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Mitochondria from distinct tissues are differently affected by 17β-estradiol and tamoxifen

Paula I. Moreira[a,b,*], José B.A. Custódio[a,c], Elsa Nunes[a,b], Paulo J. Oliveira[a,d], António Moreno[a,d,e], Raquel Seiça[b,f], Catarina R. Oliveira[a,g], Maria S. Santos[a,d,**]

[a] Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal
[b] Institute of Physiology, Faculty of Medicine, University of Coimbra, 3000-354 Coimbra, Portugal
[c] Institute of Biochemistry, Faculty of Pharmacy, University of Coimbra, 3000-295 Coimbra, Portugal
[d] Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, 3004-517 Coimbra, Portugal
[e] Institute of Marine Research, University of Coimbra, 3004-517 Coimbra, Portugal
[f] Institute of Biomedical Research on Light and Image, University of Coimbra, 3000-548 Coimbra, Portugal
[g] Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3000-548 Coimbra, Portugal

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ABSTRACT

This study was aimed to analyse and compare the bioenergetics and oxidative status of mitochondria isolated from liver, heart and brain of ovariectomized rat females treated with 17β-estradiol (E2) and/or tamoxifen (TAM). E2 and/or TAM did not alter significantly the respiratory chain of the three types of mitochondria. However, TAM significantly decreased the phosphorylation efficiency of liver mitochondria while E2 significantly decreased the phosphorylation efficiency of heart mitochondria. E2 also significantly decreased the capacity of heart and liver mitochondria to accumulate Ca^{2+} this effect being attenuated in liver mitochondria isolated from E2 + TAM-treated rat females. TAM treatment increased the ratio of glutathione to glutathione disulfide (GSH/GSSG) of liver mitochondria. Brain mitochondria from TAM- and E2 + TAM-treated females showed a significantly lower GSH/GSSG ratio. However, heart mitochondria from TAM- and E2 + TAM-treated females presented a significant decrease in GSSG and an increase in GSH/GSSG ratio. Thiobarbituric acid reactive substances levels were significantly decreased in liver mitochondria isolated from E2 + TAM-treated females. Finally, E2 and/or TAM treatment significantly decreased the levels of hydrogen peroxide produced by brain mitochondria energized with glutamate/malate. These results indicate that E2 and/or TAM have tissue-specific effects suggesting that TAM and hormonal replacement therapies may have some side effects that should be carefully considered.

1. Introduction

Several studies have shown that by replacing the estrogen lost at menopause, many of the manifestations of aging including osteoporosis, cardiovascular disease and the decline of cognitive function would be prevented. This attractive view initially led to the widespread use of hormone replacement therapy (HRT) after the menopause, but following the recent publication of the results of the Women's Health Initiative Study, this enthusiasm has diminished, leading to new clinical recommendations. Although HRT has potential benefits with regard to menopausal symptoms and in the prevention of osteoporosis-related fractures and colorectal cancer, potential risks for pulmonary embolism, stroke, coronary heart disease and breast cancer have been described [1,2].

Breast cancer is the most frequently diagnosed and the second cause of cancer death in women, thus making breast cancer a most feared disease. However, breast cancer mortality is decreasing and one of the main contributory factors to this marked improvement in survival has been the more widespread use of systemic therapy in early-stage disease [3]. Although the safety of HRT has been questioned in breast cancer survivors, it has been shown that HRT may be safe in women with receptor-negative or receptor-positive cancers in the presence of tamoxifen (TAM) [3]. Indeed, the synthetic nonsteroidal antiestrogen drug TAM is widely used in the chemotherapy of breast cancer [4] and has been proposed as a prophylactic agent against this disease. TAM undergoes extensive hepatic metabolism being 4-hydroxytamoxifen (OHTAM) and

* Corresponding author at: Institute of Physiology, Faculty of Medicine, University of Coimbra, 3000-354 Coimbra, Portugal. Tel.: +351 239480012; fax: +351 239480034.
** Corresponding author at: Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, 3004-517 Coimbra, Portugal. Tel.: +351 239834729; fax: +351 239826798.
E-mail addresses: pismoreira@gmail.com (P.I. Moreira), mssantos@ci.uc.pt (M.S. Santos).

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endoxifen its major active metabolites responsible for its antiestrogenic activity [5]. However, it is now known that under certain circumstances and in certain tissues, in addition to acting as competitive inhibitors of endogenous estrogen, estrogen antagonists can also exert estrogenic agonist properties [6–8].

Mitochondria play a central role in both cell life and death [9]. Mitochondria are essential for the production of ATP through oxidative phosphorylation, regulation of intracellular Ca^{2+} homeostasis and are the main generators of intracellular reactive oxygen species (ROS). Mitochondria also play a key role in controlling pathways that lead to apoptosis. Defects of mitochondrial function can result in the excessive production of ROS, induction of the permeability transition pore (PTP) and release of apoptotic proteins. Therefore, changes in the structural and functional characteristics of mitochondria provide a number of primary targets for drugs-induced toxicity and cell death [10]. Indeed, accumulating evidence indicates that both 17β-estradiol (E2) and TAM [11–17] modulate mitochondrial function. However, it is known that mitochondria from different tissues have a distinct behavior in the presence of the same agents [18,19], which can explain several phenomena of differential toxicity. Johnson et al. [20] reported that the mitochondrial proteome from rat brain, liver, heart and kidney present very specific characteristics indicative of differences regarding structure and metabolic activity.

In this study, we aimed to evaluate and compare the effect of E2 and/or TAM treatment on the status of liver, heart and brain mitochondria isolated from ovariectomized female rats. In our experimental set-up, ovariectomy mimicked a postmenopausal state, E2 treatment mimicked an estrogen replacement therapy, TAM treatment mimicked a postmenopausal breast cancer therapy and E2 + TAM treatment mimicked a premenopausal breast cancer therapy or the combination of both therapies in a postmenopausal state. To achieve our goal, we evaluated several mitochondrial parameters from the respiratory chain [states 2, 3 and 4 respiration and respiratory control ratio (RCR)], phosphorylation system [mitochondrial transmembrane potential ($\Delta\Psi$m), membrane depolarization induced by ADP and lag phase of repolarization], mitochondrial Ca^{2+} loading capacity, hydrogen peroxide (H_2O_2) and glutathione levels and lipid peroxidation markers [thiobarbituric acid reactive substances (TBARS) levels].

