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Ovarian failure induced by 4-vinylcyclohexene diepoxide worsens the autonomic cardiovascular response to chronic unpredictable stress in rats

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ABSTRACT

Aims: After menopause, women are more responsive to stress and more prone to exhibit hypertension, which elevates the risk of cardiac diseases. This vulnerability is due, in part, to the decline of ovarian steroids plasma levels. The 4-vinylciclohexane diepoxide (VCD) causes a gradual depletion of ovarian follicles causing loss of the normal ovarian function and a hormonal profile comparable to menopause in humans. We aimed to verify whether the ovarian failure (OF) worsens the cardiovascular autonomic response to stress.

Main methods: Rats were treated with VCD (160 mg/kg) or oil for 15 days, exposed to chronic unpredictable stress (CUS) for 10 days and studied 80 and 180 days after VCD treatment.

Key findings: 80 days after VCD-treatment, stressed rats showed increased sympathetic nerve activity, reduced parasympathetic activity and an increase in the overall spontaneous baroreflex sensitivity (BRS). 180 days after VCD treatment, BRS was impaired and the vascular sympathetic activity was increased, independently of stress exposure.

Significance: Neither 80 nor 180 days after the onset of VCD-treatment the hypertensive effects of stress were enhanced in rats. However, OF led to a worsening on different aspects of the cardiovascular response to stress, which can cause cardiovascular complications when associated with ovarian aging.

1. Introduction

Ovarian aging in female mammals is characterized by a progressive ovarian failure (OF), aging-related uterine defects and changes in the neuroendocrine axis [1]. In women, ovarian aging seems to correlate with a worsening of the hypothalamic pituitary adrenal axis (HPAA) functioning, which can lead to an extended recovery after stress [2]. However, few studies have focused on the reproductive status of women considering the physiological stress response.

Stress is a risk factor for cardiovascular diseases [3] and menopausal women are more susceptible since they present increased sympathoadrenal responsiveness, indicating an increased HPAA activity [4]. Also, young women tend to have lower levels of blood pressure compared to men at the same age [5] and women after menopause [6], suggesting a significant role of ovarian steroids on the maintenance of blood pressure. Indeed, estradiol acts as a cardiovascular protector by decreasing the expression of angiotensin II receptor type 1 (AT-1 receptors), and of angiotensin-converting enzyme (ACE) [7,8]. On the other hand, administration of losartan, an AT-1 receptor antagonist, reduces but does not normalize the arterial pressure in old hypertensive rats [9], indicating a possible concomitant baroreflex malfunctioning. Thus, although the malfunctioning of the renin-angiotensin-aldosterone system seems indisputable, the contribution of the baroreflex to hypertension in this context is much less known. A reduction in the baroreflex sensitivity can produce increased arterial pressure lability, which impairs the endothelial function and contributes to the emergence of hypertension due to an increased vascular tone [10]. Also, the removal of ovarian hormones in hypertensive rats worsens the baroreflex and changes the autonomic control of the heart [11]. The incidence of cardiovascular diseases is greater when associated with stress. Cardiovascular responses to stress are more intense when stressors are long, inescapable and unpredictable [12,13]. Although repeated exposure to

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stress can cause habituation [12], this response is absent for chronic unpredictable stress (CUS). In rats, CUS induces a decreased expression of endothelial nitric oxide synthase [14], elevated resting heart rate (HR) and reduction of the HR variability [15].

The study of menopause requires experimental models with similarities to human endocrine aspects of ovarian biology. The exposure to a chemical compound called 4-vinylcyclohexene diepoxide (VCD) leads to a gradual OF resulting in depleted follicles and retention of residual ovarian tissue, as occurs in women during menopause [16–18]. Differently from what is seen in ovariectomized rats, where there is an abrupt withdrawal of ovarian hormones, the VCD model mimics the gradual progression of perimenopause to menopause, by selectively destroying primordial and primary follicles, without affecting either the estrous cyclicity [18] or the body weight [19,20]. It accelerates the natural follicular atresia, leading over time, to increased levels of follicle-stimulating hormone (FSH), similarly to what occurs in menopausal women [16]. Also, since OF induced by VCD is installed early in life, the mechanisms underlying this process can be studied without the influence of ovarian aging. Since FSH levels increase at the end of perimenopause in women [21], the present study evaluated rats 80 days after VCD administration, when according to Reis and colleagues [22], FSH levels are not altered and would be equivalent to the perimenopause/periestropause. FSH levels start to rise on day 120 after VCD treatment [18] and, following this idea, we chose the periods of 80 and 180 days after the beginning of VCD treatment, assuming that these periods can be related in some points to perimenopause and menopause, as previously described [19]. Estradiol levels are unstable on the VCD model and its variability increases around one year after the onset of VCD treatment [18].

