Am J Transl Res 2014;6(1):91-101
www.ajtr.org /ISSN:1943-8141/AJTR1309011

Original Article

Cardiovascular autonomic dysfunction in primary ovarian insufficiency: clinical and experimental evidence

Silvia Goldmeier[1*], Kátia De Angelis[2*], Karina Rabello Casali[1], César Vilodre[3], Fernanda Consolim-Colombo[4], Adriane Belló Klein[5], Rodrigo Plentz[6], PoliMara Spritzer[3,5], Maria-Cláudia Irigoyen[1,4]

[1]Instituto de Cardiologia do Rio Grande do Sul/Fundação Universitária de Cardiologia (IC/FUC), Porto Alegre, Brazil; [2]Universidade Nove de Julho (UNINOVE), São Paulo, Brazil; [3]Division of Endocrinology, Gynecological Endocrinology Unit, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil; [4]Hypertension Unit, Heart Institute (InCor), Medical School of University of Sao Paulo, Sao Paulo, Brazil; [5]Department of Physiology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil; [6]Department of Physiotherapy, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brazil. [*]Equal contributors.

Received September 23, 2013; Accepted November 16, 2013; Epub December 1, 2013; Published January 1, 2014

Abstract: Objective: Women with primary ovarian insufficiency (POI) present an increased risk for cardiovascular disease. In this study we tested the hypothesis that POI in women under hormone therapy (HT) are associated with vascular vasodilatation attenuation and cardiovascular autonomic dysfunction and these impairments are related to changes in systemic antioxidant enzymes. Furthermore, the possibility that ovarian hormone deprivation can induce such changes and that HT cannot reverse all of those impairments was examined in an experimental model of POI. Methods: Fifteen control and 17 patients with primary ovarian insufficiency receiving HT were included in the study. To test the systemic and cardiac consequences of ovarian hormone deprivation, ovariectomy was induced in young female rats that were submitted or not to HT. Spectral analysis of RR interval and blood pressure signals were performed and oxidative stress parameters were determined. Results: POI women under HT have increased mean arterial pressure (94±10 vs. 86±5 mmHg) despite normal endothelial and autonomic modulation of vasculature. Additionally, they presented impaired baroreflex sensitivity (3.9±1.38 vs. 7.15±3.62 ms/mmHg) and reduced heart rate variability (2310±1173 vs. 3754±1921 ms^2). Similar results obtained in ovariectomized female rats were accompanied by an increased lipoperoxidation (7433±1010 vs. 6180±289 cps/mg protein) and decreased antioxidant enzymes in cardiac tissue. As it was observed in women, the HT in animals did not restore hemodynamic and autonomic dysfunctions. Conclusion: These data provide clinical and experimental evidence that long term HT may not restore all cardiovascular risk factors associated with ovarian hormone deprivation.

Keywords: Primary ovarian insufficiency, women, rats, autonomic nervous system, oxidative stress, hormones, endothelium

Introduction

Premature ovarian failure, or primary ovarian insufficiency (POI), is defined as a failure of the ovarian function before the age of 40. It is characterized by amenorrhea during four months or more, associated with sex steroid deficiency and high gonadotropin levels [1]. Prevalence of POI at reproductive age is estimated as 1% in women under the age of 40 and 0.1% in women under the age of 30 [2].

Women who experience POI are at increased risk for cardiovascular morbidity and mortality [3, 4]. Hormonal therapy (HT) in young women with POI restores normal physiology and might also have beneficial effects on the cardiovascular system. However, the magnitude of long-term risk in these patients, including cardiovascular disease (CVD) remains an unresolved issue.

Cardiovascular disease (CVD), which is the leading cause of premature death in the Western World, typically develops 10 years later in women than in men. It's well known many CVD states have been associated with alterations in the cardiovascular autonomic control, such as reduced parasympathetic modulation and increased sympathetic modulation of the heart

evaluated by different methods. Moreover, many CVD states have been associated with baroreflex impairment, the most important short-term regulator of arterial blood pressure (AP). Indeed, the measurement of heart rate variability (HRV) and baroreflex sensitivity (BRS) has been used as a tool to better quantify the markers of autonomic dysfunction [5]. Additionally, oxidative stress has been implicated in the pathophysiology of a large number of diseases, and it plays a possible mechanistic role in baroreflex dysfunction. Antioxidant substances seem to improve BRS [6, 7] and oxidative stress reduction was correlated with this reflex improvement in rats [8, 9]. In this aspect, scientists have long established that the female hormone estrogen protects against CVD, but the mechanism of action remains unclear. Studies have shown that ovarian hormones deprivation induces endothelial dysfunction, autonomic impairment and increases oxidative stress in fertile young women as well as in female ovariectomized rats [8, 10-12].

Considering these data reported above, it is reasonable to hypothesize that women with POF (or premature menopause) present an increased risk for CVD, which might be attributed to the early onset of ovarian hormone deprivation. However, until recently, very few studies have assessed the cardiovascular consequences of POF and there is no information about autonomic dysfunction and baroreflex sensitivity, as well as, the role of oxidative stress in women diagnosed as POF. In this study we tested the hypothesis that POF in women under HT are associated with vascular vasodilatation attenuation and cardiovascular autonomic dysfunction, quantified by HRV and BRS, and that these impairments are related to changes in systemic antioxidant enzymes. Furthermore, considering that HT is indicated for women after POF, the possibility that ovarian hormone deprivation can induce such changes and that HT cannot reverse all of those impairments was examined in an experimental model of POF induced by ovariectomy in young adult rats submitted or not to HT. This experimental model also allows the study of oxidative stress in cardiac tissue.

