In female rat heart mitochondria, oophorectomy results in loss of oxidative phosphorylation

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Abstract

Oophorectomy in adult rats affected cardiac mitochondrial function. Progression of mitochondrial alterations was assessed at one, two and three months after surgery: at one month, very slight changes were observed, which increased at two and three months. Gradual effects included decrease in the rates of oxygen consumption and in respiratory uncoupling in the presence of complex I substrates, as well as compromised Ca^{2+} buffering ability. Malondialdehyde concentration increased, whereas the ROS-detoxifying enzyme Mn^{2+} superoxide dismutase (MnSOD) and aconitase lost activity. In the mitochondrial respiratory chain, the concentration and activity of complex I and complex IV decreased. Among other mitochondrial enzymes and transporters, adenine nucleotide carrier and glutaminase decreased. 2-Oxoglutarate dehydrogenase and pyruvate dehydrogenase also decreased. Data strongly suggest that in the female rat heart, estrogen depletion leads to progressive, severe mitochondrial dysfunction.

Key Words

▶ estrogens
▶ heart mitochondria
▶ oophorectomy
▶ estrogen receptors
▶ gender

Introduction

Estrogens (17β-estradiol, estrone and progesterone) control diverse reproductive system functions. Their participation in other physiological processes such as cognition (Sherwin 1999), cardiovascular function (Stevenson 2000), immunity (Ahmed *et al.* 1999) and bone and mineral metabolism (Compston 2001) has been reported. Thus, estrogens are considered pleiotropic hormones. Estrogens enter the nucleus after being internalized by estrogen receptors α and β (ERα and ERβ) (Hall *et al.* 2001). In the myocardium, non-genomic pathways involving plasma membrane-bound ERs that activate protein kinase-mediated

signaling cascades have been described (Sugden & Clerk 1998, Nuedling *et al.* 1999). Each estrogen receptor is codified by a unique gene (Giguere *et al.* 1998), which possesses the characteristic functional domains of the steroid/thyroid hormone superfamily of nuclear receptors (Matthews & Gustafsson 2003).

ERα and ERβ are widely distributed. ERα is expressed primarily in the uterus, liver, kidneys and heart, whereas ERβ is expressed primarily in the ovaries, prostate, lungs, gastrointestinal tract, bladder and hematopoietic and central nervous systems. Both receptors are co-expressed in mammary glands, epididymis, thyroid, adrenals, bone and some brain regions (Orshal & Khalil 2004, Mendoza-Garcés *et al.* 2011, Knowlton & Lee 2012). In addition, both receptors have been found in mitochondria, where their functions seem to be different and even antagonistic (Pedram *et al.* 2006, Psarra & Sekeris 2008, Yang *et al.* 2009). In brain mitochondria, estrogens modulate mitochondrial functions such as oxidative phosphorylation (Wang *et al.* 2001, Duckles *et al.* 2006) and Ca^{2+} uptake (Nilsen & Diaz Brinton 2003). In mouse heart, estrogens increase mitochondrial respiratory complex IV activity (Hsieh *et al.* 2006). In monkeys and in MCF-7 human breast cancer cells, estrogens may regulate mitochondrial biogenesis and size (Irwin *et al.* 2008, Rosario *et al.* 2008). However, in rats, this response has not been observed (Mattingly *et al.* 2008).

We used oophorectomized rats as a model to study estrogenic depletion. In adipose tissue mitochondria, oophorectomy decreases oxidative capacity and antioxidant defenses (Nadal-Casellas *et al.* 2011), as well as complex IV (COX) and pyruvate dehydrogenase (PDH) activities in whole-brain mitochondria (Irwin *et al.* 2011). However, these changes have not been fully explored in heart mitochondria. Estrogen receptors have been reported in the mitochondrial inner membrane and matrix of neurons, primary cardiomyocytes, murine hippocampus cell lines and human heart cells, whereas for other steroids, such as progesterone, receptors have been found only in the outer membrane (Dai *et al.* 2013).

We observed that oophorectomy affects heart mitochondrial functions such as oxygen consumption, Ca^{2+} uptake, transmembrane potential and the expression of different mitochondrial oxidative phosphorylation-related proteins; in castrated male rats, these results are not observed (Pavón *et al.* 2012). Thus, it was decided to evaluate the post-oophorectomy time-dependent evolution of heart mitochondria function.

Materials and methods

All experiments were conducted in agreement with ethical rules and guides from the Instituto Nacional de Cardiología, México (Record N°14-865).

Animals

Sixty Wistar female rats (3 weeks old) were used in the experiments. These were randomly assigned to one of two groups: control (Ctrl, intact rats) and oophorectomized (Cast). In addition, the latter were subdivided in three groups of twenty, to be analyzed at 1st, 2nd and 3rd month after surgery. Oophorectomy was performed in three-week-old animals under pentobarbital anesthesia. After surgery, rats were housed in our animal colony and maintained under controlled light/darkness cycles (12 h each) with water and rodent chow *ad libitum*.

Isolation of heart mitochondria

Rats were killed with sodium pentobarbital (100 mg/kg i.p.), and the heart was obtained. Heart tissue was incubated for 10 min with 2 mg/g of proteinase K (Sigma, P6556). Digested samples were centrifuged at 11,951.9 *g*, and the resulting pellet was homogenized in 125 mM KCl, 1 mM EDTA, 10 mM Tris, pH 7.3 (Pavón *et al.* 2012) and centrifuged again at 478.1 *g*, to pellet debris. From the supernatant, mitochondria were separated by differential centrifugation. Protein concentration was determined by the Bradford method (Bradford 1976).

Oxygen consumption measurements

It was assayed polarographically with a Clark electrode at 25°C. Reaction medium was 125 mM KCl, 3 mM phosphate, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.3. Either 10 mM succinate + 5 µg/mL rotenone or 5 mM glutamate + 5 mM malate were added as respiratory substrates. 300 µM ADP was added to induce the phosphorylating state (state 3) as described in Pavón and coworkers (Pavón *et al.* 2012). Mitochondria were added to a final concentration of 0.5 mg prot/mL; final volume was 1.5 mL. Respiratory control (RC) was calculated as

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the quotient between the rate of oxygen consumption in state 3 (ADP-stimulated respiration) and the rate in state 4 (after ADP pulse is entirely phosphorylated and respiration shifts to resting state).

Calcium uptake

Mitochondrial Ca^{2+} uptake was measured spectrophotometrically at 675–685 nm (dual wavelength mode) at room temperature using the indicator Arsenazo III as described by Janssen and Helbing (1991). Briefly, 10 mM succinate, 5 µg/mL rotenone, 100 µM CaCl₂, 50 µM Arsenazo III, 100 µM ADP and 2 mg of mitochondrial protein were added to 2.9 mL 125 mM KCl, 3 mM phosphate, 10 mM HEPES, pH 7.3.