2. Materials and methods

2.1. Chemicals

4-(2-Hydroxymethyl)-1-piperazineethanesulfonic acid (Hepes), cyclosporin A (CsA), oligomycin, ethylene glycol-bis(β-aminoethylether)NNN′N′-tetraacetic acid (EGTA), tetraphenylphosphonium (TPP^+) were obtained from Sigma (Potugal). Placebo, tamoxifen (25 mg/pellet, 60-day release) and 17β-estradiol pellets (0.5 mg/pellet, 60-day release) were purchased from Innovative Research of America, Sarasota, FL. All the other chemicals were of the highest grade of purity commercially available.

2.2. Ovariectomy, 17β-estradiol and/or tamoxifen treatment and biochemical analyses

Female rats (12–15 weeks) from our animal colony (Laboratory Research Center, University Hospital) were housed five per cage with *ad libitum* access to food (URF1-Charles River, France) and water and maintained at a constant temperature and humidity with a 12 h light/dark cycle. To remove the main source of female hormones, the animals were bilaterally ovariectomized under anesthesia (i.m.) using ketamine chloride (88.5 mg/kg, Park Davies, USA) and chlorpromazine chloride (2.65 mg/kg, Lab. Victoria, Portugal). One week after ovariectomy, pellets of placebo, TAM and/or E2 were implanted in the neck of the animals.

Biochemical analyses of blood samples collected from cardiac puncture were performed with commercial kits used in the Clinical Laboratory of our University Hospital.

2.3. Isolation of mitochondria

Sixty days after the implant of pellets, animals were sacrificed by cervical displacement and decapitation, following procedures approved by the Institutional Animal Care and Use Committee.

Liver mitochondria were isolated by conventional methods [21], with slight modifications. Briefly, animals were sacrificed and the liver was immediately excised finely minced and homogenized in ice-cold medium containing 250 mM sucrose, 5 mM Hepes, 0.5 mM EGTA and 0.1% defatted bovine serum albumin (BSA) (pH 7.4). The homogenate was centrifuged at 2500 rpm (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 10 min at 4 °C. The resulting supernatant was spun at 10,000 rpm for 10 min (at 4 °C) to pellet mitochondria. The mitochondrial pellet was washed twice (2 × 10,000 rpm for 10 min) and suspended in the washing medium. EGTA and BSA were omitted from the washing medium.

Brain mitochondria were isolated by the method of Rosenthal et al. [22], with slight modifications, using 0.02% digitonin to free mitochondria from the synaptosomal fraction. Briefly, the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenized at 4 °C in 10 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/ml defatted BSA, pH 7.4) containing 5 mg of the bacterial protease type VIII. Single brain homogenates were brought to 30 ml and then centrifuged at 2500 rpm (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 5 min. The pellet, including the fluffy synaptosomal layer, was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 10,000 rpm for 10 min. The brown mitochondrial pellet without the synaptosomal layer was then resuspended in 10 ml of medium and centrifuged again at 10,000 rpm for 5 min. The pellet was then resuspended in 10 ml of resuspension medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4) and centrifuged at 10,000 rpm for 5 min. Finally, the mitochondrial pellet was resuspended in 200 μl of resuspension medium.

Heart mitochondria were prepared using a conventional procedure [23]. Briefly, the heart was immediately excised and finely minced in an ice-cold isolation medium containing 250 mM sucrose, 1 mM EGTA, 10 mM Hepes-KOH (pH 7.4) and 0.1% defatted BSA. The minced blood-free tissue was then resuspended in 40 ml of isolation medium containing 0.4 mg protease type VIII per g of tissue and homogenized. Exposure to concentrated protease was limited to 2–3 min in order to minimize loss of mitochondrial membrane integrity. The suspension was incubated for 1 min (4 °C) and then re-homogenized. The homogenate was then centrifuged at 9000 rpm (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 10 min (4 °C). The supernatant fluid was decanted and the pellet, essentially devoid of protease, was gently homogenized to its original volume with a loose-fitting homogenizer. The suspension was centrifuged at 200 rpm for 10 min and the resulting supernatant was centrifuged at 10,000 rpm for 10 min with a final washing medium. The pellet was resuspended using a paintbrush and repelleted twice at 8000 rpm for 10 min. EGTA and defatted BSA were omitted from the final washing medium.

Mitochondrial protein was determined by the Biuret method calibrated with BSA [24].

2.4. Mitochondrial respiration

Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode [25] connected to a suitable recorder in a thermostated water-jacketed closed chamber with magnetic stirring. The reactions were carried out at 30 °C in 1 ml of standard respiratory medium with 1 mg of liver mitochondria or 0.5 mg of heart and brain mitochondria. For liver and heart mitochondria the reaction medium is composed of 130 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM KH_2PO4, 5 mM Hepes and 0.1 mM EGTA (pH 7.4). For brain mitochondria the reaction medium was composed of 100 mM sucrose, 100 mM KCl, 2 mM KH_2PO4, 5 mM Hepes and 0.01 mM EGTA (pH 7.4). States 4 and 3 respiration were initiated with 5 mM glutamate/2.5 mM malate and ADP (150 nmol/mg protein), respectively. Respiration rates were calculated assuming an oxygen concentration of 240 nmol O_2/ml in the experimental medium at 30 °C. The respiratory state 2 (oxygen consumption in the absence of substrate), state 3 (oxygen consumption in the presence of substrate and ADP) and 4 (oxygen consumption after ADP phosphorylation) and respiratory control ratio (RCR = state 3/state 4) were calculated according to Chance and Williams [26]. ADP/O ratio is expressed by the ratio between the amount of ADP added and the oxygen consumed during the state 3 of respiration.