Methods

Evaluations in POI women

Seventeen women with POI diagnosis (POI group) and fifteen control women (C group) were included in this study. The POI patients with amenorrhea were investigated at the Gynecological Endocrinology, Division of Endocrinology, Hospital de Clinicas de Porto Alegre and had more than a 7-year follow-up from POI diagnosis. POI was defined as secondary amenorrhea before the age of 40 years, in normal female karyotype (46, XX) that showed high gonadotropin levels (FSH>40 IU/L) in at least two consecutive determinations and hypergonadotropic ovarian failure resulting from causes other than autoimmune ovarian diseases, surgery, chemotherapy or radiotherapy. Women included in our study in POI group showed at the diagnosis, as expected, increased FSH (53.9±24.15 IU/L), luteinizing hormone plasmatic levels (LH: 27.1±11.2 IU/L), and reduced estradiol plasmatic levels (17.7±14.9 pg/mL) in relation to normal standard ranges [13]. While soon after the diagnosis, the patients underwent HT, reaching estrogen plasmatic levels no lower than 50 pg/mL, the time elapsed since menstrual irregularities until the diagnosis was variable (4 months to 6 years). HT consisted of continuous oral daily conjugated estrogens plus medroxiprogesterone acetate, 14 days a month. Age-matched control women with regular cycles and users of non-hormonal contraceptive methods were selected from the same Ginecological Endocrinology Unit. All measurements in this group of patients were performed in the days 2 to 10 of the menstrual cycle. Patients presenting diabetes mellitus, hypertension, hipercholesterolemia, overweight and under pharmacological treatment or cigarette smoking or alcohol abuse were excluded from this study. All women gave written informed consent for participation in this institutional Cardiology Review Board-approved study.

Anthropometric measurements included body weight, height, waist circumference (WC, measured at the midpoint between the lower rib margin and the iliac crest) and body mass index (BMI, by the ratio of weight (Kg) and square height (m^2) determinations. Blood samples were collected by assessing an antecubital vein for biochemical and hormonal parameter determination, as well as antioxidant enzyme quantification.

Brachial flow-mediated vasodilation: Noninvasive endothelial function was assessed using brachial artery ultrasound. Analyses were per-

formed in acclimated room at the Institute of Cardiology of Porto Alegre. The ultrasound study was performed using EnVisor Series (Philips Ultrasound - Bothell, WA - USA) that is composed by an echo Doppler instrument equipped with a 7-12 MHz resolution linear and a software to image the data acquired in bi-dimensional mode with color Doppler and ECG signal. A pressure cuff was placed on the left arm and inflated up to 50 mmHg above systolic pressure during 5 minutes, immediately, the cuff was removed and the brachial artery internal diameter images were obtained and endothelium-dependent vasodilator function recorded. Ten minutes were allowed for vessel recovery. After this recovery period, a second baseline scan was performed. Glyceryl trinitrate (0.4 mg; Nitrostat, Parke-Davis, Morris Plainf, New Jersey, USA) was administered and the 4th scan of the brachial artery was performed, according to the guidelines [14].

Hemodynamic and autonomic evaluations: Hemodynamic measurements were recorded using arterial pressure signal obtained by Finapres Medical Systems devices that continuously assess the pressure wave, through a sensor placed on the patient's finger. All records were performed in a quiet and acclimatized room by a data acquisition system (CODAS, 1-kHz sampling frequency; Dataq Instruments, Inc., Akron, OH). To test the autonomic modulations, it was applied an orthostatic maneuver. Records were performed with patients resting for 30' prior to the start of recording the signal: 10' in supine position and 10' standing upright. Frequency domain analysis of heart rate variability (HRV) was performed with an autoregressive algorithm [15, 16] on the interval pulse (IP) and systolic arterial pressure (SAP) sequences (200 beats). The power spectral density was calculated for each stationary time series, using specific softwares. In this study, two spectral components were considered: low frequency (LF), from 0.04 to 0.15 Hz; and high frequency (HF), from 0.15 to 0.40 Hz. The spectral components were expressed in absolute (ms^2) and normalized units (nu). Spontaneous BRS was estimated by the alpha index, defined as the square root of the ratio between LF powers in BPV and HRV.

Biochemical measurements: A fasting blood sample was obtained for sodium (Na), potassium (K), glucose, total cholesterol, high and low-density cholesterol (HDL and LDL, respectively), triglycerides (TG), urea and creatinine quantification. Laboratory measurements were performed using automated enzymatic commercial kits (Roche, Mannheim, GE).

Antioxidant enzyme determinations: After collection, heparinized venous blood samples were washed in a solution of 9 g/L sodium chloride and centrifuged three times at 3000 g for 10 min at room temperature. White cells were discarded by aspiration and the erythrocytes diluted 1/10 in 1 mM acetic acid and 4 mM magnesium sulfate, placed in an ice bath for 10 min and centrifuged at 4200 g for 20 min at 0°C. The supernatant was used for enzymes assays. Superoxide dismutase (SOD) activity was measured in blood by rate inhibition of pyrogallol auto-oxidation at 420 nm as described previously by Marklund [17].

The enzyme activity was reported as U/mg hemoglobin. Catalase (CAT) concentration was measured by monitoring the decrease in the hydrogen peroxide concentration spectrophotometrically at 240 nm, and the results are reported as pmol of hydrogen peroxide/mg hemoglobin [18].