Enzyme activities

Citrate synthase (CS) was measured at 412 nm ($\varepsilon = 13.6$ mM/cm) in a reaction mixture containing 0.023 mg/mL acetyl-CoA, 0.1 mM DTNB (5,5′-dithio-bis-2-nitrobenzoic acid), 0.25 mM oxaloacetate, 0.05% Triton X-100 and 10 mM Tris–HCl, pH 8; mitochondria 0.03 mg prot/mL. CS activity was used for normalization of enzyme activities (Davies *et al.* 2001, Barrientos *et al.* 2009, Schwarzer *et al.* 2013). NADH:decylubiquinone oxidoreductase (complex I) activity was measured by following the fluorescence changes of NADH at 460 nm using SET buffer (250 mM sucrose, 0.2 mM EDTA and 50 mM Tris, pH 7.2), 0.155 mM NADH, 0.077 mM decylubiquinone, 10 µM antimycin A, 0.05% Triton X-100 and 0.5 mg prot/mL mitochondria (Barrientos *et al.* 2009). Rotenone (10 µM) was added to inhibit complex I and remaining inhibitor-insensitive activities were subtracted to the data. Succinate:DCPIP oxidoreductase (complex II) activity was measured spectrophotometrically at 590 nm ($\varepsilon = 15.96$ mM/cm) in SET buffer, 100 µM DCPIP, 10 mM succinate, 10 µM antimycin A, 5 µM rotenone and 0.5 mg prot/mL mitochondria (Barrientos *et al.* 2009). An OMEGA microplate reader was used to determine CS and complexes I and II activities; final volume per well was 200 µL. Complex IV activity was measured as cyanide-sensitive oxygen consumption in the presence of 5 mM ascorbate, 1 µM TMPD (tetramethyl-phenylenediamine), 10 µM antimycin A and 0.5 mg prot/mL mitochondria (Barrientos *et al.* 2009). NaCN (1 mM) was added to inhibit respiration at the end of each trace. Pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (2-OGDH) activities were measured as in Cooney and coworkers (Cooney *et al.* 1981) with slight

modifications using 125 mM KCl, 10 mM phosphate, 10 mM Tris/HCl, 5 mM MgCl₂, 0.05% Triton X-100, 2 mM NAD⁺, 0.63 mM CoA, 1 mM TPP, 1 mM DTT, 1 mM PMSF, 10 µM rotenone, pH 7.4; mitochondria 0.5 mg prot/mL. The reaction was started with either 10 mM pyruvate or 10 mM 2-oxoglutarate. Reduction of NAD⁺ ($\varepsilon = 6.22$ mM/cm) was followed in a DW2000 AMINCO OLIS spectrophotometer at 340 nm. Aconitase activity was measured as in Hausladen & Fridovich (1994). Mitochondria were solubilized by adding 0.05% Triton X-100 in 25 mM phosphate, pH 7.2. Then, 0.6 mM MnSO₄ and 10 mM citrate were added to the reaction mixture. The formation of cis-aconitate was measured at 240 nm.

Malondialdehyde by capillary zone electrophoresis

Malondialdehyde was determined as in Claeson and coworkers (Claeson *et al.* 2000). Briefly, 2 mg mitochondria were washed with methanol (1:1), centrifuged at 16,000 ***g*** for 15 min and filtered through a 0.22 µm nitrocellulose membrane. Samples were diluted (1:10) with 0.1 M NaOH and analyzed in a P/ACE MDQ (Beckman Coulter). Capillary tube was preconditioned with 0.1 M NaOH/10 min, distilled water/10 min and finally with 10 mM borate + 0.5 mM CTAB, pH 9 buffer. Separation was performed at –25 kV/4 min and absorbance was followed at 267 nm.

Western blot

Mitochondria were powdered in liquid nitrogen and dissolved in RIPA lysis buffer (PBS 1×, 1% IGEPAL NP40, 0.1% SDS and 0.05% sodium deoxycholate, pH 7.2) plus 5 mM protease inhibitor cocktail (Roche). Protein samples (40 µg) were re-suspended in loading buffer plus 5% β-mercaptoethanol and separated under denaturing conditions. Electrophoretic transfer to PVDF membranes (BioRad) was followed by overnight immunoblotting at 4°C with 1:500 diluted primary antibodies (Santa Cruz) against complex I subunit ND1; complex IV subunit COX4; ATP synthase subunit 5B (beta); adenine nucleotide translocator; pyruvate dehydrogenase subunit E1α; 2-oxoglutarate dehydrogenase; succinate dehydrogenase subunit C or glutaminase. Bands were revealed with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz). The signal was detected by chemiluminescence using an ECL-Plus system (Amersham Bioscience). Densitometry was performed using the Scion Image Software (Scion; MD, USA) and normalized against its respective loading control.

Blue native polyacrylamide gel electrophoresis (BN-PAGE) and in-gel enzymatic activities

BN-PAGE was performed as described by Schägger (2001). Briefly, mitochondrial pellet was re-suspended in sample buffer (750 mM aminocaproic acid, 25 mM imidazole, pH 7.0) and solubilized with 2 mg n-dodecyl-β-D-maltoside (lauryl maltoside, LM)/mg prot at 4°C for 30 min and centrifuged at 60,000 *g*; 4°C/25 min. Supernatants were loaded into 4–12% (w/v) polyacrylamide gradient gels. After electrophoresis, in-gel NADH oxidoreductase (NDH) and cytochrome *c* oxidase (COX) activities were performed as in Zerbetto and coworkers (Zerbetto *et al.* 1997). BN-gels not subjected to in-gel activities, were stained with Coomassie blue G-250 (Wittig *et al.* 2007). Densitometry was done using the ImageJ (1.49v) software (NIH) and normalized against its respective loading control.

Superoxide dismutase (MnSOD) activity

MnSOD activity was determined in non-denaturing gels. Solubilized mitochondria (200 µg) were loaded into 10% polyacrylamide gels. After electrophoresis, gels were incubated in 0.5 mg/mL nitrotetrazolium blue (NTB) for 30 min and then in 28 mM TEMED, 36 mM potassium phosphate, 0.28 mM riboflavin, pH 7.8, in the darkness for 10 min. Activities were revealed by exposure to UV light for 10 min. A standard curve was performed using a serial dilution (2.5, 5, 10, 15, 30 and 60 ng) of MnSOD from bovine erythrocytes (Sigma Chemical Co.). Activities were calculated as in Pérez-Torres and coworkers (Pérez-Torres *et al.* 2009).

Statistical analysis

Student's *t*-test for unpaired data was used to compare the baseline variables of the groups. ANOVA test was employed and when a significant *F* was obtained, a Newman–Keuls post-test was used to find intergroup differences. A $P < 0.05$ was considered statistically significant. For statistical analysis, we used Prism 5.0 software.

Results

Strong evidence indicates that estrogens control mitochondrial functions. Blood vessel mitochondria from oophorectomized rats (Cast) exhibit a delay in respiration that disappears upon estradiol administration (Duckles *et al.* 2006). Thus, we explored functional alterations in rat heart mitochondria at one, two and three months after oophorectomy.