2.5. Membrane potential ($\Delta\Psi m$)

The mitochondrial transmembrane potential ($\Delta\Psi$m) was monitored by evaluating transmembrane distribution of the lipophilic cation TPP^+ (tetraphenylphosphonium) with a TPP^+-selective electrode prepared according to Kamo et al. [27] using a $Ag/AgCl_2$—saturated electrode as reference (model MI 402; Microelectrodes, Inc., Bedford, NH). TPP^+ uptake has been measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Linear 1200 recorder. The voltage response of the TPP^+ electrode to log [TPP^+] was linear with a slope of 59 ± 1, in a good agreement with the Nernst equation. Reactions were carried out in a chamber with magnetic stirring in 1 ml of the standard reaction medium containing 3 μM TPP^+. This TPP^+ concentration was chosen in order to achieve high sensitivity in measurements and to avoid possible toxic effects on mitochondria [28]. The $\Delta\Psi$m was estimated by the equation:

$$\Delta\Psi\mathrm{m\,(mV)} = 59\ \log(v/V) - 59\ \log(10^{\Delta E/59} - 1),$$

as indicated by Kamo et al. [27] and Muratsugu et al. [29]. v, V, and ΔE stand for mitochondrial volume, volume of the incubation medium, and deflection of the electrode potential from the baseline, respectively. This equation was derived assuming that TPP^+ distribution between the mitochondria and the medium follows the Nernst equation and that the law of mass conservation is applicable. A matrix volume of 1.1 μl/mg protein was assumed. No correction was made for the "passive" binding contribution of TPP^+ to the mitochondrial membranes because the purpose of the experiments was to show relative changes in potentials rather than absolute values. As a consequence, we can anticipate a slight overestimation on $\Delta\Psi$m values. However, the overestimation is only significant at $\Delta\Psi$m values below 90 mV, therefore, far from our measurements.

Mitochondria (1 or 0.5 mg/ml) in the standard reaction medium supplemented with 3 μM TPP^+ were energized by the addition of 5 mM glutamate/2.5 mM malate. After a steady-state distribution of TPP^+ had been reached (ca. 1 min of recording) ADP was added and $\Delta\Psi$m fluctuations recorded.

Membrane depolarization denotes the decrease in $\Delta\Psi$m induced by ADP phosphorylation. Lag phase of repolarization denotes the time that mitochondria need to re-establish the $\Delta\Psi$m after ADP phosphorylation.

2.6. Mitochondrial calcium fluxes

Mitochondrial calcium loading capacity was measured by monitoring the changes in Ca^{2+} concentration in the reaction medium using the hexapotassium salt of the fluorescent probe Calcium Green 5-N [30]. Mitochondria (heart, 0.1 mg; liver, 0.1 mg and brain 0.2 mg) were resuspended in 2 ml of reaction medium (200 mM sucrose, 10 μM EGTA, 10 mM Tris, 1 mM KH_2PO_4) supplemented with 100 nM of Calcium Green 5-N and 0.2 μM rotenone. After establishing a base line, sequential pulses of Ca^{2+} were added differing in concentration with the type of organ. For the heart and liver mitochondria, 5 pulses of 4 nmol each were used, while for brain mitochondria, 5 pulses of 2 nmol each were used. Mitochondrial calcium uptake was initiated with the addition of succinate (1 mM). EGTA was added at the end of the experiment to calibrate the base line. The amount of calcium accumulated by mitochondria was calculated from the initial signal calibration (fluorescence vs. log [Ca]). Fluorescence was measured in a water-jacketed cuvette holder at 30 °C using a Perkin-Elmer spectrofluorometer LS-50 B with an excitation wavelength of 506 nm (slit 5 nm) and an emission wavelength of 532 nm (slit 5 nm).

2.7. Glutathione levels

Glutathione (GSH) and glutathione disulfide (GSSG) glutathione were determined with fluorescence detection after reaction of the supernatant of the H_3PO_4/NaH_2PO_4–EDTA or H_3PO_4/NaOH deproteinized mitochondria solution, respectively, with *o*-phthalaldehyde (OPT), pH 8.0, according to Hissin and Hilf [31]. In brief, mitochondria (1 or 0.5 mg) resuspended in 1.5 ml phosphate buffer (100 mM NaH_2PO_4, 5 mM EDTA, pH 8.0) and 500 μl H_3PO_4 4.5% were rapidly centrifuged at 50,000 rpm (Beckman, TL-100 Ultracentrifuge) for 30 min. For GSH determination 100 μl of the supernatant was added to 1.8 ml phosphate buffer and 100 μl OPT. After mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420 nm. For GSSG determination 250 μl of the supernatant was added to 100 μl of *N*-ethylmaleymide (NEM) and incubated at room temperature for 30 min. After the incubation 140 μl of the mixture was added to 1.76 ml NaOH (100 mM) buffer and 100 μl OPT. After mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420 nm and 350 nm emission and excitation wavelengths, respectively (slits 5, 5). The GSH and GSSG contents were determined from comparisons with a linear reduced or oxidized glutathione standard curve, respectively.

2.8. Lipid peroxidation

The extent of lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay, according to a modified procedure as described elsewhere [32]. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nmol TBARS/mg protein.

2.9. Hydrogen peroxide production

The rate of H_2O_2 production in isolated mitochondria was determined fluorimetrically using a modification of the method

Table 1
Characterization of placebo-, 17β-estradiol (E2)-, tamoxifen (TAM)- and E2 + TAM-treated ovariectomized female rats.

	Placebo	E2	TAM	E2 + TAM
Body weight gain (%)	50.7 ± 8.6	15.1 ± 2.8**	19.1 ± 2.8**	43.1 ± 8.2
WBC count ($\times 10^9$/l)	7.5 ± 1.2	6.4 ± 1.0	5.8 ± 0.5	8.1 ± 0.9
RBC count ($\times 10^{12}$/l)	8.4 ± 0.2	7.4 ± 0.3*	7.9 ± 0.2	7.6 ± 0.1
PLT count ($\times 10^9$/l)	481.8 ± 99.0	252.3 ± 72.8	421.8 ± 53.8	492.3 ± 58.5
AST (IU/l)	201.0 ± 18.0	176.0 ± 22.5	187.7 ± 30.8	168.7 ± 8.0
ALT (IU/l)	52.7 ± 3.1	52.2 ± 3.6	74.2 ± 15.7	57.0 ± 4.8
ALP (IU/l)	106.3 ± 8.2	68.0 ± 7.4	133.8 ± 18.7#	106.8 ± 16.3
γ–GT (IU/l)	0.58 ± 0.2	0.38 ± 0.2	0.58 ± 0.2	0.36 ± 0.2
Total cholesterol (mg/dl)	87.0 ± 3.2	95.8 ± 3.6	81.4 ± 5.1	89.5 ± 12.4
HDL cholesterol (mg/dl)	55.4 ± 1.5	62.5 ± 3.6	53.6 ± 3.9	59.0 ± 7.9
Triglycerides (mg/dl)	126.6 ± 11.5	379.3 ± 42.1***	109.1 ± 16.9	198.4 ± 28.7