Evaluations in a rat experimental model of POI

Experiments were performed on 21 female virgin Wistar rats (192±4 g) from the Animal Shelter of Sao Judas University, Sao Paulo, Brazil, receiving standard laboratory chow and water *ad libitum*. The animals were housed in individual cages in a temperature-controlled room (22°C) with a 12-h dark-light cycle. All surgical procedures and protocols used were approved by the Experimental Animal Use Committee of the Sao Judas University and were conducted in accordance with National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. The rats were randomly assigned to one of three groups: control-sham (S, n=7), experimental POI induced by ovariectomy (EPOI, n=7) and EPOI + estrogen therapy (EPOI+ET, n=7). POI was induced by ovariectomy at 10 weeks of age under anesthesia (Ketamine 80 mg/kg + Xylazin 12 mg/Kg) [8, 11, 19]. Control-sham rats (S group) were submitted to an ovariectomy sham procedure. Seven days after ovariectomy and under the same anesthesia, the EPOI+ET rats were subcutaneously implanted with a pellet releasing

1.5 mg/day 17β-estradiol (Innovative Research of America, Toledo, OH over an 8-week period). As reported recently, the concentrations of 17β-estradiol decreases in ovariectomized rats and increases after 17β-estradiol pellets implantation in these animals [11, 12]. In this study, the estrogen concentration, measured by immunoassay, was non-detectable in EPOI group, and the estrogen concentration was similar between S and EPOI+ET groups (39±7 and 57±15 pg/ml, respectively).

Cardiovascular assessments: Nine weeks after experimental POI induction, 2 catheters were implanted into the carotid artery and jugular vein (PE-10) of the anesthetized rats (Ketamine 80 mg/kg + Xylazin 12 mg/Kg) for direct measurements of AP and drug administration, respectively. During experiments, rats received food and water *ad libitum*; the rats were conscious in their cages and allowed to move freely during the hemodynamic experiments. Vaginal secretion was collected and was observed under a light microscope for the determination of the estrous cycle phases. Given the short-time of the ovulatory phase, all evaluations in S rats were performed during the non-ovulatory phases of estrous cycle (metaestrous and dyestrous). The arterial catheter was connected to a transducer (Blood Pressure XDCR, Kent© Scientific, Litchfield, CT), and AP signals were recorded over a 30-minute period by an analog-to-digital converter board (CODAS, 2-kHz sampling frequency; Dataq Instruments, Inc., Akron, OH) [8, 19]. Increasing doses of phenylephrine (0.25 to 32 μg/kg) and sodium nitroprusside (0.05 to 1.6 μg/kg) were given to produce AP responses ranging from 5 to 40 mmHg. A 3-5 minute interval between doses was necessary for AP to return to baseline. BRS was evaluated by a mean index [8, 19].

Oxidative stress profile: After hemodynamic evaluations, animals were killed by decapitation, the heart (ventricles) was immediately removed, rinsed in saline, trimmed to remove fat tissue and visible connective tissue. This tissue was placed in ice-cold buffer and was homogenized in an ultra-Turrax blender using 1 g of tissue for 5 mL of 150 mmol/L potassium chloride and 20 nmol/L phosphate buffer, pH 7.4. The homogenates were centrifuged at 600 g for 10 min at -2°C. Chemiluminescence (CL) assay was carried out with a LKB Rack Beta Liquid Scintillation Spectrometer 1215 (LKB Producer AB, Bromma, Sweden) in the out-of-coincidence mode at room temperature (25-27°C). The supernatants were diluted in 140 mmol/L KCl, 20 mmol/L phosphate buffer, pH 7.4, and added to glass tubes, which were placed in scintillation vials; 3 mM tert-butyl hydroperoxide was added and CL was determined up to the maximal level of emission [8, 20]. CAT concentration was measured spectrophotometrically by monitoring the decrease in H_2O_2 concentration over time. Aliquots of the samples were added to 50 mM phosphate buffer in a quartz cuvette. After determining the baseline of the instrument, H_2O_2 was added to a final concentration of 10 mM in 0.9 ml and absorbance was measured at 240 nm [8, 17]. SOD activity was determined in the homogenates by measuring the inhibition of the rate of autocatalytic adrenochrome formation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine-NaOH, pH 10.5 [8, 18]. Proteins were assayed using the method of Lowry et al. [21].

Statistical analysis

Data are presented as the mean ± SD. Part 1. Comparisons between the two groups for general characteristics, biochemical evaluations, vascular function and antioxidant enzymes were performed using Student's unpaired *t* tests or Mann Whitney test. The spectral analysis parameters were analyzed using Two Way Repeated Measures of ANOVA (One Factor Repetition) followed by Student-Newman-Keuls test. Part 2. One Way ANOVA was used to compare the groups, followed by the Student-Newman-Keuls test. The significance level for all tests was established as $P<0.05$.

Results

Evaluations in POI women

Table 1 shows the baseline characteristics of studied women groups. There were no differences in age and in anthropometric measurements between control and POI groups. No differences were observed either in fasting serum levels of total cholesterol, HDL and LDL cholesterol, triglycerides, glucose, or in sodium, potassium, creatinine and urea levels between both groups.

Table 2 shows the endothelium-dependent (FMD) and independent (GTN-induced) vasodi-

Table 1. Characteristics of control (C) and premature ovarian failure (POI) groups

	C	POI	*P* value
Age (years)	38±9	37±9	0.76
BMI (kg/m^2)	26±3	26±5	0.74
WC (cm)	91±7	92±11	0.76
Na (mEq/L)	141±3	139±3	0.07
K (mEq/L)	4.3±0.4	4.2±0.5	0.54
Glucose (mg/mL)	87±7	85±8	0.44
Total Cholesterol (mg/dL)	187±35	196±31	0.41
LDL (mg/dL)	135±28	129±26	0.66
HDL (mg/dL)	51±9	52±7	0.75
TG (mg/dL)	84±33	82±33	0.83
Urea (mg dL)	25±3	27±6	0.25
Creatinine (mg/dL)	0.59±0.06	0.65±0.10	0.07

Data are reported as means ± SD. (Student t test). BMI: body mass index, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate, Na: sodium, K: potassium, HDL: high density cholesterol, LDL: low density cholesterol; TG: triglycerides.