Oxygen consumption measurements were performed to analyze the progressive effect of castration on rat heart mitochondrial oxidative phosphorylation (OXPHOS) system (Table 1). Respiratory substrates used were succinate or glutamate–malate. Succinate-dependent oxygen consumption and respiratory controls (RC) were similar in oophorectomized (Cast) groups and in non-oophorectomized controls (Table 1 and Supplementary Fig. 1, see section on supplementary data given at the end of this article). By contrast, in the presence of glutamate–malate, respiratory coupling gradually decreased from the 1st month after surgery, whereas state 4 increased up to two times (Table 1 and
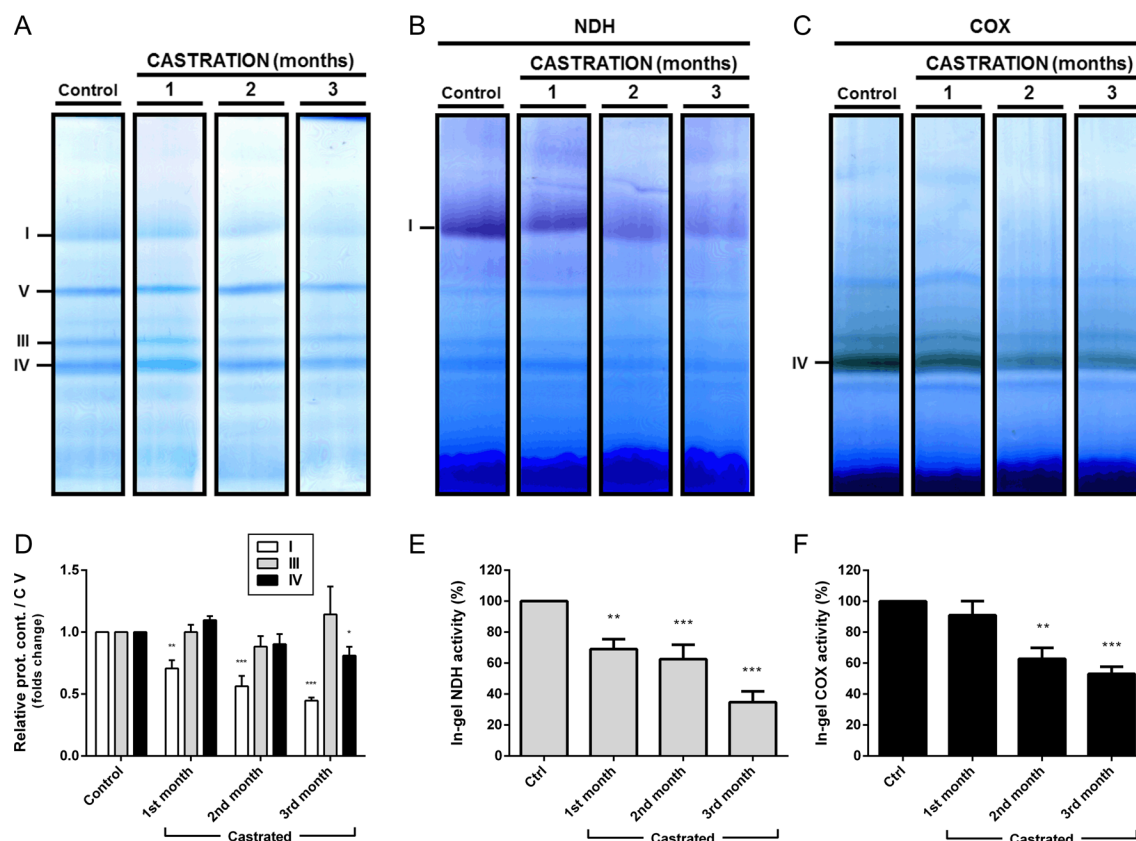
Table 1 Oxygen consumption and respiratory controls in isolated heart mitochondria from control (Ctrl) and castrated (Cast) female rats at different times after surgery.

Condition	+ Glutamate–malate			+ Succinate–rotenone		
	State 4	State 3	RC	State 4	State 3	RC
1st month						
Ctrl	36 ± 10	178 ± 42	5.2 ± 1.2	54 ± 9	153 ± 31	2.8 ± 0.2
Cast	56 ± 8	192 ± 36	3.4 ± 0.5*	70 ± 8	186 ± 35	2.7 ± 0.6
2nd month						
Ctrl	32 ± 6	152 ± 23	4.8 ± 1.1	71 ± 23	190 ± 36	2.8 ± 0.8
Cast	92 ± 19**	164 ± 27	1.9 ± 0.6***	70 ± 14	172 ± 19	2.5 ± 0.5
3rd month						
Ctrl	33 ± 6	165 ± 16	5.1 ± 1.1	49 ± 13	141 ± 29	3.0 ± 0.9
Cast	62 ± 13*	96 ± 16**	1.6 ± 0.4***	54 ± 19	157 ± 40	3.1 ± 0.9

