sham-operated control rats ($P < 0.01$; Fig. 2). Exercise training prevented the increase in anxiety in OVX rats treated with PPT or DPT ($P < 0.05$), but not in saline-treated animals. The level of anxiety in OT-treated sedentary or exercised OVX rats was not different from that of the sham-operated rats.

Changes in heart rate induced by I/R in the experimental groups were recorded before the ischaemia and throughout the ischaemia and reperfusion periods (Table 2). Baseline heart rates were significantly depressed

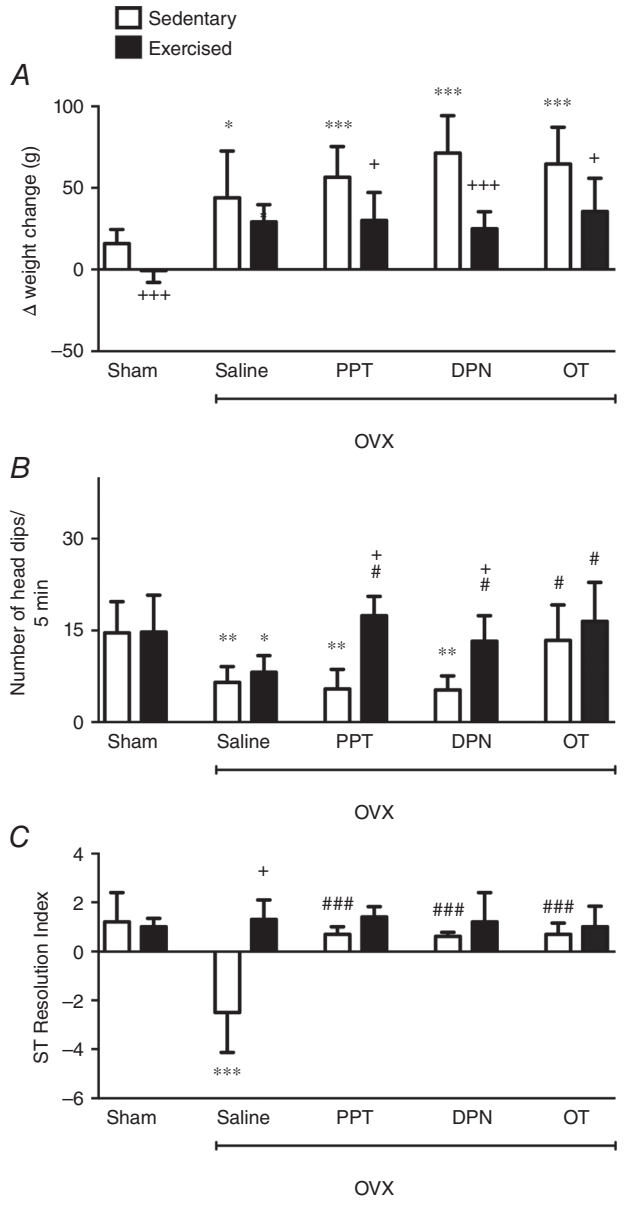

Figure 2. Body weight changes, anxiety scores and ST resolution indices in the experimental groups
Body weight changes throughout the experimental procedures (*A*) and the number of head dips, indicating anxiety scores (*B*), measured before the induction of myocardial ischaemia–reperfusion in sham-operated or OVX, sedentary or exercised (4 weeks) rats, which were treated for 2 weeks with saline, PPT, DPN or OT. After the 30 min ischaemia and 60 min reperfusion periods, the ST resolution index (*C*) was calculated as the ST segment elevation (in millimetres) during coronary artery occlusion divided by the ST segment elevation (in millimetres) at 60 min of reperfusion. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.01$ compared with respective sham-operated group. $^{+}P < 0.05$ and $^{+++}P < 0.01$ compared with respective sedentary group. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.01$ compared with respective saline-treated group. Each subgroup consisted of eight rats.

in the sham-operated or OT-treated OVX rats that had previously exercised ($P < 0.001$ and $P < 0.05$) with respect to the corresponding sedentary rats. During the 30 min ischaemia, heart rates were significantly elevated in both exercised and non-exercised sham-operated groups, as well as in the sedentary saline-treated OVX rats ($P < 0.01$–0.001), and these high heart rates were maintained during the reperfusion period ($P < 0.001$). However, in the PPT-, DPN- or OT-treated groups, ischaemia-induced tachycardia was either lower ($P < 0.05$) or not evident. Likewise, the elevations in the heart rates of the PPT-, DPN- or OT-treated sedentary groups observed during the reperfusion period were not significantly different from their baseline recordings, but were elevated in the corresponding exercised groups ($P < 0.05$–0.001). Similar to its use in clinical practice and research, ST resolution was used to assess the degree of reperfusion in rats with acute myocardial infarction. The ST resolution indices of sedentary or exercised rats were similar in the sham-operated groups (Figs 2 and 3). In the sedentary OVX rats treated with saline, the ST resolution index was negative, indicating no resolution ($P < 0.001$), whereas ST resolution was evident in the exercised saline-treated OVX rats. The ST resolution indices were similar in PPT-, DPN- or OT-treated OVX rats that had either exercised or not exercised.