Table 2. Arterial vascular function in control (C) and premature ovarian failure (POI) groups

	C	POI	*P* value
FMD (%)	13.7±5.5	14.2±7.1	0.84
GTN (%)	24.2±8.5	22.1±11.7	0.54

Data are reported as means ± SD. (Student t test). FMD: flow-mediated dilatation, GTN: glyceryl trinitrate mediated vasodilatation.

Table 3. Antioxidant enzymes determinations in erythrocytes of control (C) and premature ovarian failure (POI) groups

	C	POF	*P* value
CAT (pg/mg Hb)	3.15±1.06	6.48±3.55*	0.04
SOD (USOD/mg Hb)	3.15±0.58	3.06±1.20	0.33

Data are reported as means ± SD. *p<0.05 vs. C group (Student t test). CAT: catalase enzyme concentration, SOD: superoxide dismutase enzyme activity, Hb: hemoglobin.

latation in studied women groups. The FMD and GTN-induced responses were similar between C and POI groups, indicating that both groups had normal arterial vasodilatation.

The CAT concentration was increased in POI group as compared with C group, while no changes were observed in SOD activity (**Table 3**).

Evaluation of beat to beat BP signals was able to show a small, but significant increase in diastolic and mean arterial pressure in POI as compared with C women in supine position, which was sustained while standing. No differences were observed in SAP while HR was lower in POI than in control in both supine and standing situation (**Table 4**). HRV total variance was lower in POI women in the supine position in relation to C women. This reduction in HRV was not additionally decreased in response to the standing maneuver in POI group, since only the C group reduced HRV total variance in response to orthostatism maneuver (**Table 4**, **Figure 1**). The absolute LF component of HRV was similar between POI and C women, while both groups presented increased normalized values of LF component, decreased absolute and normalized values of HF component and an increase in LF/HF ratio, comparing supine with standing situation. The studied groups showed no statistical difference in SAPV. Both studied groups responded similarly to standing maneuver, showing an increase in SAPV total variance and in its LF componentrelated to vascular sympathetic modulation. However, spontaneously BRS expressed by alpha index was reduced in the POI women compared with C women in the supine condition. Furthermore, the reduction in this index after standing maneuver was observed only in C group.

Evaluation in a rat experimental model of POI

As described in **Table 5**, EPOI rats presented higher MAP compared to S rats, and HT did not decrease MAP. HR was similar among the groups. Experimental POI caused a decrease in tachycardic response evoked by baroreceptor activation during AP falls ($p<0.001$). HT did not change tachycardic response ($p>0.54$). The bradycardic response to AP rises was similar among S, EPOI, EPOI+ET groups ($p>0.16$) (**Figure 2**).

Myocardium oxidative stress assessed by membrane lipid peroxidation was increased after experimental POI and was restored by HT. These changes were accompanied by significant alterations in antioxidant enzymes in this tissue. The CAT concentrations and the SOD

Table 4. Hemodynamic and autonomic evaluations in control (C) and premature ovarian failure (POI) groups

	C		POI	
	Supine	*Standing*	Supine	*Standing*
SAP (mmHg)	125±9	123±11	122±12	121±13
DAP (mmHg)	65±5	68±7	72±8*	77±12*
MAP (mmHg)	86±5	87±9	94±10*	96±11*
HR (bpm)	71±8	84±9‡	64±1*	76±14*,‡
HRV - var (ms^2)	3754±1921	1860±1035‡	2319±1173*	1455±766
- f_{LF} (Hz)	0.10±0.03	0.10±0.02	0.09±0.03	0.09±0.03
- LF (ms^2)	844±490	636±542	578±400	420±391
- %LF (nu)	43.9±19.9	79.1±13.0‡	39.6±16.9	70.5±20.7‡
- f_{HF} (Hz)	0.27±0.05	0.24±0.04	0.29±0.05	0.29±0.05*
- HF (ms^2)	857±474	194±170‡	821±803	150±86‡
- %HF (nu)	56.1.3±19.1	20.9±13.0‡	60.4±16.9	29.5±20.7‡
- LF/HF	1.3±0.7	5.8±4.2‡	0.8±0.5	4.5±4.0‡
SAPV - var ($mmHg^2$)	14.6±5.1	34.3±20.7‡	15.1±5.5	34.8±22.9‡
- LF ($mmHg^2$)	21.5±12	95.7±60.4‡	35.9±9.34	74.7±44.6‡
- HF ($mmHg^2$)	17.0±8.7	16.2±12.1	23.1±17.8	18.1±14.1
α Index (ms/mmHg)	7.15±3.62	2.87±1.60‡	3.9±1.38*	2.56±3.23

Data are reported as means ± SD. *p<0.05 vs. C group in the same condition. ‡p<0.05 vs. supine condition in the same group. MAP: mean arterial pressure, DAP: diastolic arterial pressure, SAP: systolic arterial pressure, HR: heart rate, HRV: heart rate variability, SAPV: systolic arterial pressure variability, var: total variance, f: spectrum frequency, LF: low frequency component, HF: high frequency component.

enzyme activity reduced after POI and were restored with HT (**Table 6**).

Discussion

There are important insights in the present study. Women with POI under HT who were followed up for more than 7 years presented normal endothelial function and vascular autonomic modulation (LF SPV) with an increase in the systemic antioxidant enzymes. However, they showed increased MAP (although in normality range), impaired HRV and BRS at supine position, reinforcing the complexity of mechanisms involved in ovarian hormone deprivation and cardiovascular function relationship. By using an experimental approach, it was possible to reproduce a very similar animal model of human ovarian hormone deprivation and to study the effects of the lack of estrogens on cardiovascular variables in animals, since a group of patients with POI without HT is difficult to recruit. Indeed, MAP increase and the BRS impairment observed in ovariectomized rats were associated with an increase in oxidative stress and a decrease in antioxidant enzymes in cardiac tissue. The use of estrogen therapy in ovariectomized rats could restore the cardiac oxidative profile, although it was not able to normalize hemodynamic and autonomic dysfunctions, confirming the data we have obtained from POI women under HT.