Oxygen consumption was measured at 25°C, incubating mitochondria in 1.5 mL of a medium containing 125 mM KCl, 3 mM phosphate, 2 mM MgCl$_2$, 10 mM HEPES, pH 7.3 and either 5 mM glutamate + 5 mM malate or 10 mM succinate + 5 µg/mL rotenone as substrates. To induce phosphorylating state (state 3), 300 µM ADP was added to the reaction chamber. Mitochondrial respiratory control (RC) is defined as the ratio between the rate of oxygen consumption in phosphorylating and non-phosphorylating states (RC = state 3/state 4). Values of oxygen consumption are expressed as ngAO/min.mg prot. Data of six independent experiments are shown as the mean ± S.D. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ with respect to each Ctrl value.
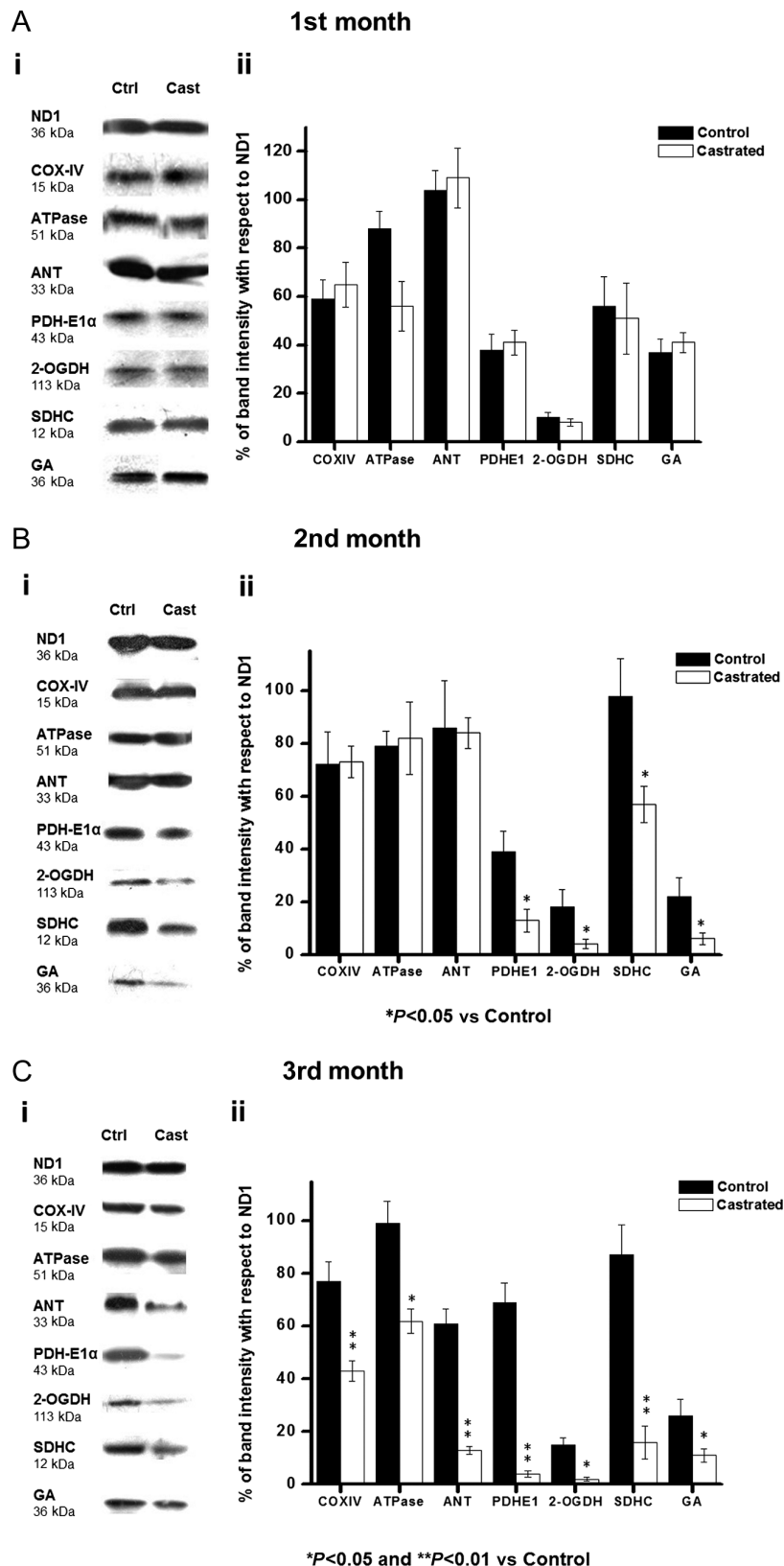
Figure 1

Progressive mitochondrial protein contents modifications during the first three months after oophorectomy. Panel A, one month; panel B, two months; panel C, third month after castration. Left panels (i) western blot analysis of proteins from intact (Ctrl) and castrated (Cast) female rat mitochondria. ND1, NADH ubiquinone oxidoreductase (complex I); COX IV, cytochrome *c* oxidase subunit 4; ATPase, ATP synthase subunit 5B (beta); ANT, adenine nucleotide translocase; PDH-E1α, pyruvate dehydrogenase subunit E1; 2-OGDH, α-ketoglutarate dehydrogenase; SDHC, succinate dehydrogenase subunit B; GA, glutaminase. Right panels (ii), variations in protein contents compared to the control ND1. Representative blots and data from three independent experiments; *$P < 0.05$ and **$P < 0.01$.

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Figure 2
Progressive effects of castration on heart mitochondrial OxPhos complexes I, III, IV and V from female rats. Lanes are from control (Ctrl) and 1-, 2- and 3-month castrated rat heart mitochondrial samples. Isolated mitochondria were solubilized with lauryl-maltoside (LM) 2 mg/mg protein before electrophoretic separation. (A) Different samples were resolved by BN-PAGE in a 4–12% polyacrylamide gradient gel and were subjected to Coomassie staining. (B) In-gel NADH dehydrogenase activity (NDH); 1 mM NADH and 0.5 mg/mL Nitrotetrazolium blue chloride (NTB). (C) In-gel cytochrome *c* oxidase activity (COX); 0.04% diaminobenzidine and 0.02% cytochrome *c*. (D, E and F) Densitometry analysis of different protein bands from panels A (complexes I, III, IV and V), B (complex I in-gel activity (NDH)) and C (complex IV in-gel activity (IV)), respectively; $*P < 0.05$, $**P < 0.01$, $***P < 0.01$. Representative figures from 3 independent gels. Respiratory chain complexes of interest are marked as I and IV. ATP synthase (V) was used as loading control. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-16-0161.

Supplementary Fig. 1). At the 3rd month after surgery, state 3 decreased near to half (Table 1). These results suggested a dysfunction of complex I, which is highly sensitive to stress and can be regulated by estrogens (Chen *et al.* 2009).

Previously, in rats tested at the 4th month after castration, we detected changes in different mitochondrial OXPHOS-related proteins such as cytochrome *c* oxidase, ATP synthase, adenine nucleotide translocase (ANT), pyruvate dehydrogenase subunit 1 (PDH-E1α), 2-oxoglutarate dehydrogenase (2-OGDH), succinate dehydrogenase subunit C (SDHC) and glutaminase (GA). These data led to measure the activity and contents of these proteins at different times after oophorectomy (Fig. 1).

At the 1st month, Cast rats showed similar contents of mitochondrial OXPHOS-related proteins as those of Ctrl (Fig. 1A). Then, at 2nd and 3rd months, some of these proteins gradually changed their expression (Fig. 1B and C). For example, at the 2nd month, there was a perceptible decrease in 2-OGDH, SDHC and GA (Fig. 1B) and later on the decrease was more evident (from 0.5 to 5 times approximately) for most proteins, particularly for PDH (Fig. 1C). Besides, it was of interest to determine if these low levels of protein expression correlated with changes in OXPHOS complexes function; therefore, these enzymes were explored.