Many risk factors can promote cardiovascular problems in women, including oophorectomy and menopause. The lack or the fluctuation of estrogen increases sensitivity to stress [23], which can lead to a higher vulnerability to other issues like mood disorders and cardiovascular problems. The mechanisms by which the cardiovascular system can be affected by this context are still unclear since no studies have been performed relating the effects of the ovarian failure on cardiovascular responses to stress.

For this purpose, the aim of the current study was to evaluate whether female rats submitted to VCD-induced OF display a worsening on the cardiovascular responses to a chronic exposition to stress, 80 and 180 days after treatment. We hypothesized that the association between CUS and OF will unbalance the autonomic control of heart rate, which may lead to the development of hypertension in rats with OF.

2. Materials and methods

2.1. Induction of Ovarian failure and chronic unpredictable stress protocol

Female Wistar rats, at post-natal day (PND) 21, from the animal care facility of Federal University of Santa Catarina were housed in white propylene cages, at 21 ± 2 °C, an inverted 12 h dark/light cycle (light from 6:00 am to 6:00 pm/dark from 6:00 pm to 6:00 am) and food and water ad libitum.

The treatment with VCD (160 mg/kg, i.p) or corn oil (placebo-control groups) was initiated at PND 28 and continued for 15 consecutive days, according to [19,24,25]. Experiments were accomplished 80 ± 3 days (226 ± 16 g) or 180 ± 3 days (255 ± 20 g) after the onset of VCD treatment. Ten days before surgery, animals were submitted to a CUS protocol. Thus, animals were placed in the following study groups: CTL80 (control group, $n = 15$), VCD80 ($n = 12$); CUS80 (stressed control group, $n = 17$); CUS + VCD80 (stressed + VCD80 group, n = 15); CTL180 (control group, $n = 14$), VCD180 (n = 14); CUS180 (stressed control group, $n = 16$) and CUS + VCD180 (stressed + VCD180 group, $n = 13$). The CUS protocol [26–28] consisted of a random application of different stressors for ten days, twice a day (adapted from [29]). The stressors stimuli used were: Immobilization for 60 min; cold exposure (4 °C– 60 min); forced swimming (5 min); water and food deprivation (12 h) and dark/light cycle inversion (24 h of light or 24 h of dark). None visual sign of stress was measured.

2.2. Measurements of cardiovascular responses

All animals were submitted to surgery for implantation of catheters into the femoral artery and vein [30,31]. Rats were anesthetized (ketamine, 90 mg·kg^{-1} + xylazine, 10 mg·kg^{-1}, i.p.) and a polyethylene catheter was implanted into the left femoral artery and vein (PE-10/PE-50, IBD Company, USA). 24 h later, rats did not exhibit signs of stress. Then, the arterial catheter was connected to a pressure transducer (AVS Project, Brazil) coupled to an amplifier (AECAD 04P, AVS Projects, São Carlos, Brasil) and a recording system (Powerlab, ADInstruments, Australia). Beat-by-beat time series with diastolic arterial pressure (DAP), systolic arterial pressure (SAP), mean arterial pressure (MAP), HR and pulse interval (PI) values were generated from the pulsatile arterial pressure. Parameters were recorded 30 min before and during the studied reflex at a sampling rate of 1 kHz for two subsequent days. On the first day, the basal recording of cardiovascular parameters was analyzed, as well as the baroreflex induced by vasoactive drugs and part of the autonomic tone in response to atropine and propranolol administration. On the second day, the contribution of the sympathetic nervous system to vasculature as well as the responsiveness of α 1-adrenergic vascular receptors was evaluated.