Little is known about the effect of estrogen deprivation on cardiovascular risk in young women with POI (or premature menopause). In this aspect, it is important to highlight that natural menopause (>50 years of age) is associated with endothelial dysfunction [22, 23]. POI has been also associated with significant endothelial dysfunction, which is already restored following six months of cyclical HT [24]. The results of the present study, confirm the role of estrogen therapy in vascular function, since our data demonstrated that POI women that were chronically treated with HT (>7 years) presented similar dependent and independent endothelium vasodilation when compared with aged matched healthy control women.

In the present study heart homogenates of POI rats presented increased oxidative stress, characterized by a high index of membrane lipoperoxidation accompanied by a reduction in

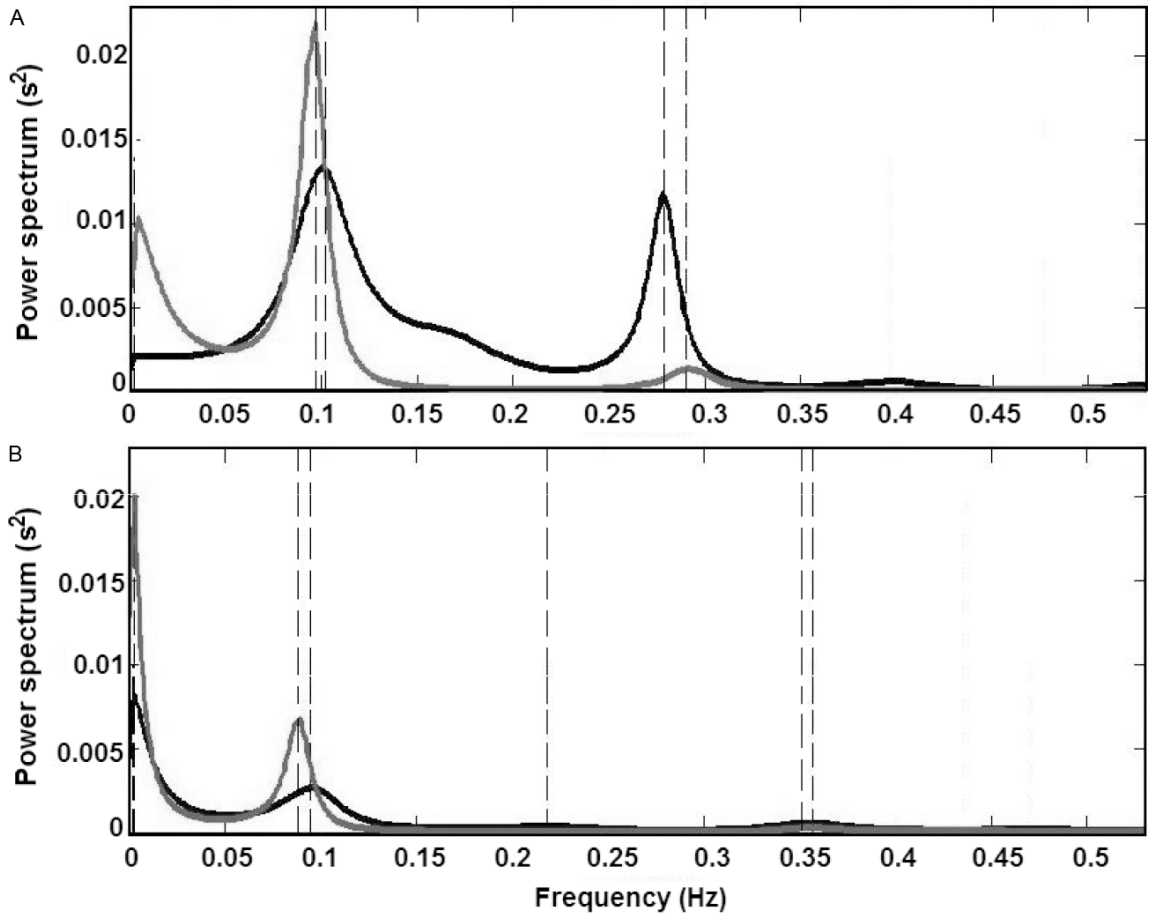

Figure 1. Examples of power spectrum obtained by spectral analysis, applied to IP series of control group (A) and POI group (B) in supine condition (black line) and after sympathetic maneuver (standing condition) (grey line).

Table 5. Hemodynamic evaluations in sham (S), experimental premature ovarian failure (EPOI) and experimental premature ovarian failure+estrogen therapy (EPOI-ET) rats

	S	EPOI	EPOI+ET	*P* value
MAP (mmHg)	106±4	123±8*	121±9*	0.01
HR (bpm)	358±27	359±31	369±30	0.74

Data are reported as means ± SD. *p<0.05 vs. S group (One Way ANOVA). MAP: mean arterial pressure, HR: heart rate.

CAT concentration and SOD activity. These changes were likely followed by a nitric oxide (NO) bioavailability increase [25, 26]. A previous study of our group demonstrated reduced SOD and unchanged CAT after ovariectomy in myocardium of female rats [27]. Since SOD detoxifies the superoxide anion, which inactivates NO [28], the increased myocardium SOD activity in POI rats submitted to HT in the present study may be associated with an increased NO bioavailability and consequently with an improvement in endothelial function. A previous study has reported that a free radical scavenger (N-acetyl-L-cysteine) reverted endothelial dysfunction in the aortic rings of ovariectomized rats [29]. Hernandez et al. showed that estrogen administration increases vascular conductance in ovariectomized rats, relating this effect to an increase in NO synthesis and/or oxidative stress prevention, thus improving endothelial function [11].