In all the rats, plasma CK-MB concentrations were measured as the cardiac markers of I/R injury. Plasma CK-MB concentrations were similar in saline-treated OVX and sham-operated sedentary rats, whereas lower levels were measured in the respective exercised groups ($P < 0.05$ and $P > 0.05$; Fig. 4). PPT or DPN treatments did not have additional effects on exercise-induced reduction in CK-MB, whereas OT further suppressed plasma CK-MB concentrations in the exercised groups ($P < 0.05$). However, in the absence of exercise, PPT and OT were also inhibitory on I/R-induced elevation in CK-MB concentrations ($P < 0.05$). Plasma concentrations of cTnI, an early predictor of myocardial infarction, were measured in all the groups. The cTnI concentrations in all the sedentary and exercised groups were similar (Fig. 4), except for the sham-operated rats, which demonstrated significantly higher cTnI concentrations when they had exercised ($P < 0.05$). In the sham-operated rats, plasma concentrations of PAI-1 were similar in both the sedentary and exercised rats (Fig. 4). The PAI-1 concentration was increased significantly in the saline-treated OVX sedentary rats ($P < 0.01$), but this increase was reversed when the rats had exercised ($P < 0.01$). This inhibitory effect of exercise on the PAI-1 concentration was not changed further by either PPT or DPN, but OT had an additional suppressive effect ($P < 0.05$). Likewise, in the absence of exercise, when the sedentary OVX rats were treated with DPN, PPT or OT,

Table 2. Heart rate data calculated from standard limb lead II recordings using a computerized data acquisition system

Group		Baseline HR (beats min^{-1})	Average HR during 30 min ischaemia (beats min^{-1})	Average HR during 60 min reperfusion (beats min^{-1})
Sham operated	Sedentary	247.7 ± 53.7	407.7 ± 52.6***	442.5 ± 47.8***
	Exercised	200.7 ± 6.4†††	339.5 ± 69.9***	427.4 ± 9.0***
Saline treated, OVX	Sedentary	264.0 ± 64.3	354.7 ± 28.2**	403.6 ± 45.6***
	Exercised	259.3 ± 56.3	317.5 ± 84.6	418.0 ± 36.4***
PPT treated, OVX	Sedentary	322.8 ± 100.9‡	349.8 ± 56.6*‡‡‡	399.5 ± 63.9
	Exercised	283.8 ± 67.9	375.8 ± 94.2	402.2 ± 94.3**
DPN treated, OVX	Sedentary	373.2 ± 103.8	245.4 ± 58.7	358.3 ± 82.1
	Exercised	299.8 ± 105.5	350.2 ± 55.3*	431. 5 ± 29.8*
OT treated, OVX	Sedentary	347.2 ± 73.4‡	370.7 ± 43.9	373.2 ± 103.6
	Exercised	259.3 ± 56.3†	317.5 ± 84.6	418.0 ± 36.4***

Heart rate (HR) recordings were made before the ischaemia (baseline, 10 min) and throughout the ischaemia (30 min) and reperfusion (60 min) periods. Myocardial ischaemia–reperfusion was induced in all the sham-operated or ovariectomized (OVX), sedentary or exercised (4 weeks) rats that were treated for the last 2 weeks with saline, oestrogen receptor-α agonist (PPT), oestrogen receptor-β agonist (DPN) or oxytocin (OT). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with corresponding baseline recording. †$P < 0.05$ and †††$P < 0.001$ compared with respective sedentary group. ‡$P < 0.05$ and ‡‡‡$P < 0.001$ compared with respective saline-treated group. Each subgroup consisted of eight rats.

similar reductions in PAI-1 concentrations were observed ($P < 0.01$–0.001).

When compared with sham-operated sedentary rats, plasma TNF-α concentrations were significantly increased in the OVX sedentary group ($P < 0.05$), and exercise in both sham-operated and OVX rats depressed the TNF-α concentrations ($P < 0.001$ and $P < 0.01$; Fig. 5). Treatments with DPN or PPT had no additional effect on the inhibitory effect of exercise in OVX rats, but OT treatment along with exercise depressed TNF-α concentrations of the OVX rats further ($P < 0.05$). In the absence of exercise, treatments with DPN, PPT or OT also depressed the TNF-α concentrations ($P < 0.05$–0.001) compared with those of saline-treated OVX rats. When compared with sham-operated rats, plasma IL-6 and IL-8 concentrations were found to be higher in the OVX saline-treated rats that had exercised or stayed sedentary ($P < 0.05$; Fig. 5). PPT, DPN or OT treatments reduced IL-6 concentrations significantly when they were accompanied with exercise training ($P < 0.05$), whereas only OT resulted in the suppression of IL-6 in sedentary OVX rats ($P < 0.05$). Likewise, only OT treatment was effective in reducing elevated plasma IL-8 concentrations of sedentary OVX rats ($P < 0.05$).