WBC – white blood cells; RBC – red blood cells; PLT – platelets; AST – aspartate aminotransferase; ALT – alanine aminotransferase; ALP – alkaline phosphatase; γ-GT – γ-glutamyl transferase; HDL – high density lipoprotein.
The values are expressed as mean ± S.E.M. of five to six animals.
* $p < 0.05$ when compared with placebo-treated ovariectomized female rats.
** $p < 0.01$ when compared with placebo-treated ovariectomized female rats.
*** $p < 0.001$ when compared with placebo-treated ovariectomized female rats.
# $p < 0.05$ when compared with E2-treated ovariectomized female rats.

Table 2
Effect of 17β-estradiol (E2) and/or tamoxifen (TAM) on respiratory chain parameters of liver (A), heart (B) and brain (C) mitochondria.

	State 2	State 3	State 4	RCR	ADP/O
A. Liver mitochondria					
Placebo	5.92 ± 0.56	46.64 ± 3.39	6.97 ± 0.64	6.23 ± 1.17	3.59 ± 0.96
E2	6.55 ± 0.62	57.89 ± 5.44	8.86 ± 0.81	6.55 ± 1.08	2.97 ± 0.61
TAM	6.12 ± 1.95	45.13 ± 7.47	6.62 ± 0.89	6.69 ± 1.54	2.95 ± 0.65
TAM + E2	5.93 ± 1.19	46.02 ± 4.52	6.12 ± 1.18	8.15 ± 2.44	2.97 ± 0.56
B. Heart mitochondria					
Placebo	25.05 ± 5.28	137.79 ± 12.13	54.45 ± 5.03	2.73 ± 0.26	3.04 ± 0.25
E2	28.10 ± 7.35	125.70 ± 16.46	46.61 ± 6.65	2.75 ± 0.12	3.37 ± 0.22
TAM	24.49 ± 4.00	127.39 ± 10.01	46.81 ± 4.66	2.84 ± 0.29	2.39 ± 0.16
TAM + E2	25.08 ± 6.95	111.04 ± 8.29	43.42 ± 5.14	2.70 ± 0.23	2.94 ± 0.26
C. Brain mitochondria					
Placebo	9.22 ± 1.36	30.28 ± 3.06	10.96 ± 0.97	2.61 ± 0.12	3.97 ± 0.26
E2	10.99 ± 0.86	27.89 ± 1.54	11.52 ± 0.85	2.49 ± 0.18	3.69 ± 0.10
TAM	9.84 ± 0.10	29.31 ± 1.63	12.59 ± 1.74	2.56 ± 0.47	3.67 ± 0.23
TAM + E2	9.25 ± 0.24	28.73 ± 1.22	10.82 ± 1.16	2.69 ± 0.43	3.55 ± 0.13

The values are expressed as mean ± S.E.M. of 6–7 animals. States 2, 3 and 4 = nmol O_2/min/mg protein; RCR = state 3/state 4; ADP/O = nmol ADP/nAtO/min/mg protein.

described by Barja [33]. Briefly, mitochondria were incubated at 30 °C with 5 mM glutamate/2.5 mM malate in 1.5 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 145 mM KCl, 30 mM Hepes, 0.1 mM homovalinic acid and 6 U/ml horseradish peroxidase. The incubation was stopped at 10 min with 0.5 ml of cold 2 M glycine buffer containing 25 mM EDTA and NaOH, pH 12. The fluorescence of supernatants was measured at 312 nm as excitation wavelength and 420 nm as emission wavelength. The rate of peroxide production was calculated using a standard curve of H_2O_2.

Table 3
Effect of 17β-estradiol (E2) and/or tamoxifen (TAM) on oxidative phosphorylation parameters of liver (A), heart (B) and brain (C) mitochondria.

	Membrane potential (−mV)	Membrane depolarization (−mV)	Lag phase of repolarization (min)
A. Liver mitochondria			
Placebo	212.39 ± 1.82	26.12 ± 0.95	0.69 ± 0.01
E2	211.15 ± 1.83	26.67 ± 1.32	0.76 ± 0.03
TAM	212.39 ± 2.72	30.90 ± 0.45*	0.84 ± 0.07*
TAM + E2	217.56 ± 1.49	29.01 ± 1.56	0.61 ± 0.07#,$
B. Heart mitochondria			
Placebo	204.89 ± 1.62	27.11 ± 1.39	0.49 ± 0.22
E2	205.42 ± 3.01	25.86 ± 1.33	0.68 ± 0.04**
TAM	198.52 ± 2.46	33.06 ± 2.11	0.53 ± 0.03
TAM + E2	196.80 ± 2.07	28.73 ± 1.58	0.76 ± 0.12**
C. Brain mitochondria			
Placebo	171.80 ± 2.74	29.90 ± 1.71	0.69 ± 0.01
E2	172.30 ± 3.43	32.10 ± 2.67	0.76 ± 0.03
TAM	169.00 ± 3.58	27.20 ± 3.10	0.84 ± 0.07
TAM + E2	170.80 ± 1.11	29.30 ± 1.795	0.62 ± 0.07

The values are expressed as mean ± S.E.M. of 6–7 animals.
* $p < 0.05$ when compared with placebo-treated ovariectomized female rats.
** $p < 0.01$ when compared with placebo-treated ovariectomized female rats.
# $p < 0.05$ when compared with E2-treated ovariectomized female rats.
$ $p < 0.05$ when compared with TAM-treated ovariectomized female rats.