It is noteworthy that our data have shown a higher level of antioxidant activity in POI women under HT than in normal controls. This increase may be related to a compensatory local vascular mechanism related to control of sympathetic activity. A recent review has discussed the important interaction between peripheral sympathetic activity and endothelial function in cardiovascular profile. It has been suggested that

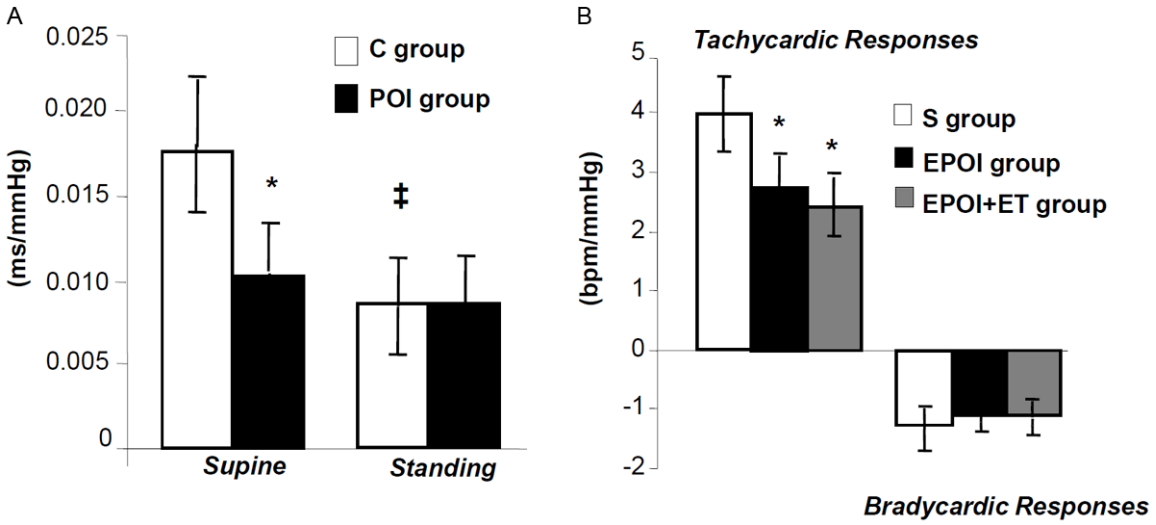

Figure 2. Baroreflex sensitivity. A: Alpha index in control (C) and premature ovarian failure (POI) women groups. B: Tachycardic and bradycardic responses to arterial pressure changes in sham (S), experimental premature ovarian failure (EPOI) and experimental premature ovarian failure+estrogen therapy (EPOI-ET) rats. *p<0.05 vs. C group or S group. ‡p<0.05 vs. supine condition in the same group.

Table 6. Oxidative stress profile in sham (S), experimental premature ovarian failure (EPOI) and experimental premature ovarian failure+estrogen therapy (EPOI-ET) rats

	S	EPOI	EPOI+ET	*P* value
CL (cps/mg protein)	6180±289	7433±1010*	5898±806†	0.004
CAT (pmol/mg protein)	3.84±0.17	3.10±0.68*	4.32±0.72†	0.037
SOD (USOD/mg protein)	43±3.24	29±3.50*	47±7.51†	0.0001

Data are reported as means ± SD. *P<0.05 vs. S group; †P<0.05 vs. EPOI group. (One Way ANOVA). CL: chemiluminescence; CAT: catalase enzyme concentration, SOD: superoxide dismutase enzyme activity.

endothelial function could counteract a higher peripheral sympathetic activity in order to maintain cardiac output and AP in normal range values [30]. Although we have not measured direct sympathetic activity in POI women, the present data demonstrate a decrease in BRS and HRV associated with a slightly but significant increase in AP, even during HT. However, it is important to note that a variable time elapsed between the menstrual dysfunction that preceded the beginning of amenorrhea and the starting of HT in our POI patients. Indeed as previously reported, approximately 50% of POI patients presented a history of oligomenorrhea or dysfunctional uterine bleeding before definitive POI [31]. Therefore, it is possible to speculate that the duration of time preceding the diagnosis of POI and, in consequence, the time of hypoestrogenic status, may have exerted an influence on the results of the present study. Moreover, while it was not possible to obtain a POI group without HT for long term, because of obvious ethical reasons, the fact that HT revert, at least in great part, the cardiovascular dysfunction in POI women seems to be an adequate evidence of the role of normal estrogen concentration in cardiovascular physiology in young women with hypogonadism. Although endothelial function was correlated to atherosclerosis development and consequently increased cardiovascular risk in POI women, other risk factors must be considered in this scenario. The enhanced incidence of CVD in this population can also be related to changes in AP and its regulation, independently or, at least, not only related to endothelial dysfunction. In rats and mice, AP values after ovarian hormones deprivation were higher when compared to those observed in healthy control female rats [8, 11, 12, 19, 31, 32]. Corroborating those findings, we showed elevated MAP values in rats submitted to POI in relation to the sham group. Despite the HT, the female rat group supplemented with 17β-estradiol (EPOI+ET group) also presented increased AP values as compared to sham female controls. Furthermore, women with POI undergoing long

term HT in the present study presented higher MAP (although in the normality range) in relation to control women. A previous study had also shown unchanged AP after long term HT in women [33].

HT has been described as a strategy to protect females from cardiac death probably by improving vagal activity [33]. In accordance with this clinical observation, previous data of our group has shown that rats supplemented with 17β-estradiol had an exacerbated vagal tonus when compared to sedentary ovariectomized control rats, reaching values similar to control females [12]. Experimental studies in rats have demonstrated that the sympathetic inhibition, by presynaptic control mechanisms, is more potent in females than males and these mechanisms are attenuated by ovariectomy [34].