2.3. Arterial pressure/HR variability and spontaneous baroreflex sensitivity

The PI and SAP variability analyses were performed using computer software (CardioSeries v2.4– http://www.danielpenteado.com). Beat-by-beat series recordings were converted to data points every 100 ms using cubic spline interpolation (10 Hz). Prior to the calculation of the spectral density, data were visually inspected and the non-stationary segments were disregarded. The interpolated series were divided into half-overlapping sequential sets of 512 data points (51.2 s). The spectra of the SAP and PI variability was integrated into the low (LF; 0.2–0.75 Hz) and high (HF; 0.75–3 Hz) frequency bands.

The spontaneous baroreflex sensitivity (BRS) was assessed in the time-domain by means of the sequence analysis technique using the same software (CardioSeries v2.4), which scanned beat-by-beat time series of SAP and PI searching for sequences of 4 consecutive beats in which increases in SAP were followed by PI lengthening (up sequence) and decreases in SAP were followed by PI shortening (down sequence), with a linear correlation higher than 0.85. The slope of the linear regression lines between SAP and PI was taken as a measure of BRS [32].

2.4. Autonomic tone and intrinsic heart rate

After the baroreflex evaluation (first day) and the basal cardiovascular parameters recordings (second day), parasympathetic and sympathetic tone, and intrinsic heart rate (IHR) were measured by the response to atropine (2 mg/kg, iv) and propranolol (4 mg/kg, iv) with a maximum volume of 0.2 mL per injection. First day: atropine was administered in half of the animals, and 15 min later propranolol was administered for IHR evaluation, while in the other half, the order of administration was reversed. Second day: the sequence of administration was reversed for each rat. The sympathetic tone was measured as the difference between maximum HR after atropine administration and IHR. The parasympathetic tone was determined as the difference between the lowest HR after propranolol injection and IHR [33].

2.5. Baroreflex sensitivity

On the first day, 30 min after the basal recording, pharmacological stimulation of the baroreflex was performed. All drugs used to stimulate the baroreflex were injected in a small volume corresponding to 200 μL.

To determine the baroreflex sensitivity, reflex variations in HR were elicited by changes in MAP induced by a bolus intravenous injection of phenylephrine (PE; 8 μg/kg; Sigma, St. Louis, MO, USA) or sodium nitroprusside (SNP; 32 μg/kg; Sigma, St. Louis, MO, USA). The baroreflex index was calculated as the DHR/DMAP ratio (bpm/mmHg) for bradycardia and tachycardia reflexes. On the second day, hexamethonium, a ganglionic blocker, was injected (25 μg/kg; Sigma, St. Louis, MO, USA) and the cardiovascular parameters were recorded for the next 5 min. The magnitude of the fall in MAP induced by the ganglionic blockade was used to evaluate the contribution of the sympathetic nervous system to the maintenance of the baseline MAP [34]. After the ganglionic block, to determine the responsiveness of α 1 -adrenergic vascular receptors, seven increasing doses of phenylephrine at concentrations of 1, 10, 100, 150 and 200 μg/kg were administered, with a 5-min interval between doses. The responsiveness was calculated as the maximum response to phenylephrine minus the average of ten seconds before the administration of each dose.

2.6. Hormonal assay

After recording cardiovascular parameters, all rats were euthanized by anesthetic overdose (urethane-1.3 g/kg, i.p.); blood samples were collected by cardiac puncture and centrifuged at 2500 ×*g*. Plasma was frozen at −20 °C until the assay. Plasma progesterone was determined using MP kits (MP Biomedicals; Orangeburg-NY, USA) and estradiol was determined by chemiluminescence according to manufacturer's specifications. Plasma corticosterone radioimmunoassay (RIA) was carried out using specific standard and antibody (Sigma Co., USA) and tritiated hormone (Perkin Elmer- NET399001MC - (1,2,6,7-3H(N))-Corticosterone, 1 mCi (37 MBq)), after plasma extraction using ethanol [35]. The minimal detectable level for progesterone and corticosterone were 0.02 ng/mL and 0.08 ng/mL, respectively. The intra-assay error was 5% for progesterone and 4.5% for corticosterone.