Histopathological analysis of the I/R-injured heart tissues revealed that bleeding, inflammatory cell infiltration and disorganization of cardiac muscle fibres were severe (Fig. 6*A*–*E*) and the microscopic scores high (Fig. 7) in the sham-operated and saline-treated OVX groups that were sedentary in comparison to control heart tissue (Fig. 6*K*). Bleeding, inflammatory cell infiltration and disorganization of cardiac muscle fibres were found to be moderate in the OT-treated sedentary OVX group, but all these histopathological parameters were severe in DPN- or PPT-treated sedentary OVX groups (Figs 6*A*–*E* and 7). In the sham-operated and saline-treated OVX groups that had exercised, a moderate disorganization of cardiac muscle fibres was present along with severe bleeding and inflammatory cell infiltration (Fig. 6*F*–*J*), with significantly lower scores in the saline-treated OVX group (Fig. 7; $P < 0.01$). All these parameters were moderate in OT-, DPN- or PPT-treated groups that had exercised, with a significantly ($P < 0.01$) reduced score in the PPT-treated group.

Discussion

Basal transthoracic echocardiography measurements of the randomly grouped rats and their anxiety levels recorded at the beginning of the experimental protocol were not different (data not shown). The results of the present study demonstrated that OVX increased weight gain and anxiety in the sedentary rats before they underwent an ischaemic injury, whereas exercise prevented weight gain without altering the level of anxiety. The ER agonists and OT had inhibitory effects on both weight gain and anxiety, when they were accompanied with regular exercising. Echocardiographic analyses revealed that OT, given in the absence or presence of exercise, increased the left ventricular diastolic dimensions and ejection fraction, suggesting an improvement in left ventricular function before ischaemia was induced. In addition to OT, all the treatments resulted in increased left ventricular diameter when they were accompanied by exercise training, but the increases in EF were not significant. Upon the induction of myocardial I/R in the OVX rats, the cardiac injury marker CK-MB was depressed