Antioxidant therapy by increasing the bioavailability of NO in sinus node may influence the autonomic control [35] preventing the autonomic dysfunction caused by free radicals [6] and improving the effectiveness of the cardiac response [25]. In this sense, the HT- induced effects in antioxidant enzymes (SOD and CAT in rat hearts and in the patients blood cells) could have contributed to the improvement in cardiac autonomic/modulation, as previously demonstrated by antioxidant therapy. Importantly, clinical data of the present study demonstrated normal autonomic balance and also similar LF component of HRV, but reduced total power of HRV in POI women under HT. Considering that reduced BRS and HRV may increase the potential risk to cardiovascular disease and death [5, 36, 37], the results of this study point out to the limited effects of HT in POI women.

However, it is important to consider the fact that the impairment in BRS and in HRV is associated with both higher AP and severity of CVD [5]. Thus, the identification of the mechanisms underlying depressed BRS has important clinical implications, as well as, the study of therapies to improve this cardiovascular reflex. A recent study has examined the changes of BRS during the menstrual cycle and reported an increase of BRS in phases of estrogen preponderance, whereas progesterone seemed to antagonize this effect [38]. Hunt et al. have demonstrated that long term estrogen replacement therapy in postmenopause women has effects on cardiovascular regulation, as evidenced by an increase in vascular sympathetic baroreflex gain, that may not be reflected in resting AP or in cardio-vagal baroreflex gain [39]. We observed increased AP and reduced tachycardiac response evoked by baroreflex in female rats submitted to POI in the present study, and these changes were not reverted by HT. Importantly, the results of the present study also showed that women with POI submitted to HT presented higher AP, baroreflex dysfunction and reduced total HRV in the supine position and also after the a sympathetic activation maneuver (standing) in relation to control women. These data provide evidence in an experimental model of POI and in women with POI that long term HT cannot restore all cardiovascular risk factors associated with ovarian hormones deprivation. In this sense, alternative therapies must be investigated.

Acknowledgements

This study was supported by Projeto Casadinhos Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS)/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - InCor-USP/ICFUC, Conselho Nacional de Pesquisa e Desenvolvimento (CNPq), Fundação de Amparo a Pesquisa do Instituto de Cardiologia do Rio Grande do Sul (FAPIC) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP-2012/20141-5). K.R.C. held a postdoctoral scholarship from CAPES. K.D.A., P.S., F.C.C., A.B.K. and M.C.I. are recipients of CNPq-BPQ fellowships.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Maria Cláudia Irigoyen, Hypertension Unit, Heart Institute (InCor), Av. Dr. Eneas de Carvalho Aguiar, 44 – Subsolo, São Paulo, São Paulo, Brazil – 05403-000. Tel: +55 11 2661 5006; Fax: +55 11 3085 7887; E-mail: hipirigoyen@incor.usp.br

References

[1] Goswami D and Conway GS. Premature ovarian failure. Horm Res 2007; 68: 196-202.

[2] Coulam CB, Adamson SC and Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol 1986; 67: 604-606.

[3] Jacobsen BK, Knutsen SF and Fraser GE. Age at natural menopause and total mortality and