The amount of complexes I, III, IV and V was determined by BN-PAGE (Fig. 2A). After oophorectomy, a progressive decrease in complex I was observed (Fig. 2A) and evidenced further by densitometry (Fig. 2D). At the 3rd month, a slight decrease in complex IV content was also present (Fig. 2A and D). As there are only subtle changes in the amount of complexes III and V (Fig. 2A

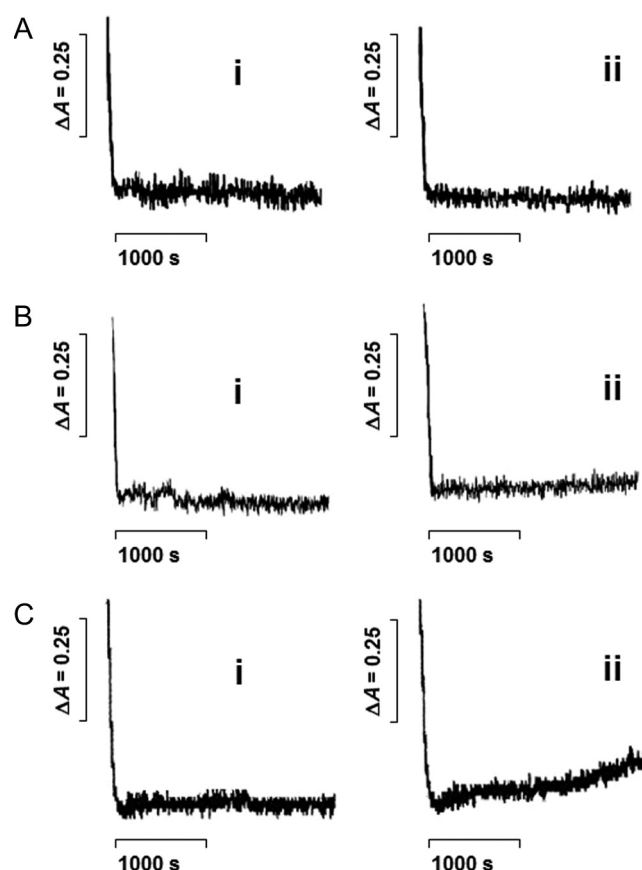**Table 2** Effect of castration on the mitochondrial enzyme activities at different times (months) after surgery.

Enzyme	Condition	Activity (%)	Activity (%)/CS activity (%)
Citrate synthase	Control	100 ± 10[a]	
	Castrated		
	1st month	95 ± 9	
	2nd month	90 ± 12	
	3rd month	85 ± 12	
Complex I	Control	100 ± 6[b]	1
	Castrated		
	1st month	90 ± 6	0.95
	2nd month	62 ± 10**	0.69**
	3rd month	31 ± 8***	0.36***
Complex II	Control	100 ± 13[c]	1
	Castrated		
	1st month	94 ± 15	0.99
	2nd month	83 ± 5	0.92
	3rd month	84 ± 16	0.98
Complex IV	Control	100 ± 13[d]	1
	Castrated		
	1st month	91 ± 21	0.96
	2nd month	50 ± 12**	0.56**
	3rd month	47 ± 8***	0.55***
Pyruvate dehydrogenase	Control	100 ± 14[e]	1
	Castrated		
	1st month	94 ± 12	0.99
	2nd month	54 ± 10**	0.60**
	3rd month	10 ± 2***	0.12***
2-Oxoglutarate dehydrogenase	Control	100 ± 12[f]	1
	Castrated		
	1st month	102 ± 10	1.07
	2nd month	48 ± 6***	0.53***
	3rd month	30 ± 4***	0.35***

100% of activity corresponds to: [a]444.13 ± 43.9 nmol DTNB/min·mg prot; [b]627.2 ± 54.6 nmol NADH/min·mg prot; [c]141.6 ± 18.9 nmol DCPIP/min·mg prot; [d]620 ± 83.3 ngAO/min·mg prot; [e]34.5 ± 4.7 nmol NADH/min·mg prot; [f]121.8 ± 14.4 nmol NADH/min·mg prot. Activities were measured at room temperature (~25°C). In PDH, OGDH and complex II determinations, rotenone 10 µM was added to prevent the oxidation of the NADH or reverse electron transfer by complex I. Data from three-six independent experiments. **$P<0.01$, ***$P<0.001$ with respect to each control value.

and D), the last one was used as loading control. Only one control (1st month) is shown in Fig. 2 as no differences were observed throughout the three months (data not shown). Furthermore, in-gel activities for complexes I (Fig. 2B) and IV (Fig. 2C) decreased as post-castration time increased. Once again, densitometry analysis confirmed the differences in both NDH (Fig. 2E) and COX (Fig. 2F) activities at two and three months after castration.

Individual activities of the enzymes that decreased after oophorectomy were determined to verify our findings. Citrate synthase (CS) activity was almost the same at different post-castration times; only a slight decrease at the 3rd month was detected (Table 2).

Therefore, to discard the effects of different yield or stability of mitochondria on enzyme activities, data were also normalized to their respective CS activities. Complex I activity decreased as post-oophorectomy time increased in a similar way as observed by in-gel staining (Table 2). In addition, complex II activity did not change in any case (Table 2). The activities of complexes III and V were not determined as their respective relative contents did not change (Fig. 2A). PDH and 2-OGDH activities were also determined spectrophotometrically. At the 1st month after oophorectomy, no differences were found in enzyme activities, although beginning on the 2nd month, both

Figure 3
Effect of castration on Ca²⁺ transport by heart mitochondria isolated from female rats at different castration times. Mitochondrial protein (2 mg) was added to 3 mL of a medium containing 125 mM KCl, 10 mM succinate, 10 mM HEPES, 3 mM phosphate, 100 µM ADP, 100 µM CaCl₂, 5 µg rotenone and 50 µM arsenazo III. Arsenazo III absorbance changes were followed at 675–685 nm; room temperature. Panel A, trace (i) shows intact female mitochondria from 1 month; trace (ii) shows castrated female mitochondria from 1 month; panel B trace (i) shows intact female mitochondria from 2 months; trace (ii) shows castrated female mitochondria from 2 months; panel C trace (i) shows intact female mitochondria from 3 months; trace (ii) shows castrated female mitochondria from 3 months. Representative traces from 10 independent experiments.