2.10. Statistical analysis

The results are presented as mean ± S.E.M. of the indicated number of experiments. Statistical significance between the different assays was made using the one-way analysis of variance (ANOVA) followed by the Tukey post-test, for multiple comparisons. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Characterization of animals

TAM- and E2-treated females presented a significantly lower body weight gain when compared with placebo-treated females, the experimental group that presented the highest body weight gain (Table 1). Nevertheless, females treated with TAM plus E2 presented a body weight gain similar to that of placebo-treated females. E2-treated females also presented a significant decrease in red blood cells and a significant increase in triglycerides (Table 1). However, these effects were significantly attenuated when females were treated with E2 plus TAM (Table 1). TAM-treated females presented a significant increase in alkaline phosphatase (ALP) when compared with E2-treated females that presented the lowest levels of ALP (Table 1). No statistical differences were found in the other parameters that were evaluated.

3.2. The oxidative phosphorylation efficiency of liver mitochondria is decreased by tamoxifen while that of heart mitochondria is decreased by 17β-estradiol

No statistical alterations were found in the respiratory chain parameters of liver, heart and brain mitochondria isolated from all groups of experimental animals (Table 2). However, liver mitochondria isolated from TAM-treated females presented a significant increase in membrane depolarization and lag phase of repolarization (Table 3). Concerning heart mitochondria, E2 induced a significant increase in lag phase of repolarization. The phosphorylation system of brain mitochondria was not altered by E2 and/or TAM treatment (Table 3).

3.3. The capacity of liver and heart mitochondria to accumulate calcium is decreased by 17β-estradiol

Liver and heart mitochondria isolated from E2-treated females presented a lower capacity to accumulate Ca^{2+} when compared to liver and heart mitochondria isolated from placebo-treated females (Fig. 1A and B). While mitochondrial calcium loading capacity in liver returned to control values in females treated with TAM plus E2, the same was not observed with heart mitochondria (Fig. 1B). The capacity of brain mitochondria to accumulate Ca^{2+} remained statistically unchanged in all groups of experimental animals (Fig. 1C). The presence of cyclosporine-A, the specific PTP inhibitor, increased calcium loading capacity in all groups, without any difference between them (data not shown).

3.4. Tamoxifen interferes with mitochondrial glutathione levels

Liver mitochondria isolated from TAM-treated females presented a significantly higher GSH/GSSG ratio when compared with liver mitochondria from placebo-treated females (Fig. 2A). However, heart mitochondria isolated from TAM- and E2 + TAM-treated females presented significantly lower levels of GSSG when compared with placebo- and E2-treated females that resulted in a slight increase in the GSH/GSSG ratio in these two experimental groups (Fig. 2B). Concerning brain mitochondria, E2 and TAM

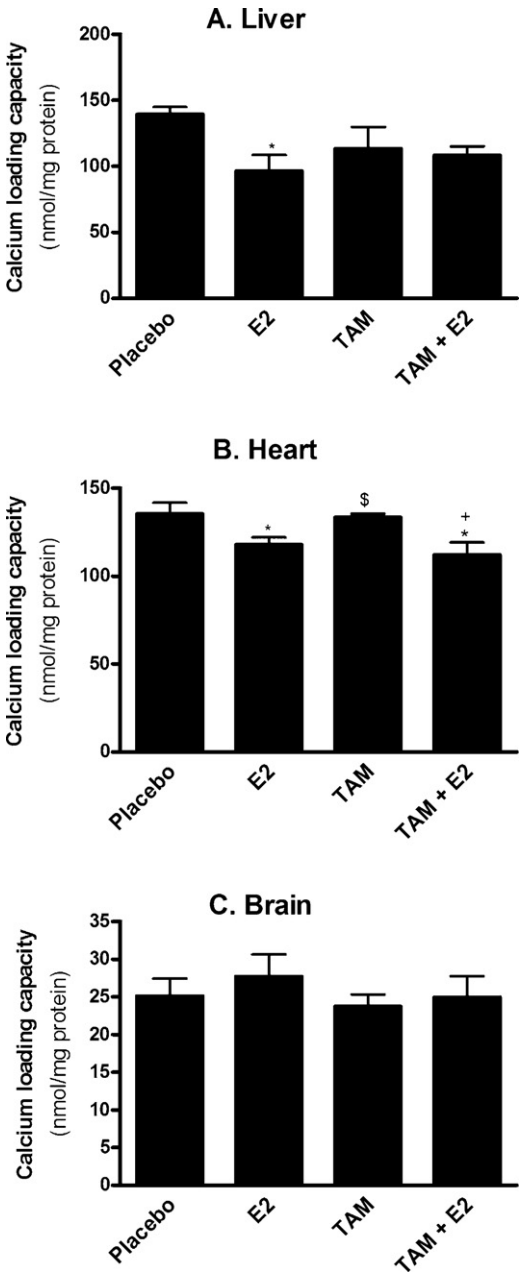

Fig. 1. Effects of 17β-estradiol (E2) and/or tamoxifen (TAM) on calcium loading capacity of liver, heart and brain mitochondria. E2 decreased the capacity of liver and heart mitochondria to accumulate calcium. Data shown represent mean ± S.E.M. from 6 animals for each experimental group studied. $^{*}p < 0.05$ when compared with the mitochondria isolated from placebo-treated female rats. $^{\$}p < 0.05$ when compared with the mitochondria isolated from E2-treated female rats. $^{+}p < 0.05$ when compared with the mitochondria isolated from TAM-treated female rats.

treatment induced an increase in GSSG levels although only significant in TAM- and E2 + TAM-treated females when compared with placebo-treated females (Fig. 2C). Accordingly, GSH/GSSG ratio of brain mitochondria decreased significantly in TAM- and E2 + TAM-treated females when compared with placebo-treated females (Fig. 2C).