mortality from ischemic heart disease: The adventist health study. J Clin Epidemiol 1999; 52: 303-307.
[4] Hu FB, Grodstein F, Hennekens CH, Colditz GA, Johnson M, Manson JE, Rosner B and Stampfer MJ. Age at natural menopause and risk of cardiovascular disease. Arch Intern Med 1999; 159: 1061-1066.
[5] La Rovere MT, Bigger JT Jr, Marcus FI, Mortara A and Schwartz PJ. Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction. Atrami (autonomic tone and reflexes after myocardial infarction) investigators. Lancet 1998; 351: 478-484.
[6] Li Z, Mao HZ, Abboud FM and Chapleau MW. Oxygen-derived free radicals contribute to baroreceptor dysfunction in atherosclerotic rabbits. Circ Res 1996; 79: 802-811.
[7] Studinger P, Mersich B, Lenard Z, Somogyi A and Kollai M. Effect of vitamin e on carotid artery elasticity and baroreflex gain in young, healthy adults. Auton Neurosci 2004; 113: 63-70.
[8] Irigoyen MC, Paulini J, Flores LJ, Flues K, Bertagnolli M, Moreira ED, Consolim-Colombo F, Bello-Klein A and De Angelis K. Exercise training improves baroreflex sensitivity associated with oxidative stress reduction in ovariectomized rats. Hypertension 2005; 46: 998-1003.
[9] Bertagnolli M, Campos C, Schenkel PC, de Oliveira VL, De Angelis K, Bello-Klein A, Rigatto K and Irigoyen MC. Baroreflex sensitivity improvement is associated with decreased oxidative stress in trained spontaneously hypertensive rat. J Hypertens 2006; 24: 2437-2443.
[10] Mercuro G, Podda A, Pitzalis L, Zoncu S, Mascia M, Melis GB and Rosano GM. Evidence of a role of endogenous estrogen in the modulation of autonomic nervous system. Am J Cardiol 2000; 85: 787-789.
[11] Hernandez I, Delgado JL, Diaz J, Quesada T, Teruel MJ, Llanos MC and Carbonell LF. 17beta-estradiol prevents oxidative stress and decreases blood pressure in ovariectomized rats. Am J Physiol Regul Integr Comp Physiol 2000; 279: R1599-1605.
[12] Flues K, Paulini J, Brito S, Sanches IC, Consolim-Colombo F, Irigoyen MC and De Angelis K. Exercise training associated with estrogen therapy induced cardiovascular benefits after ovarian hormones deprivation. Maturitas 2010; 65: 267-271.
[13] Benetti-Pinto CL, Bedone AJ and Magna LA. Evaluation of serum androgen levels in women with premature ovarian failure. Fertil Steril 2005; 83: 508-510.
[14] Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R; International Brachial Artery Reactivity Task Force. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: A report of the international brachial artery reactivity task force. J Am Coll Cardiol 2002; 39: 257-265.
[15] Malliani A, Pagani M, Lombardi F and Cerutti S. Cardiovascular neural regulation explored in the frequency domain. Circulation 1991; 84: 482-492.
[16] Montano N, Porta A, Cogliati C, Costantino G, Tobaldini E, Casali KR and Iellamo F. Heart rate variability explored in the frequency domain: A tool to investigate the link between heart and behavior. Neurosci Biobehav Rev 2009; 33: 71-80.
[17] Aebi H. Catalase in vitro. Methods Enzymol 1984; 105: 121-126.
[18] Marklund S and Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974; 47: 469-474.
[19] Flores LJ, Figueroa D, Sanches IC, Jorge L and Irigoyen MC, Rodrigues B, De Angelis K. Effects of exercise training on autonomic dysfunction management in an experimental model of menopause and myocardial infarction. Menopause 2010; 17: 712-717.
[20] Gonzalez Flecha B, Llesuy S and Boveris A. Hydroperoxide-initiaded chemiluminescence: An assay for oxidative stress in biopsies of heart, liver, and muscle. Free Rad Biol Med 1991; 10: 1093-1100.
[21] Lowry OH, Rosenbrough NT and Farr AL. Protein measurements with the folin phenol reagent. J Biol Chem 1951; 193: 265-275.
[22] Herrington DM, Espeland MA, Crouse JR 3rd, Robertson J and Riley WA, McBurnie MA, Burke GL. Estrogen replacement and brachial artery flow-mediated vasodilation in older women. Arterioscler Thromb Vasc Biol 2001; 21: 1955-1961.
[23] de Kleijn MJ, Wilmink HW, Bots ML, Bak AA, van der Schouw YT, Planellas J, Engelen S, Banga JD and Grobbee DE. Hormone replacement therapy and endothelial function. Results of a randomized controlled trial in healthy postmenopausal women. Atherosclerosis 2001; 159: 357-365.
[24] Kalantaridou SN, Naka KK, Papanikolaou E, Kazakos N, Kravariti M, Calis KA, Paraskevaidis EA, Sideris DA, Tsatsoulis A, Chrousos GP and Michalis LK. Impaired endothelial function in young women with premature ovarian failure: Normalization with hormone therapy. J Clin Endocrinol Metab 2004; 89: 3907-3913.

[25] Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A and Salvetti A. Age-related reduction of no availability and oxidative stress in humans. Hypertension 2001; 38: 274-279.
[26] Chowdhary S and Townend JN. Role of nitric oxide in the regulation of cardiovascular autonomic control. Clin Sci (Lond) 1999; 97: 5-17.
[27] Barp J, Araujo AS, Fernandes TR, Rigatto KV, Llesuy S, Belló-Klein A and Singal P. Myocardial antioxidant and oxidative stress changes due to sex hormones. Braz J Med Biol Res 2002; 35: 1075-1081.
[28] Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sato T and Inoue M. Does superoxide underlie the pathogenesis of hypertension? Proc Natl Acad Sci U S A 1991; 88: 10045-10048.
[29] Delgado JL, Landeras J, Carbonell LF, Parrilla JJ, Abad L, Quesada T, Fiol G and Hernandez I. Effect of n-acetyl-cysteine on vascular endothelelium function in aorta from oophorectomized rats. Gen Pharmacol 1998; 32: 23-27.
[30] Wallin BG. Interindividual differences in muscle sympathetic nerve activity: a key to new insight into cardiovascular regulation? Acta Physiol (Oxf) 2007; 190: 265-75.
[31] Meskhi A and Seif MW. Premature ovarian failure. Curr Opin Obstet Gynecol 2006; 18: 418-426
[32] Heeren MV, De Sousa LE, Mostarda C, Moreira E, Machert H, Rigatto KV, Wichi RB, Irigoyen MC and De Angelis K. Exercise improves cardiovascular control in a model of dislipidemia and menopause. Maturitas 2009; 62: 200-204.
[33] Moien-Afshari F, Kenyon E, Choy JC, Battistini B, McManus BM and Laher I. Long-term effects of ovariectomy and estrogen replacement treatment on endothelial function in mature rats. Maturitas 2003; 45: 213-223.
[34] Du XJ, Riemersma RA and Dart AM. Cardiovascular protection by oestrogen is partly mediated through modulation of autonomic nervous function. Circ Res 1995; 30: 161-165.
[35] Du XJ, Dart AM, Riemersma RA and Oliver MF. Sex difference in presynaptic adrenergic inhibition of norepinephrine release during normoxia and ischemia in the rat heart. Circ Res 1991; 68: 827-835.
[36] Mullan BA, Young IS, Fee H and McCance DR. Ascorbic acid reduces blood pressure and arterial stiffiness in type 2 diabetes. Hypertension 2002; 40: 804-809.
[37] Heart rate variability: standards of measurement, physiological interpretation, and clinical use. Task Force of the European Society of Cardiology and The North American Society of Pacing and Electrophysiology. Circulation 1996; 93: 1043-1065.
[38] Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J; Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 2002; 3: 321-333.
[39] Hunt BE, Taylor JA, Hamner JW, Gagnon M and Lipsitz LA. Estrogen replacement therapy improves baroreflex regulation of vascular sympathetic outflow in postmenopausal women. Circulation 2001; 103: 2909-2914.