A

B
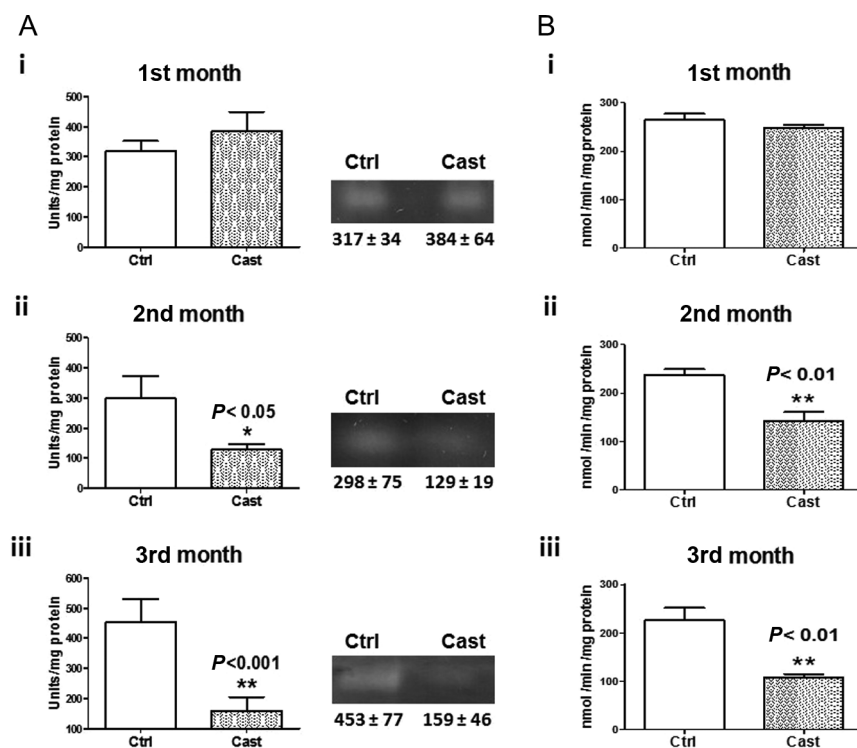
Figure 4

Superoxide dismutase (MnSOD) and aconitase activities in heart mitochondria from control and castrated female rats. Panel A figure (i) shows MnSOD activity in heart mitochondria from control (Ctrl) and castrated (Cast) rats after 1st month; figure (ii) shows MnSOD activity in Ctrl and Cast rats at the 2nd month; figure (iii) shows MnSOD activity in Ctrl and Cast at the 3rd month. Representative figures from 5 independent gels; images are representative of 10 separate experiments. Panel B trace (i) shows aconitase activity in Ctrl and Cast heart mitochondria at the 1st month; trace (ii) shows aconitase activity at the 2nd month and trace (iii) shows aconitase activity at the 3rd month. The results are expressed as the mean ± S.D. from 10 different experiments. Unpaired *t*-test was used for statistical analysis. *$P < 0.05$, **$P < 0.01$.

decreased (Table 2). This was more evident at the 3rd month after surgery where PDH activity decreased almost 10 times and 2-OGDH almost 3 times (Table 2).

Ca^{2+} overaccumulation is considered as another effect of oophorectomy on mitochondria (Pavón *et al.* 2012). Therefore, it was interesting to study whether this parameter changed in heart mitochondria. As Fig. 3A shows, at 1st month, there was no difference between Ctrl (trace i) and Cast (trace ii). At the 2nd month, a minimal difference was present (Fig. 3B, i and ii). Nonetheless, 3rd month Cast mitochondria exhibited a mild loss in the capacity to retain Ca^{2+} (Fig. 3C). After 45 min, these mitochondria released about a 30% of Ca^{2+} (Fig. 3C).

Heart mitochondria are equipped with effective ROS scavenging systems. Dysfunctions in these systems are directly related to cardiovascular disease (Matthews & Gustafsson 2003, Nilsen & Diaz Brinton 2003, Orshal & Khalil 2004, Psarra & Sekeris 2008, Irwin *et al.* 2011). Earlier observations have evidenced the influence of estrogens on the expression and function of antioxidant proteins such as MnSOD (Baños *et al.* 2005a,b). These evidences raise the possibility of oxidative damage in Cast rats. To test if this condition was present, MnSOD and aconitase activities were quantified (Fig. 4 panels A, B) and malondialdehyde levels were measured (Fig. 5). In regard to MnSOD activity, it was found that at the 1st month after oophorectomy, there were no differences

between Ctrl and Cast groups (Fig. 4A, i). However, at the 2nd month, activity was lower in Cast group, which was statistically significant (Fig. 4, ii). This difference in MnSOD activity was even more obvious at the 3rd month (Fig. 4, iii).
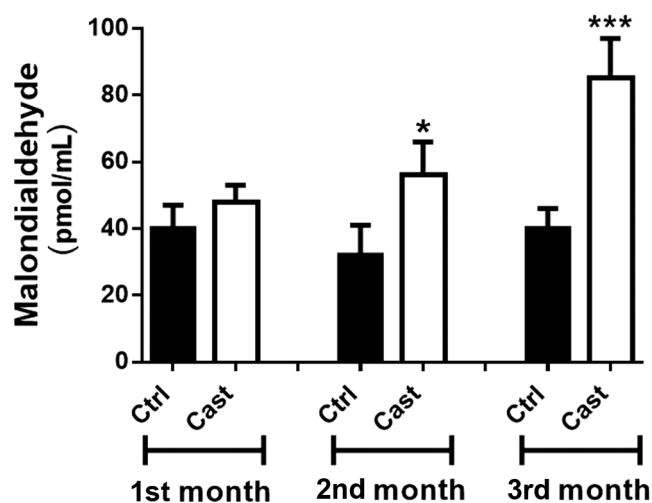
Figure 5

Lipoperoxidation expressed as malondialdehyde generation in heart mitochondria from Ctrl and Cast female rats. The results are expressed as mean ± S.D. for 10 different samples per group analyzed. 2 mg of protein were used and malondialdehyde was separated at −25 kV/4 min at 267 nm. Results were expressed as pmol/mL. *$P < 0.05$, ***$P < 0.001$.
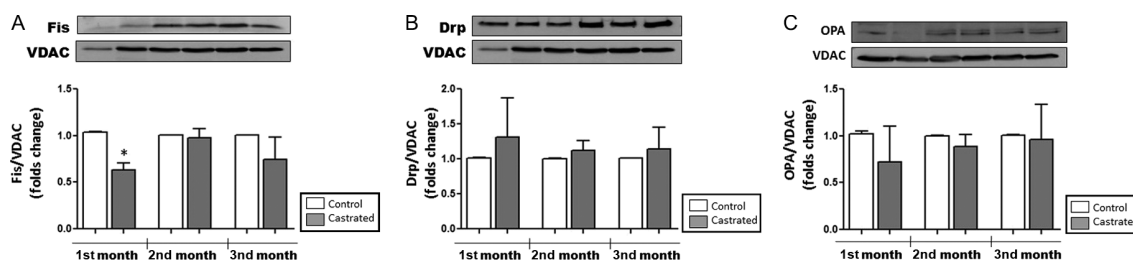
Figure 6

Western blot detection of proteins Fis-1, Drp-1 and OPA-1. Panel A shows content of Fis-1 in each experimental group; Panel B shows the content of Drp-1 and Panel C, OPA-1 content. In all cases, 30 µg of each sample were loaded per lane. VDAC was used as loading control. Bars represent mean ± s.e.m. of 3 independent experiments; *$P < 0.05$.

In a previous study, we found evidence that estrogens may control the expression of some proteins involved in OXPHOS (Pavón *et al.* 2012). Thereby, a dysfunction in the respiratory chain should also involve the overproduction of ROS. These ROS would inactivate enzymes containing iron–sulfur centers, e.g. aconitase and complexes I, II and III. Aconitase inactivation is an appropriate marker of the superoxide production on the matrix side (Muller *et al.* 2004). No differences were detected at the 1st month (Fig. 4B, i), whereas inhibition was present from the 2nd month (40%) (Fig. 4B, ii) and increased during the 3rd month (54%) (Fig. 4B, iii). Thus, higher production of ROS or defective detoxification mechanisms will damage lipids, proteins and DNA. An indicator of oxidative damage is malondialdehyde (MDA), whose level increased in the Cast group beginning at the 2nd month and becoming even higher at the 3rd month (Fig. 5).