2.7. Ovarian histology

Rats were perfused with 4% paraformaldehyde (PFA) and ovaries were separated, immersed in 4% PFA for 24 h, embedded in paraffin, cut in 5 μm serial sections, processed and stained with hematoxylin and eosin. The number of healthy antral follicles, atretic follicles, and corpus luteum was counted in every ten sections.

2.8. Statistical analysis

All data were analyzed by Two-Way ANOVA, using "VCD-treatment" and "stress" as the two factors, followed by the Bonferroni post hoc test. The outliers were removed considering the interval containing 95% of data, by calculating the mean ± 2× standard deviation of the mean (SDM). Statistical analyses were conducted using the Prism Statistical software 6.0 (GraphPad Software Inc., San Diego, Calif., USA). Differences were considered statistically significant if $p < 0.05$ and the results are presented as mean ± standard error of the mean (SEM).

3. Results

Regarding the animals evaluated 80 days after VCD treatment, there was no significant effect of VCD or stress on the body weight, the relative weight of adrenals and DAP in the studied groups (Table 1). All data presented one degree of freedom. The mean of MAP (p = 0,001; F1,35 = 11.99, η^2 = 25.42%) and SAP (p < 0,0001; F1,36 = 24.25; η^2 = 39.54%), were increased, confirming the effect of stress, independently of the treatment with VCD (Table 1). Conversely, when rats were analyzed 180 days after VCD treatment, there was a significant interaction between the factors "VCD" and "stress" (p = 0.02; F1,53 = 5.20; η^2 = 7.43%; η_p^2 = 0.09), revealing that VCD-treated animals presented a reduction in body weight only when submitted to stress (Table 1). Moreover, stress reduced this parameter (p = 0.001; F1,53 = 11.54; η^2 = 16.48%) and increased the relative weight of adrenals (p = 0.006, F1,50 = 8.19; η^2 = 13.66%), regardless of the VCD treatment (Table 1). Regarding cardiovascular parameters after 180 days, stress increased the MAP (p = 0.005, F1,49 = 8.50; η^2 = 14.69%) as well as the DAP (p < 0.0005, F1,49 = 13.91; η^2 = 20.52%). Besides that, VCD treatment significantly increased the DAP (p = 0.01; F1,49 = 6.31; η^2 = 9.31%), regardless the exposition to stress. In contrast, there was no significant variation on the SAP or HR among the studied groups (Table 1).

Spectral variability of the HR in rats evaluated 80 days after VCD treatment exhibited a significant interaction between factors "VCD" and "stress", showing that VCD-treated rats presented an increased LF band (p = 0.004; F1,38 = 9.90; η^2 = 18.45%; η_p^2 = 0.190; Fig. 1A), a decreased HF band (p = 0.004; F1,38 = 8.90; η^2 = 18.45%; η_p^2 = 0.190; Fig. 1C) and an increased LF/HF ratio (p = 0.01; F1,36 = 6.49; η^2 = 14.97%; η_p^2 = 0.153; Fig. 1D), but only when submitted to CUS. However, the analysis of rats evaluated 180 days after VCD treatment showed that none of the following cardiovascular parameters were significantly affected by VCD nor stress: the power of the LF band (Fig. 1B), HF band (Fig. 1D) and LF/HF ratio (Fig. 1E).

Fig. 2 shows that there was no effect or interaction between VCD and stress regarding the power of the LF band of SAP among the groups studied 80 days after VCD treatment and their controls (Fig. 2A). Cardiac autonomic tone evaluated by pharmacological blockade showed that stressed groups had a higher sympathetic tone compared to controls (p = 0,03; F1,34 = 5.01; η^2 = 12.81%; Fig. 2C), while the parasympathetic tone was similar between the groups (Fig. 2E). The IHR was higher in the VCD-treated rats compared to their respective vehicle groups (P = 0.001; F1,69 = 10.94; η^2 = 13.08%; Fig. 2G). Regarding rats evaluated 180 days after VCD treatment, data showed that the power of the LF band of SAP was significantly affected by VCD nor stress (Fig. 2B), but stressed animals had a higher sympathetic tone compared to their respective controls (p = 0.02; F1,45 = 5.12; η^2 = 9.83% Fig. 2D), while the parasympathetic tone was similar between the groups (Fig. 2F). The IHR was lower in stressed rats compared to their respective controls (P = 0.009; F1,94 = 7.04; η^2 = 6.81%; Fig. 2H).