3.5. Tamoxifen plus 17β-estradiol decrease lipid peroxidation in liver mitochondria

TBARS levels were used to determine the extension of lipid peroxidation (PX) induced by the pro-oxidant pair ADP/Fe^{2+}. Fig. 3A shows that liver mitochondria isolated from E2 + TAM females in

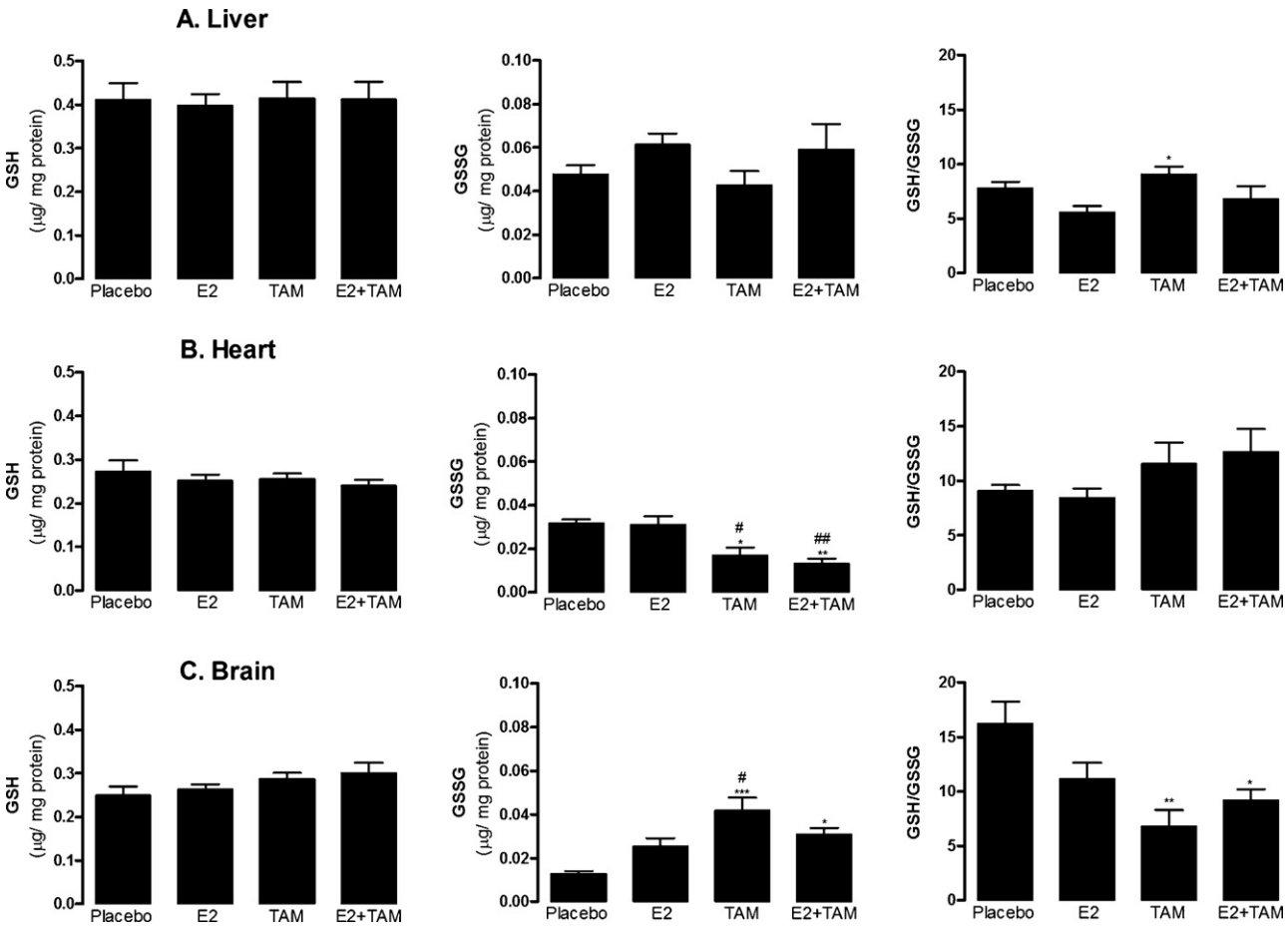

Fig. 2. Effects of 17β-estradiol (E2) and/or tamoxifen (TAM) treatments on glutathione levels of liver, heart and brain mitochondria. TAM treatment altered glutathione levels. Data shown represent mean ± S.E.M. from 6 animals for each experimental group studied. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ when compared with the mitochondria isolated from placebo-treated female rats. $^{\#\#}p < 0.01$; $^{\#}p < 0.05$ when compared with the mitochondria isolated from E2-treated female rats.

the presence of ADP/Fe^{2+} produced significantly lower levels of TBARS when compared with liver mitochondria isolated from the other groups of experimental animals. No significant alterations were observed in brain mitochondria (Fig. 3B). Heart mitochondria were not tested because the amount of mitochondria obtained per heart did not allow us to test all the parameters including lipid peroxidation.

3.6. Tamoxifen and/or 17β-estradiol decrease(s) the production of H_2O_2 in brain mitochondria energized with a substrate for mitochondrial complex I

No significant alterations were found in liver mitochondria isolated from all groups of experimental animals (Fig. 4A). Although the production of H_2O_2 by brain mitochondria energized with succinate, a substrate for complex II, did not present statistical alterations between the different experimental groups (Fig. 4B), brain mitochondria isolated from E2, TAM- and E2 + TAM-treated females and energized with glutamate/malate, a substrate for complex I, produced significantly lower levels of H_2O_2 when compared with placebo-treated females (Fig. 4B). The production of H_2O_2 in heart mitochondria was not evaluated because the amount of sample obtained was insufficient to perform this experiment.

4. Discussion

The present study clearly shows that liver, brain and heart mitochondria are differently affected by E2 and/or TAM treatments. Concerning brain mitochondria, all the treatments decrease the capacity of these organelles to produce H_2O_2, when energized with a complex I substrate, despite the decrease in GSH/GSSG ratio induced by TAM treatment. In contrast, E2 treatment exerts a negative impact on heart mitochondria by increasing the repolarization lag phase and decreasing the Ca^{2+} loading capacity of mitochondria. However, TAM treatment increases the GSH/GSSG ratio of heart mitochondria. With regard to liver mitochondria, E2 treatment decreases the Ca^{2+} loading capacity of mitochondria and TAM treatment increases the lag phase of repolarization. However, TAM increases liver mitochondrial GSH/GSSG ratio and its combination with E2 decreases lipid peroxidation levels. Interestingly, E2 and TAM treatments induce alterations in blood biochemistry and body weight.