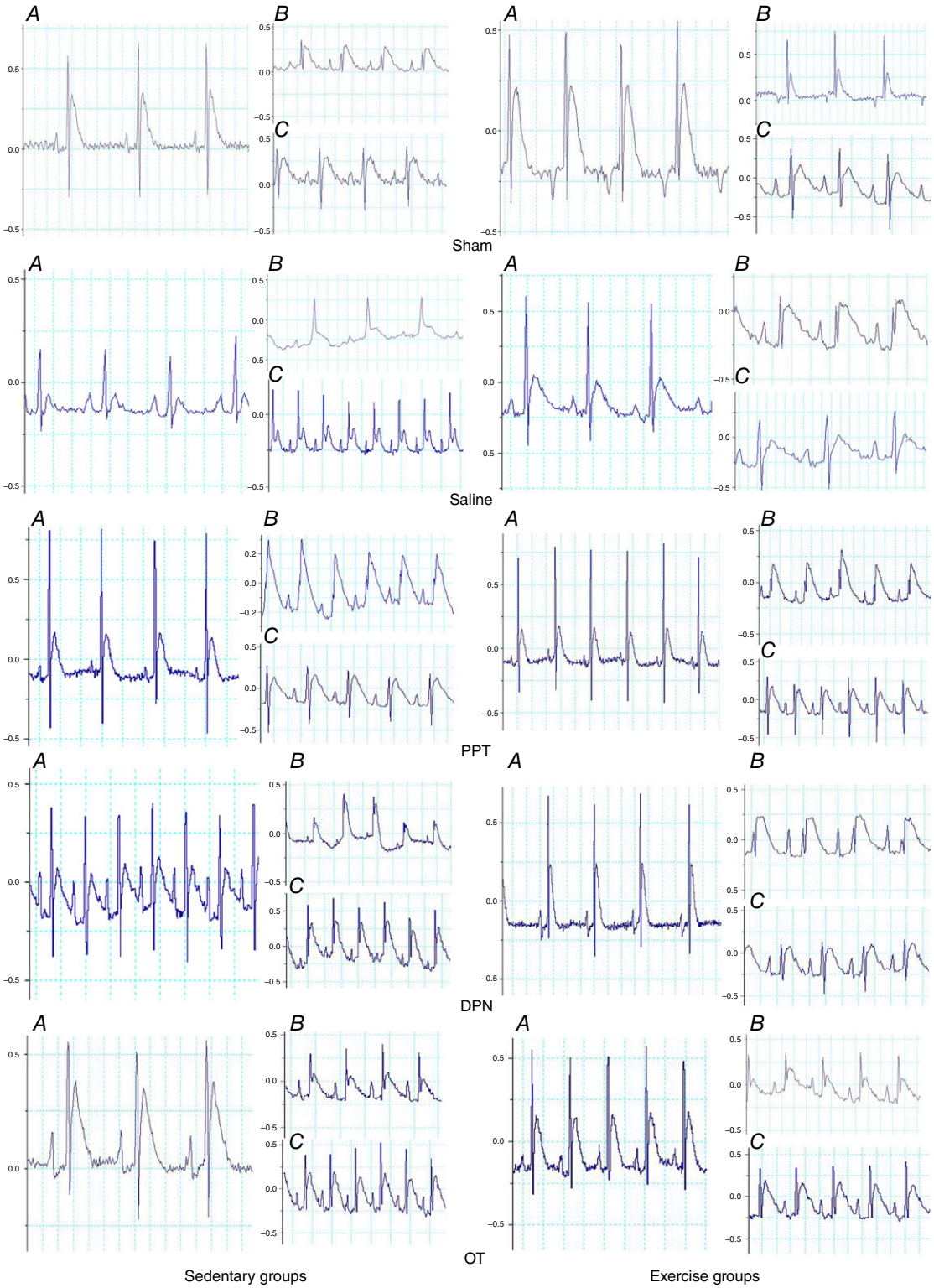

Figure 3. Representative ECG recordings of sham-operated or OVX sedentary or exercised (4 weeks) rats, which had been treated for the previous 2 weeks with saline, PPT, DPN or OT
All rats underwent a 30 min myocardial ischaemia followed by a 60 min reperfusion period. The selected recordings were obtained as follows: during the baseline period (in the fifth minute after the electrodes were placed; *A*); during ischaemia (25–30 min of coronary artery occlusion; *B*); and during reperfusion (50–60 min of reperfusion; *C*).

by both PPT and OT, whereas DPN, PPT and OT were equally effective in reducing the OVX-enhanced elevation in the plasma PAI-1 concentration. The increased TNF-α concentration resulting from myocardial I/R in OVX rats was also suppressed by DPN, PPT or OT, and having exercised had a similar effect. Likewise, the high IL-6 concentration in OVX rats after I/R was diminished by all the treatments when given in conjunction with exercise.

In the present study, OVX resulted in significant weight gain, as previously verified by Flues *et al.* (2010), and exercise training reduced the body weight gain observed in

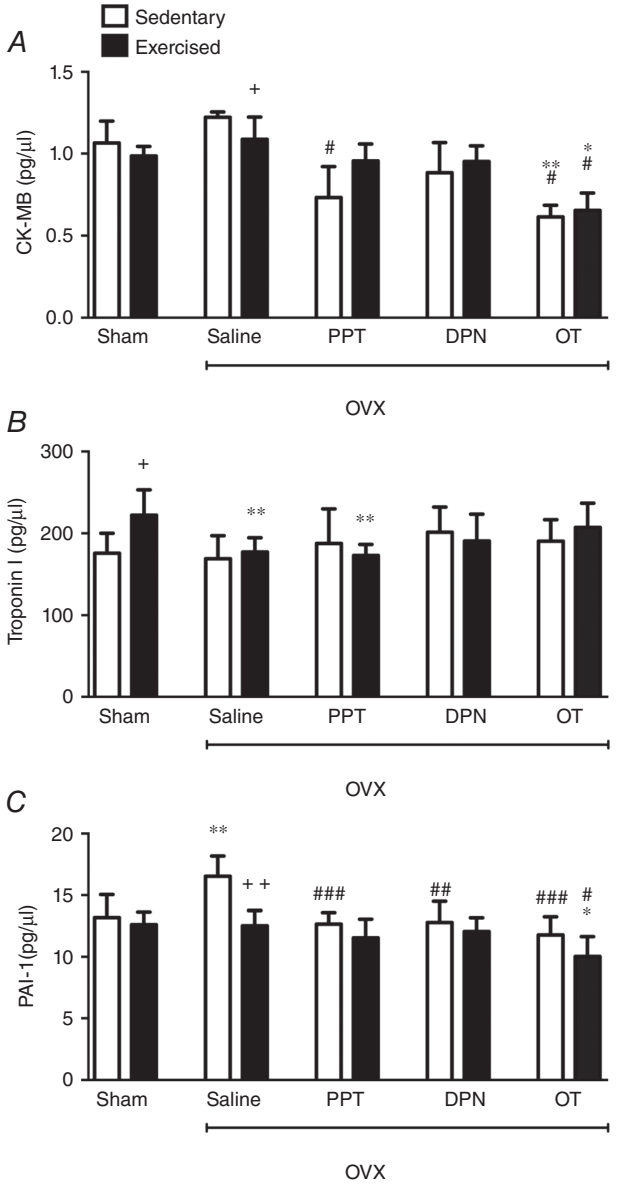

Figure 4. Plasma concentrations of creatine kinase muscle–brain (CK-MB; *A*), cardiac troponin I (*B*) and plasminogen activator inhibitor-1 (PAI-1; *C*) in sham-operated or OVX, sedentary or exercised (4 weeks) rats, which were treated for the last 2 weeks with saline, PPT, DPN or OT
All rats underwent a 30 min myocardial ischaemia followed by a 60 min reperfusion period. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the respective sham-operated group. $^{+}P < 0.05$ and $^{++}P < 0.01$ compared with respective sedentary group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.01$ compared with respective saline-treated group. Each subgroup consisted of eight rats.