All previous data indicated that oophorectomy leads to dysfunctional mitochondria and increased levels of oxidative damage. Both effects have also been implicated in abnormal mitochondrial dynamics (fusion and fission) (Ong & Hausenloy 2010, Wohlgemuth *et al.* 2014). In an effort to determine if oophorectomy is associated with mitochondrial dynamics, we measured the expression of the fission-associated proteins Fis-1 and Drp-1, the fusion-associated protein OPA-1 and the apoptosis-related proteins Bcl-2 and Bax. Figure 6 shows no significant changes in the

expression of the dynamic-related proteins at any time after castration. In fact, only Fis-1 decreased at the first month, but increased back to Ctrl levels at the second and third month. In addition, we found a decrease in expression of Bcl-2 after the first month of castration, whereas an increase was detected at the third month (Fig. 7).

Mitochondrial dysfunction can result from a decrease in protein content (Chen *et al.* 2004, 2009) and activity of OXPHOS enzymes (Stirone *et al.* 2005). These may affect the important functions for the cell (e.g. Ca^{2+} uptake, metabolite transport, and so forth) and mitochondrial biogenesis (Mattingly *et al.* 2008). All such changes would presumably lead to a decrease in metabolites oxidation. Under our experimental conditions, it is probable that most of them were present and higher at the 3rd month after oophorectomy.

Discussion

After the 1st month post-oophorectomy, isolated mitochondria in the presence of glutamate–malate exhibited a slight decrease in respiratory coupling compared to the controls (Table 1). At the 2nd and 3rd months, respiratory coupling in Cast groups was almost 40% and a decrease in complex I content and activity was detected (Fig. 2A, B, Tables 1 and 2). By contrast, using succinate–rotenone, respiratory activities and coupling
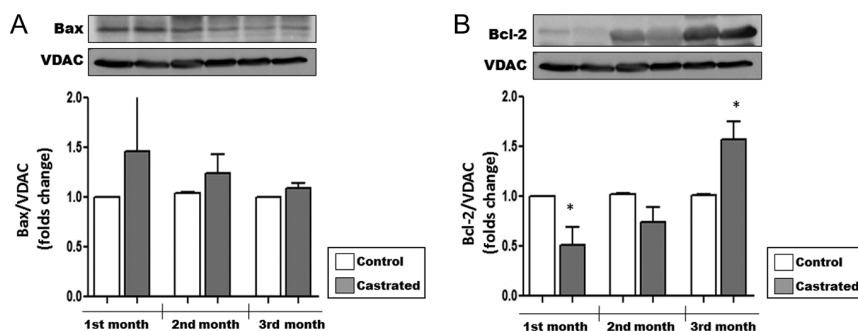
Figure 7

Western blot detection of proteins Bax and Bcl-2. Panel A shows the content of Bax in each experimental group. Panel B shows content of Bcl-2 in each experimental group. VDAC was used as loading control. Bars represent mean ± s.e.m. of 3 independent experiments; *$P < 0.05$.

were not different between Ctrl and Cast groups at any time after surgery. Individual complex II activities normalized to CS were not affected in Cast groups (Table 2) even if SDHC subunit expression decreased ~40% (Fig. 1). COX IV and ATPase β subunits decreased, suggesting a general decrease in OXPHOS-related proteins. A decrease in complex IV content (~85%) and activity (~55%) was observed, whereas complexes III and V were constant (Fig. 2 and Table 2).

Although the amount and activity of complex IV decreased, electron flux through complexes I and II was not limited. In complex I-dependent respiration, flux control mostly lies on complexes I and III, whereas in complex II-dependent respiration, control lies on complexes III and IV (Bianchi *et al.* 2004). The stoichiometry for complexes I:II:III:IV is 1:1.5:3:6–7, respectively (Schägger & Pfeiffer 2001); thus, a partial decrease in complexes II and IV contents would not be expected to modify respiratory activity as would for instance, complex I deficiency. After oophorectomy, complex IV activity decreased up to 300 ngAO/min·mg prot (~50% of the V_{max}); nevertheless, this value was still higher than the control respiratory rate in state 3 (Table 1). Thus, in Cast samples, succinate oxidation would not decrease as complex IV is still in excess compared to the other three complexes. Conversely, in the NADH–O_2 reaction, glutamate and malate were oxidized through different pathways to produce NADH and feed the respiratory chain via complex I. That is, electron flux was mostly limited by complex I. In addition to the gradual loss of complex I contribution (Fig. 2 and Table 2), we found a lower activity in two of NAD-dependent dehydrogenases from the Krebs cycle: PDH and 2-OGDH (Fig. 1 and Table 2), which must have limited even further the rate of electron transfer through this pathway. Further studies are required to explore these individual pathways in heart mitochondria after oophorectomy.

Furthermore, it has been described that the transcription of the mRNA encoding for complex I subunits ND1, NDUFS7 and NDUFS8 might be regulated by estrogens (Too *et al.* 1999, Noguchi *et al.* 2002, Chen *et al.* 2009). Thus, in the absence of estrogens, a decrease in complex I content and activity would be expected as observed here (Fig. 2A and B). Remarkably, expression of subunit ND1 did not change after castration (Fig. 1). ND1 is a mitochondrial DNA-encoded protein, whereas NDUFS7 and NDUFS8 are codified by nuclear genes (Chen *et al.* 2009). The last two subunits are associated with each other and are also known to be part of the catalytic site for ubiquinone (Sánchez-

Caballero *et al.* 2016). We have not analyzed yet the expression of nuclear-encoded subunits, which are probably more susceptible to estrogenic regulation than the mitochondrial-encoded proteins as other nuclear proteins were clearly downregulated after oophorectomy (e.g. SDHC, COX IV, GA and PDH-E1α) (Fig. 1). For instance, GA expression is upregulated via estrogen-related receptor alpha (ERRα during cell differentiation (Huang *et al.* 2016)). Estrogen receptors are known to play a crucial role in the transcriptional control of mitochondrial function and energy metabolism (Hsieh *et al.* 2006, Huang *et al.* 2016).

Inability to regulate matrix solutes is among the first alterations in damaged mitochondria. Here, Cast mitochondria exhibited dysfunction in Ca^{2+} accumulation at the 3rd month after surgery (Fig. 3C). The inability of isolated heart mitochondria to hold Ca^{2+} and its further release may be due to MPTP activation and transmembrane potential depletion, a condition fully achieved at the 4th month (Hunter *et al.* 2012, Pavón *et al.* 2012). Different stress conditions such as ischemia, hypoxia, oxidative stress and cytotoxic drugs were identified as inducers of MPTP. A link between estrogen deficiency and MPTP activation is suggested, but then again, the mechanism remains obscure.

Cardiac mitochondria exist in two functionally distinct populations: subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) (Palmer *et al.* 1977). SSM are released by tissue homogenization leaving behind skinned myocytes; the liberation of IFM from skinned myocytes requires a brief exposure to a protease. Aging (Fujioka *et al.* 2011, Suh *et al.* 2003) and caloric restriction (Hofer *et al.* 2009) studies have shown that age-related decline in mitochondrial capacity affects IFM, whereas SSM located beneath the plasma membrane remain unaffected. Changes in morphology and disposition in IFM without estrogens were reported previously (Zhai *et al.* 2000); the SSM population was not studied by these authors. Based on these observations, it would be very interesting to determine whether changes occur only in the IFM population.