We observed that E2 treatment increases plasma triglycerides (Table 1). This result is in accordance with the PEPI trial that showed that triglycerides increased 13.9% among women treated with conjugated equine estrogen [34]. E2 also decreases the amount of red blood cells, which could be related with the increase in plasma triglycerides (Table 1). Previous studies have shown that high plasma triglyceride levels lead to enhanced red blood cells aggregation and changes in their flow behavior in microcirculation [35,36]. TAM treatment alters alanine aminotransferase (Table 1), which is an expected result since TAM is extensively metabolized in the liver [37]. Accordingly, Liu et al. [38] reported that patients with breast cancer treated with TAM had increased aspartate aminotransferase and/or alanine aminotransferase plasma concentrations. Furthermore, E2 and TAM treatments decrease body weight gain when

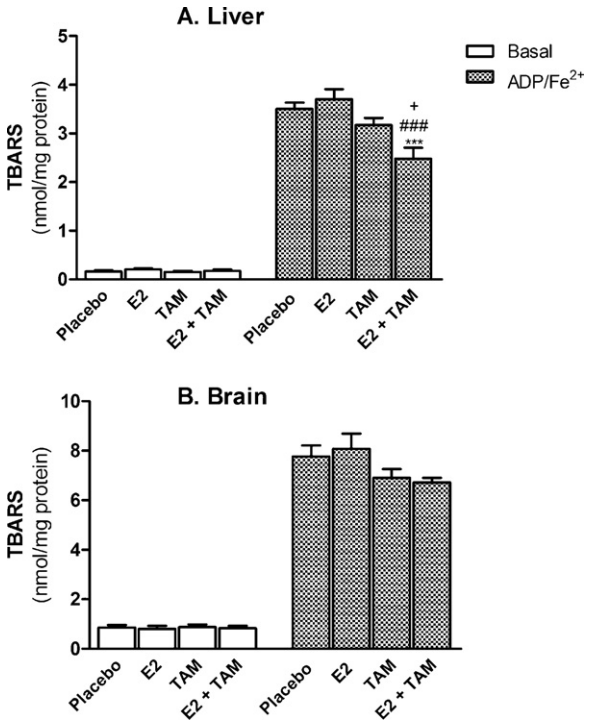

Fig. 3. Effects of 17β-estradiol (E2) and/or tamoxifen (TAM) treatments on thiobarbituric acid reactive substances (TBARS) levels. TAM plus E2 decrease lipid peroxidation in liver mitochondria. $^{***}p < 0.001$ when compared with the mitochondrial isolated from placebo-treated female rats. $^{\#\#\#}p < 0.01$ when compared with the mitochondrial isolated from E2-treated female rats. $^{+}p < 0.05$ when compared with the mitochondria isolated from TAM-treated female rats.

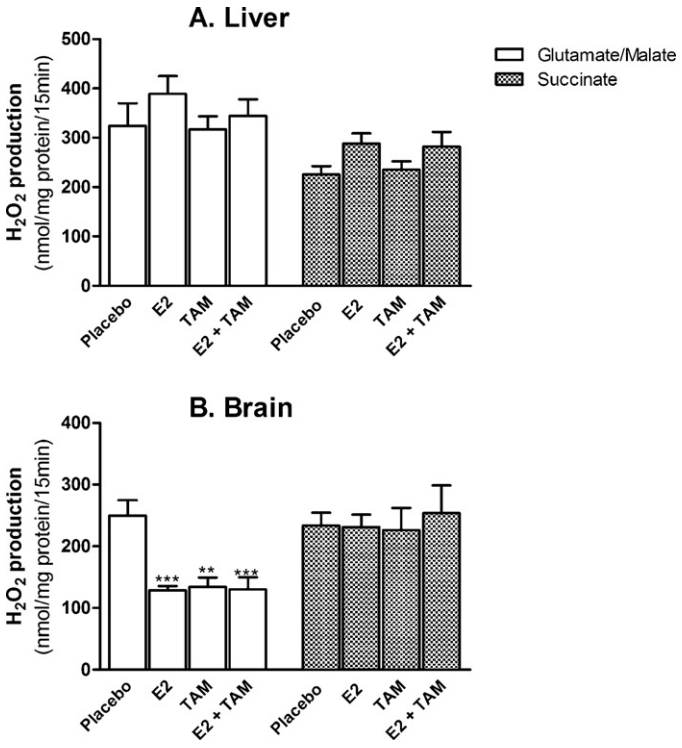

Fig. 4. Effects of 17β-estradiol (E2) and/or tamoxifen (TAM) on mitochondrial hydrogen peroxide (H_2O_2) production. TAM and/or E2 decrease(s) the production of H_2O_2 in brain mitochondria energized with glutamate/malate, a substrate of mitochondrial complex I. Data shown represent mean ± S.E.M. from 6 animals for each experimental group studied. $^{***}p < 0.001$; $^{**}p < 0.01$ when compared with mitochondria isolated from placebo-treated female rats.

compared to placebo-treated females (Table 1). These results are in accordance with previous studies that report that E2 deficiency caused by ovariectomy results in increased food intake accompanied by a sustained elevation of body weight, whereas administration of E2 reverses these changes [39,40]. Indeed, it has been shown that menopause is associated with a decrease in the resting metabolic rate that reduces the calorie usage and hence increases body weight [41]. Furthermore, previous studies also reported that treatment of ovariectomized rats with TAM mimics the effects of E2 and causes significant decreases in food intake and body weight [42]. The decreases in body weight are mainly reflected in a decreased body fat content and, similar to the effects of E2, TAM decreases parametrial white adipose tissue wet weight and lipoprotein lipase activity [42]. Lopez et al. [43] reported that the anorectic effect of TAM is associated with the accumulation of malonyl-CoA in the hypothalamus and inhibition of fatty acid synthase expression specifically in the ventromedial nucleus of the hypothalamus.