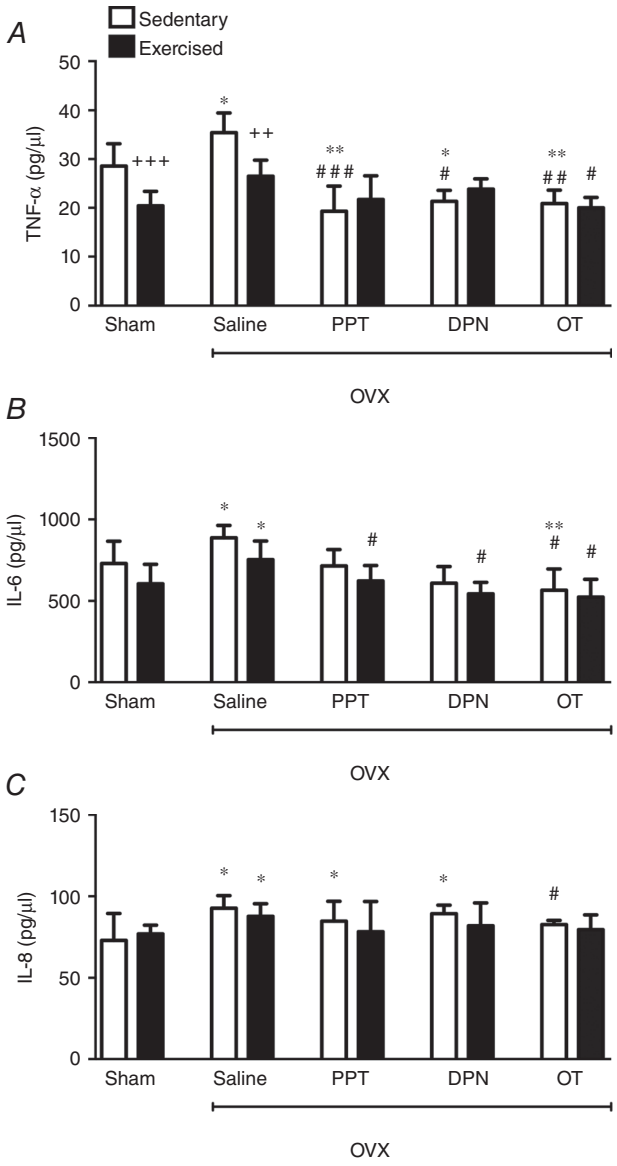

Figure 5. Plasma concentrations of tumour necrosis factor-α (TNF-α; *A*), interleukin-6 (IL-6; *B*) and interleukin-8 (IL-8; *C*) in sham-operated or OVX, sedentary or exercised (4 weeks) rats, which were treated for the last 2 weeks with saline, PPT, DPN or oxytocin
All rats underwent a 30 min myocardial ischaemia followed by a 60 min reperfusion period. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with respective sham-operated group. $^{++}P < 0.01$, $^{+++}P < 0.001$ compared with respective sedentary group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.01$ compared with respective saline-treated group. Each subgroup consisted of eight rats.