Moreover, it has been described that oxidative stress in Cast mitochondria and antioxidant systems, such as MnSOD, depends on estrogens (Baños *et al.* 2005*a*,*b*, Pedram *et al.* 2006, Bellanti *et al.* 2013). Thus, estrogen loss probably impairs SOD activity increasing ROS (Borras *et al.* 2007), as in fact we found (Figs 4 and 5). ROS damaged aconitase and increased malondialdehyde. Also, key enzymes involved in mitochondrial bioenergetics, such as 2-oxoglutarate dehydrogenase (OGDH), were

probably affected by ROS (Gibson *et al.* 2000, Starkov *et al.* 2004, Martin *et al.* 2005).

In regard to the possibility of disequilibrium in apoptosis, we evaluated the expression of two members of the Bcl-2 protein family, which regulate apoptosis. We measured Bax, which is a pro-apoptotic protein and Bcl-2 that is an anti-apoptotic protein. Although Bax remained constant, Bcl-2 levels decreased at the 1st month after castration and then increased at the 3rd month (Fig. 7). However, no increase in apoptosis was observed (data not shown).

Our findings provide an important overview of the cardioprotective effect of estrogens on mitochondrial bioenergetics and dynamics. At menopause, the decrease in estrogens may contribute to cardiac vulnerability by playing important roles in intracellular energy and redox-dependent intracellular signaling. Mitochondrial contents have to adapt to cellular growth rate and meet cell requirements. In this landscape, estrogens could orchestrate a comprehensive cardiac transcriptional program including use of substrates, production and transport of ATP and modulation of antioxidant enzymes (Noguchi *et al.* 2002, Baños *et al.* 2005*a,b*, Klinge 2008). Estrogen levels in rats vary and seem to affect mitochondrial functions. In rats, it is known that steroidogenesis by testes or ovaries are reactivated at 30–45 days of postnatal life (Banu & Aruldhas 2002) reaching their maximum levels of estrogens at puberty at 10 weeks of age (Ojeda *et al.* 2007). To avoid hormonal influences and isolate estrogen depletion-related damage, three-week-old rats were used. Our animals will not be exposed to estrogens in their lifetime unless these are provided exogenously. Thus, our model is not exactly equivalent to menopause.

Sexual hormones affect diverse non-reproductive tissues including immune, central nervous and skeletal systems, as well as cells from liver, skin and kidneys (Smith *et al.* 1994, Carani *et al.* 1997, Kovats 2012, 2015, Koss *et al.* 2015, Khalid & Krum 2016, Khan & Ansar Ahmed 2016). There is a variety of biological effects, many of which bear no clear relationship to their primary reproductive functions. Particularly in rodents, estrogens have many actions that may affect the body weight and adiposity independently of feeding patterns, including energy expenditure, gastrointestinal function, basal metabolism, growth and body composition. For example, estrogen deprivation decreases triiodothyronine (Thomas *et al.* 1986). Thyroid hormones and estrogens exhibit overlapping functions and cross-modulate genes involved in reproduction and

sexual behavior (Vasudevan *et al.* 2001). On the other hand, estrogens prevent hypertension by modulating the renin–angiotensin–aldosterone system (RAAS), acting not only on the kidney, heart and vasculature but also on the central nervous system (Sullivan 2008, Sandberg & Ji 2012, O'Donnell *et al.* 2014). Estrogens also modulate pituitary growth hormone (GH) secretion and signaling (Sinha *et al.* 1979, Kerrigan & Rogol 1992, Baik *et al.* 2011, Fernández-Pérez *et al.* 2013). Thus, after menopause or oophorectomy, a precipitous decline in insulin levels and sensitivity is present, parallels an increase in fat mass and elevations in circulating inflammatory markers, low-density lipoproteins (LDL), triacylglycerols and fatty acids, i.e. estrogen deprivation leads to metabolic syndrome (Pfeilschifter *et al.* 2002, Sites *et al.* 2002, Carr 2003, Toth *et al.* 2006). Estrogens have also been linked to cholecystokinin, increasing its satiation action (Asarian & Geary 2006). Low estrogen levels promote increased body weight and adiposity (Mauvais-Jarvis *et al.* 2013). This was also observed in our experimental groups (1st month Ctrl 75 ± 10 g vs Cast 85 ± 13 g; 2nd month Ctrl 109 ± 14 g vs Cast 137 ± 14 g; 3rd month Ctrl 216 ± 21 g vs Cast 269 ± 13 g).

Our study evaluated the progression of the oophorectomy-evoked changes in cardiac mitochondrial OXPHOS functions. These modifications were fully established only after three months of castration and not at 2 weeks as in mitochondria from other organs (Li *et al.* 2009, Cavalcanti-de-Albuquerque *et al.* 2014). These effects clearly mimic those observed in human menopause (Barrett-Connor 2013). Thus, our data provide strong evidence in favor of estrogen substitution therapy (Al-Safi & Santoro 2014, Whayne & Mukherjee 2015).

Surgical castration is generally favored as a model of menopause. However, 4-vinylcyclohexene diepoxide (VCD) has been recently proposed to reproduce 'menopausal conditions' as it destroys preantral ovarian follicles preserving ovaries (Hoer *et al.* 2001, Mayer *et al.* 2002). VCD increases markers of oxidative damage and inflammation (in liver and kidney) and also caspases 9 and 3 and other side effects (Abolaji *et al.* 2016). In heart, these secondary effects have not been discarded, so, we advocate surgery over VCD as a model to study estrogen depletion.

In conclusion, in rat heart mitochondria, estrogen deprivation gradually leads to (a) decreased contents and function of aerobic metabolism-related proteins such as complex I, complex IV, ATPase-b, ANT, PDH-E1α, 2KGDH, SDHC and GA; (b) impaired mitochondrial

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Ca²⁺ transport; (c) decreased ROS-detoxifying enzyme activities and (d) increased lipoperoxidation (MDA). By contrast, it is suggested that fusion and fission were not affected, as only small and reversible changes in proteins Fis-1, Drp-1 and OPA-1 were detected (Fig. 6). In addition, mitochondrial biogenesis probably was not affected as CS activity did not change after castration (Table 2). All oophorectomy effects were progressive; at month 1, some were hardly detectable and gradually became more evident at months 2 and 3. Our evidence suggests that estrogens regulate mitochondrial function (Hall *et al.* 2001, Duckles *et al.* 2006, Pedram *et al.* 2006, Psarra & Sekeris 2008, Yang *et al.* 2009), probably through transcriptional changes (Orshal & Khalil 2004, Klinge 2008, Mattingly *et al.* 2008) that lead to loss of OXPHOS.

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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