Mitochondria are organelles essential for generating energy that fuels normal cellular function and, at the same time, are the major intracellular source of free radicals and the primary determinants of cell death [44]. Besides the crucial role of energy production, mitochondria are also determinants in cells death. As such, mitochondria sit at a strategic position in the hierarchy of cellular organelles determining the metabolic status of a cell or its complete termination.

Nilsen et al. [45] treated ovariectomized females with a single injection of E2. Twenty-four hours after E2 exposure the authors observed an increase in respiratory control ratio, elevated cytochrome c oxidase expression and activity and a reduction in free radical generation in brain mitochondria. A recent study showed that daily oral administration of E2 or genistein, a phytoestrogen, for six weeks protects against ovariectomy-induced neurodegeneration by attenuating oxidative stress, lipid peroxidation and the mitochondria-mediated apoptotic pathway in a region- and dose-dependent manner [46]. It was also shown that the treatment of human brain microvascular endothelial cells with E2 during 24 h increases mitochondrial cytochrome *c* protein and mRNA and significantly reduces the production of superoxide [47]. Accordingly, we observed that E2 treatment reduces the production of H_2O_2 when mitochondria are energized with a complex I substrate (Fig. 4). However, we did not observe any significant alteration in brain mitochondrial respiratory chain and phosphorylation system (Tables 2 and 3) and TBARS levels (Fig. 3). These discrepancies are probably due to the different E2 treatments and experimental models used in each study. We observed a protection against H_2O_2 production when mitochondria were energized with a substrate for complex I and no alterations when mitochondria were energized with a substrate for complex II (Fig. 4) probably because E2 and/or TAM are avoiding the production of superoxide ion at the source of ROS production, the mitochondrial complex I. Given the fact that the primary source of reducing equivalents to the mitochondrial respiratory chain is NADH rather than succinate, this is an important finding.

Various secondary prevention trials, including the Women's Health Initiative (WHI), assessing the effects of hormone therapy on coronary artery disease, showed no benefits and a trend towards early harm [48]. However, this has been a controversial issue and, several research groups are decided to explore and clarify this situation. Our present study shows that E2 treatment affects heart mitochondria by increasing the lag phase of repolarization, which can be a sign that heart mitochondria from treated animals take more time to phosphorylate ADP than heart mitochondria from control animals (Table 3). The capacity of heart mitochondria to accumulate Ca^{2+} was also affected (Fig. 1). These results support the idea that HRT could have deleterious effects on the bioenergetics of

cardiac mitochondria. In contrast, Morkuniene et al. [49] reported that the pre-perfusion of hearts with 100 nM E2 prevents the loss of cytochrome *c* from mitochondria, its accumulation in cytosol, and inhibition of respiration during ischemia. More recently, Liou et al. [50] reported that 6–7 month-old ovariectomized female rats injected intraperitoneally with 10 μg/kg/day E2 for 10 weeks are protected against ovariectomy-induced cardiac Fas-dependent and mitochondria-dependent apoptotic pathways. As said above, we consider that discrepancies between studies are due to differences in the experimental protocols.

Previous in vitro studies from our laboratory demonstrated that E2 alters the respiratory chain and phosphorylation system activity and increase the probability of the Ca^{2+}-induced PTP opening in liver mitochondria [16,17]. Although in the present study we did not observe any significant alteration induced by E2 treatment in the respiratory chain and phosphorylation system (Tables 2 and 3), we observed a decrease in the capacity of mitochondria to accumulate Ca^{2+} (Fig. 1), which can be originated from a higher susceptibility to the PTP opening, as confirmed by the inhibitory effect of cyclosporin-A. We also demonstrated previously that TAM impairs the respiratory chain and phosphorylation system of liver mitochondria [16,17]. Accordingly, in this study we observed that TAM treatment decreases the phosphorylation efficiency of liver mitochondria, although we did not observe significant alterations in the respiratory chain (Tables 2 and 3). The divergences between this and the previous studies are probably due to the concentrations of E2 and/or TAM that reach liver mitochondria in vivo. It is well known that E2 and TAM are extensive metabolized in vivo thus the concentrations that reach mitochondria are, probably, lower than the doses used in our in vitro studies. Curiously, we observed that TAM treatment increases GSH/GSSG ratio of heart and liver mitochondria and decreases the same ratio in brain mitochondria (Fig. 2). The increase in GSH/GSSG ratio in liver mitochondria could be related with the decrease in lipid peroxidation observed in mitochondria isolated from TAM + E2-treated females (Fig. 3). GSH plays a critical role in the regeneration of vitamin E. The major function of vitamin E is to work as a chain-breaking antioxidant in a fat soluble environment thus protecting polyunsaturated fatty acids within membrane phospholipids and in plasma lipoproteins. The decrease of GSH/GSSG ratio observed in brain mitochondria could be related with the decrease of H_2O_2 production in brain mitochondria energized with a complex I substrate (Fig. 4). Indeed, GSH is a key antioxidant that acts as a potent free radical scavenger. GSH is also the co-substrate of antioxidant enzyme glutathione peroxidase. Intracellular GSH is converted into GSSG by glutathione peroxidase, which catalyzes the reduction of H_2O_2 and various hydroperoxides. Konyalioglu et al. [51] reported that raloxifene (another selective estrogen receptor modulator) treatment significantly increases the levels of GSH in brain and heart tissues of ovariectomized females although no significant differences are found in liver tissue.

5. Conclusion

We demonstrated for the first time that the impact of E2 and/or TAM treatments on mitochondrial function is tissue-specific. In general E2 has beneficial effects on brain mitochondria supporting the idea that estrogens have a potential for sustaining neurological health and prevention of neurodegenerative diseases associated with mitochondrial dysfunction. Although liver mitochondria is mildly affected by E2 treatment, the most deleterious effects are observed in heart mitochondrial function suggesting that estrogens should not be recommended, at least, to individuals with cardiac problems. Concerning TAM treatment, our results indicate that this agent may interfere with liver and brain mitochondrial function although the degree of toxicity would be dependent on predisposing conditions as the general metabolic condition of the patient. The data from the present manuscript indicate that further studies are thus necessary to correctly evaluate the toxicity of HRT on the different organs and determine the risk/benefit for each individual.

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