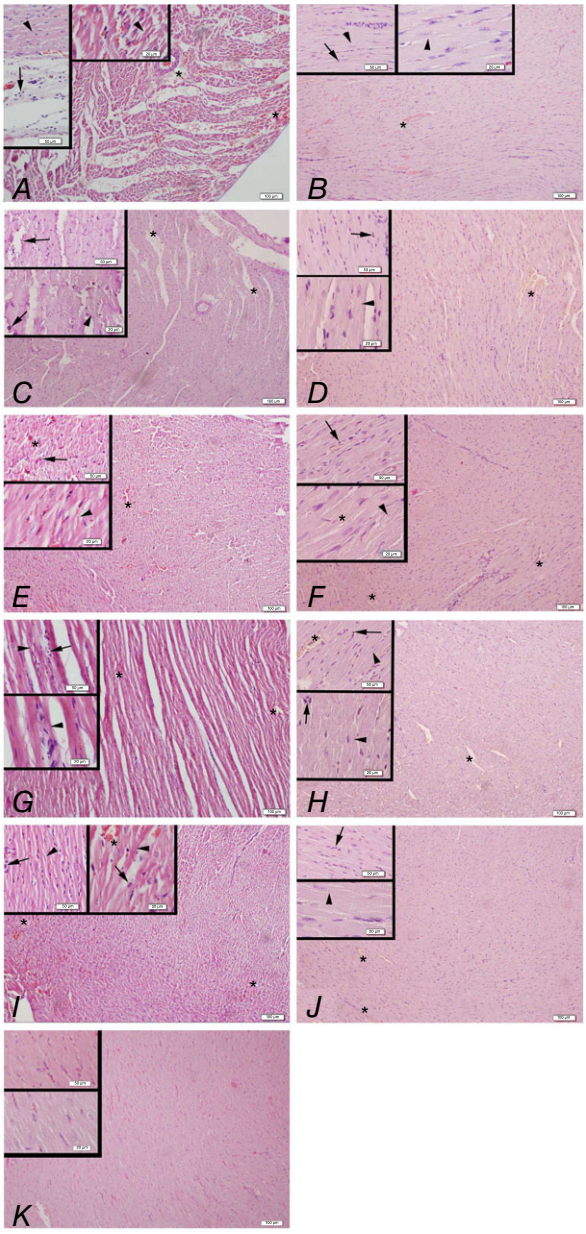

Figure 6. Representative photomicrographs of the experimental groups and the intact cardiac tissue
In the experimental groups (*A–J*), rats had undergone 30 min myocardial ischaemia followed by 60 min reperfusion. For comparison, intact cardiac tissue without ischaemia–perfusion (*K*) illustrates the regular organization of muscle fibres. Bleeding (*), inflammatory cell infiltration (arrows) and muscle fibre disorganization (arrowheads) were more severe in sham-operated sedentary (*A*), sham-operated exercised (*B*), saline-treated OVX sedentary (*C*), DPN-treated OVX sedentary (*E*), DPN-treated OVX exercised (*F*), PPT-treated OVX sedentary (*G*) and oxytocin (OT)-treated OVX sedentary (*I*) groups, with respect to moderate bleeding and inflammatory cell infiltration in saline-treated OVX exercised (*D*), PPT-treated OVX exercised (*H*) and OT-treated OVX exercised (*J*) groups. Haematoxylin and Eosin staining.

OVX rats. Epidemiological studies have shown that regular exercise improves cardiovascular health, reduces the risk of mortality resulting from CHD (Dimmeler & Zeiher, 2003) and provides protection against the development of myocardial infarction (Brown & Moore, 2007), with a greater survival rate in physically active individuals compared with sedentary counterparts (Xavier *et al.* 2016). It was shown that 5–8 weeks of swimming exercise in rats reduced the infarct size by 24–39% (Calvert & Lefer, 2013), which is in parallel with epidemiological evidence indicating the preconditioning effect of exercise against myocardial infarction (Frasier *et al.* 2011). By promoting an increase in free radical production and cytokine release in the heart (Salo *et al.* 1991) and by activating intrinsic radical scavengers (Marini *et al.* 2007, 2008), exercise appears to precondition the heart to forthcoming injurious events. In response to exercise-induced oxidative stress, myocardial antioxidants and myocardial heat shock proteins are increased, and collateral coronary arteries are developed (Marini *et al.* 2007, 2008). In the present study, 4 weeks of exercise training in OVX rats resulted in increased left ventricular diameter, facilitated the ST resolution, suppressed the plasma PAI-1 and TNF-α concentrations, and reduced the cardiac microscopic damage scores, demonstrating the protective effect of exercise on the development and severity of myocardial infarction. Exercise training was demonstrated to increase hypothalamic OT and OT receptor (OTR) expressions in rats (Martins *et al.* 2005), and reduced cardiac expressions of OTR, ANP and brain natriuretic peptide in OVX rats were normalized or enhanced by exercise training,

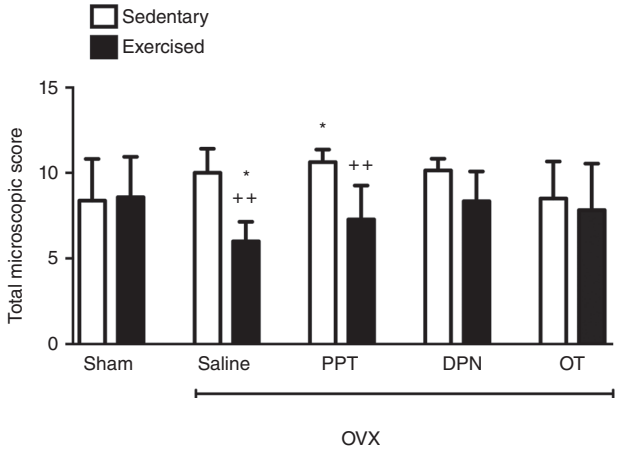

Figure 7. Microscopic damage scores in the cardiac tissues of sham-operated or OVX, sedentary or exercised (4 weeks) rats, which were treated with saline, PPT, DPN or OT
All rats underwent 30 min myocardial ischaemia followed by a 60 min reperfusion period. $^{*}P < 0.05$ compared with respective sham-operated group. $^{++}P < 0.01$ compared with respective sedentary group. Each subgroup consisted of eight rats.

supporting the contention that the upregulation of the OT and ANP systems may play a role in exercise-induced cardioprotection (Gutkowska *et al.* 2007).

Oxytocin can be considered as a novel pharmacological agent for cardioprotection, because OT regulates blood volume and reduces cardiac contractility by increasing the release of ANP from cardiac atria (Gutkowska *et al.* 1997) and promotes the differentiation of stem cells to mature cardiac myocytes by the upregulation of the OTR (Paquin *et al.* 2002). In contrast, the cardiac gene expressions of OTR, ANP, brain natriuretic peptide were decreased in OVX rats, suggesting that the detrimental effects of OVX may be associated with the downregulation of these genes (Gutkowska *et al.* 2007). It was demonstrated in rats with myocardial I/R that OT pretreatment reduces myocardial infarct size and ventricular arrhythmias via nitric oxide production and protein kinase C activation and by maintaining the oxidant–antioxidant balance (Faghihi *et al.* 2012). In parallel with that, we have previously shown that OT ameliorates oxidative renal or colonic damage through its antioxidant effect by inhibition of the free radical waterfall cascade and secretion of cytokines (Tuğtepe *et al.* 2007; Çetinel *et al.* 2010). Based on the structure of the OTR gene promoter region, it has been proposed that the pro-inflammatory cytokines may function as negative modulators of OTR gene transcription (Sidaway, 2015). Accordingly, Gutkowska *et al.* (2014) have suggested that the improvement of cardiac contractile function in response to OT treatment may be associated with reduced TNF-α expression in the injured myocardium, because the myocardial TNF-α concentration is rapidly increased and contributes to the development of contractile dysfunction during myocardial ischaemia (Dörge *et al.* 2002). In agreement with the aforementioned studies, the results of the present study showed that OT treatment in OVX rats potently improved the EF before the induction of infarct, and the levels of pro-inflammatory cytokines measured after the I/R insult were suppressed in OT-treated OVX rats, suggesting that OT may exert an alternative effect by maintaining anti-inflammatory–pro-inflammatory cytokine balance within the injured heart. Recently, cardioprotective effects of OT in the myocardium of rats after I/R were suggested to involve the downregulation of the myocardial inflammatory response, reactive oxygen species and neutrophil-dependent myocardial apoptosis (Al-Amran & Shahkolahi, 2014), as well as the modulation of mitochondrial ATP-dependent potassium channels and permeability transition pores (Alizadeh *et al.* 2012). In a rabbit model of myocardial I/R, post-infarct treatment with OT exerted antifibrotic and angiogenic effects, suggesting the long-term beneficial effects of OT on cardiac function and remodelling (Kobayashi *et al.* 2009). By activating cardiac cholinergic neurons (Mukaddam-Daher *et al.* 2001), OT was suggested to inhibit cardiac sympathetic nerve activity and improve the EF in rats subjected to myocardial I/R, while the initially downregulated cardiac OTRs in the infarcted heart were activated by OT infusion (Jankowski *et al.* 2010). In conjunction with these studies, the present results demonstrated that OT pretreatment reduced anxiety and supported the cardiac ejection function and, thereby, prepared the heart for a cardiac insult in OVX rats. Following the I/R injury, OT pretreatment resulted in a better ST resolution index along with depressed plasma CK-MB, PAI-1, TNF-α and IL-6 responses.

The increased incidence of CHD after menopause (Wenger, 2002) indicates the protective effects of endogenous oestrogens on the cardiovascular system. Exogenously administered E_2 was shown to reduce the myocardial infarct size and the occurrence of I/R-induced ventricular arrhythmias in several experimental models (Hale *et al.* 1996), and its supplementation in OVX rats was shown to attenuate myocardial infarction and ventricular dysfunction after coronary artery occlusion (Zheng *et al.* 2011). Genistein, a naturally occurring isoflavonic phytoestrogen, was also proved to have significant cardioprotective effects on the *ex vivo* perfused hearts of OVX rats (Al-Nakkash *et al.* 2009). In contrast to these positive effects, others have shown that neither oestrogen withdrawal nor its replacement changed the incidence of dysrhythmias or myocardial infarct size in female rats undergoing a coronary occlusion (McNulty *et al.* 2000). Likewise, a large clinical trial has shown that taking an oestrogen–progestin combination increased the cardiovascular risk for women, and has suggested that the use of hormone replacement therapy should not be initiated or continued for primary prevention of CHD (Rossouw *et al.* 2002). Moreover, the administration of non-human hormones results in vascular complications, such as thrombosis in veins and coronary arteries of postmenopausal women (Meyer *et al.* 2006). Therefore, specific ER agonists that would avoid these complications were investigated for their protective potentials in mediating dilatation of coronary arteries (Traupe *et al.* 2007). In rats, both ERα and ERβ have been shown to improve myocardial I/R injury and contribute to myocardial protection (Vornehm *et al.* 2009). Selective ERα activation with PPT was shown to reduce the ischaemic injury and myocardial infarct size in aged female rats (Novotny *et al.* 2009) and in OVX rabbits (Booth *et al.* 2005), whereas male ERα knockout mice were shown to have severe myocardial damage after I/R (Zhai *et al.* 2000*a*), suggesting that ERα plays a cardioprotective role. In contrast, ERβ, but not ERα, was shown to play a role in the protection of the female heart (Gabel *et al.* 2005) verified by the prevention of reperfusion-induced arrhythmias via the selective ERβ agonist DPN, whereas the selective ERα agonist PPT had no significant influence (Wang *et al.* 2010). In

accordance with that, increased mortality from myocardial infarction in ERβ-knockout mice was accompanied by aggravated clinical and biochemical markers of heart failure (Pelzer *et al.* 2005). The ERβ agonist DPN prevented the I/R-induced increase in the incidence of ventricular premature beats and ventricular tachycardia, whereas the ERα agonist PPT had no significant effect after severe myocardial ischaemia in rats (Wang *et al.* 2010). Moreover, the selective ERβ agonist DPN was proved to prevent myofibroblast formation and cardiac fibrosis (Pedram *et al.* 2010), while post-ischaemic administration of PPT or DPN significantly attenuated myocardial dysfunction in isolated, perfused hearts of adult male rats (Vornehm *et al.* 2009), In contrast, acute treatment with DPN had no effect on functional recovery after I/R injury in aged female rats (Tomicek *et al.* 2013). Regarding all these conflicting results on the cardioprotective effects of ERs, the present findings revealed that pretreatment with either of the agonists appears to have similar efficiency in protecting the heart of OVX rats against I/R injury. Both ERα and Erβ agonists reduced the plasma concentrations of TNF-α and PA-1, while PPT had an additional inhibitory effect on the plasma CK-MB response. In contrast, exercise *per se* had inhibitory effects on TNF-α and PA-1 concentrations, and when DPN or PPT pretreatment was accompanied by exercise training, the plasma changes accomplished by either exercise or treatment were not altered further.

Considerable evidence indicates that emotional factors and chronic stressors promote the development and clinical manifestations of CHD (Rozanski *et al.* 2005) and that exercise modifies psychosocial risk factors and reduces depressive symptoms (Blumenthal *et al.* 1999). It is well documented that the symptoms of depression and anxiety are heightened during the menopause (Soares & Cohen, 2001), and physical exercise may prevent or reduce anxiety and depression in postmenopausal women (Villaverde Gutiérrez *et al.* 2012). Administration of E_2 to rats was associated with a significantly decreased anxiety-like behaviour and an antidepressant-like effect in female rats (Walf & Frye, 2009), which may involve its inhibitory effect on the stress-induced cortisol response (Takuma *et al.* 2007). Recently, the ERβ agonist, DPN, was shown to exert an anxiolytic effect in rats, whereas an OT antagonist has blocked the effects of DPN on anxiety, suggesting the interaction of OT and ERβ in modulating anxiety-like behaviours (Kudwa *et al.* 2014). The results of the present study demonstrated that ovariectomy resulted in increased anxiety in both sedentary and exercised rats. However, pretreatment with DPN, PPT or OT had no anxiolytic effects in non-exercised rats, but a combination of exercise training with either of the treatments abolished the anxiety of OVX rats.

Early studies using ST segment resolution demonstrated that patients with rapid ST resolution had smaller infarcts than those with persistent ST elevation (Barbash *et al.* 1990), suggesting the use of ST resolution in the prediction of infarct size. In the present study, reduced ST resolution in the non-exercised OVX rats was partly recovered in the exercised groups, while treatment with OT, PPT or DPN had similar supportive effects on the recovery of ST segment elevation during the reperfusion period. Likewise, as an indicator of functional recovery after infarction, plasma PAI-1 concentrations were studied in the experimental groups. Plasminogen activator inhibitor-1, at physiological concentrations, plays an important role in inhibiting angiogenesis by controlling proteolytic activity and cell migration (Devy *et al.* 2002). In patients with myocardial infarction, plasma concentrations of PAI-1 were increased (Shimizu *et al.* 2015), and reducing cardiac PAI-1 activity was suggested to improve functional recovery after infarction by protecting against cardiomyocyte apoptosis (Xiang *et al.* 2005). In accordance with this information, the present findings show that the plasma PAI-1 concentration was relatively increased in sedentary OVX rats, while either exercising prior to myocardial ischaemia or treatment with PPT, DPN or OT significantly suppressed the PAI-1 concentrations. Our findings also support clinical studies, which report that E_2 inhibits PAI-1 and induces tissue plasminogen activator in postmenopausal women (Brown *et al.* 2002). Our results indicate that treatment with oestrogen agonists or oxytocin or exercise had similar beneficial effects on cardiac injury parameters of the ischaemic heart, although exercise did not have an additional protective effect on the inflammatory cytokines and PAI-1 concentration afforded by either pharmacological treatment.

At the end of the 2 h post-infarct period, plasma cTnI concentrations were found to be high in all the rats when compared with the concentrations measured in previous studies (Aydin *et al.* 2014), which supports the notion that cTn1 is an early predictor, within the first 8 h, of myocardial infarct. Cardiac troponin I was further increased in the non-OVX rats that had exercised, suggesting a non-specific skeletal muscle troponin response as a result of chronic exercise. Although the present findings did not show any effect of treatment regimens on cTnI concentrations, PPT was shown to reduce cTnI concentrations in the sera of rabbits with I/R injury (Booth *et al.* 2005), which may be because of differences in the experimental protocols.

In conclusion, given the controversies regarding hormone replacement therapy and the use of ER agonists, the results of the present study demonstrate that both agonists, as well as oxytocin, in conjunction with exercise may be considered as effective new therapeutics to protect against myocardial ischaemia in postmenopausal women, and further experimental and clinical studies are required

to elucidate their roles in the primary and secondary prevention of coronary heart disease.

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Additional information

Competing interests

None declared.

Author contributions

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