The BRS showed a significant interaction between the factors "VCD" and "stress" in rats evaluated 80 days after VCD treatment (p = 0.03; F1,33 = 5.01; η^2 = 12.42%; η_p^2 = 0.125; Fig. 3A). Indeed, VCD-treatment led to an increase in the overall baroreflex sensitivity (all baroreflex sequences), but only in stressed animals. The BEI was similar among the studied groups (Fig. 3B), as expected. The reflex bradycardia provoked by phenylephrine administered at a dose of 08 μg/kg, was different among the groups due to the interaction of "VCD" and "stress" (p = 0.01; F1,34 = 6; η^2 = 13.93%; η_p^2 = 0.150; Fig. 3C), showing an improvement of the reflex bradycardia only in stressed rats treated with VCD and analyzed 80 days later. Regarding the rats studied 180 days after VCD treatment, neither the BRS (Fig. 3B) nor the baroreflex effectiveness index (Fig. 3D) were significantly affected by VCD nor stress. In contrast, VCD reduced the reflex bradycardia evoked by phenylephrine (08 μg/kg; p = 0.02; F1,46 = 5.22; η^2 = 9.83%; Fig. 3F), independently of stress exposure.

After 80 days, there was an improvement of the reflex tachycardia (evoked by sodium nitroprusside) only in VCD-treated animals, independently of exposure to CUS (p = 0.03; F1,35 = 4.56; η^2 = 11.04%; Fig. 4A). After removal of autonomic tone (evoked by hexamethonium), the fall in the MAP did not differ significantly among the studied groups (25 mg/kg i.v.; Fig. 4C). The α1-adrenergic receptor responsiveness to vessels was similar between experimental groups compared to their respective controls (Fig. 4E).

When rats were analyzed 180 days after VCD/vehicle treatment, we observed that, independently of stress exposure, VCD reduced the reflex tachycardia evoked by sodium nitroprusside (32 μg/kg; p = 0.04;

Table 1
Body weight (BW), mean arterial pressure (MAP), diastolic arterial pressure (DAP), Systolic arterial pressure (SAP) and heart rate (HR) in rats 80 or 180 days after the initiation of the treatment with VCD) or vehicle, exposed to unpredictable chronic stress (CUS) or not. **80**: $^{a}P < 0.002$ compared to not stressed groups; **180**: $^{a}P < 0.006$ compared to not stressed groups; $^{\#}P < 0.03$ compared with VCD180 group.

Groups 80	BW	Adrenals	MAP	DAP	SAP	HR
Vhc 80	227 ± 16	0,02 ± 0,002	109 ± 10	96 ± 9	129 ± 13	363 ± 37
CUS 80	226 ± 13	0,03 ± 0,005	119 ± 5[aa]	98 ± 7	147 ± 8 [aaaa]	387 ± 37
VCD 80	218 ± 12	0,02 ± 0,003	110 ± 8	94 ± 8	133 ± 10	377 ± 38
CUS-VCD 80	226 ± 9	0,02 ± 0,005	119 ± 9 [aa]	97 ± 11	148 ± 8 [aaaa]	362 ± 51

Groups 180	BW	Adrenals	MAP	DAP	SAP	HR
Vhc 180	262 ± 18	0,023 ± 0,005	106 ± 15	87 ± 13	131 ± 20	363 ± 9
CUS 180	255 ± 20 [aa]	0,027 ± 0,006 [a]	116 ± 11 [aa]	98 ± 10 [aaaa]	141 ± 14	356 ± 45
VCD 180	280 ± 25	0,022 ± 0,005	109 ± 6	94 ± 5	137 ± 12	366 ± 49
CUS-VCD 180	247 ± 24[aa #]	0,026 ± 0,005 [a]	116 ± 9 [aaaa]	104 ± 11 [aaaa]	139 ± 10	373 ± 22

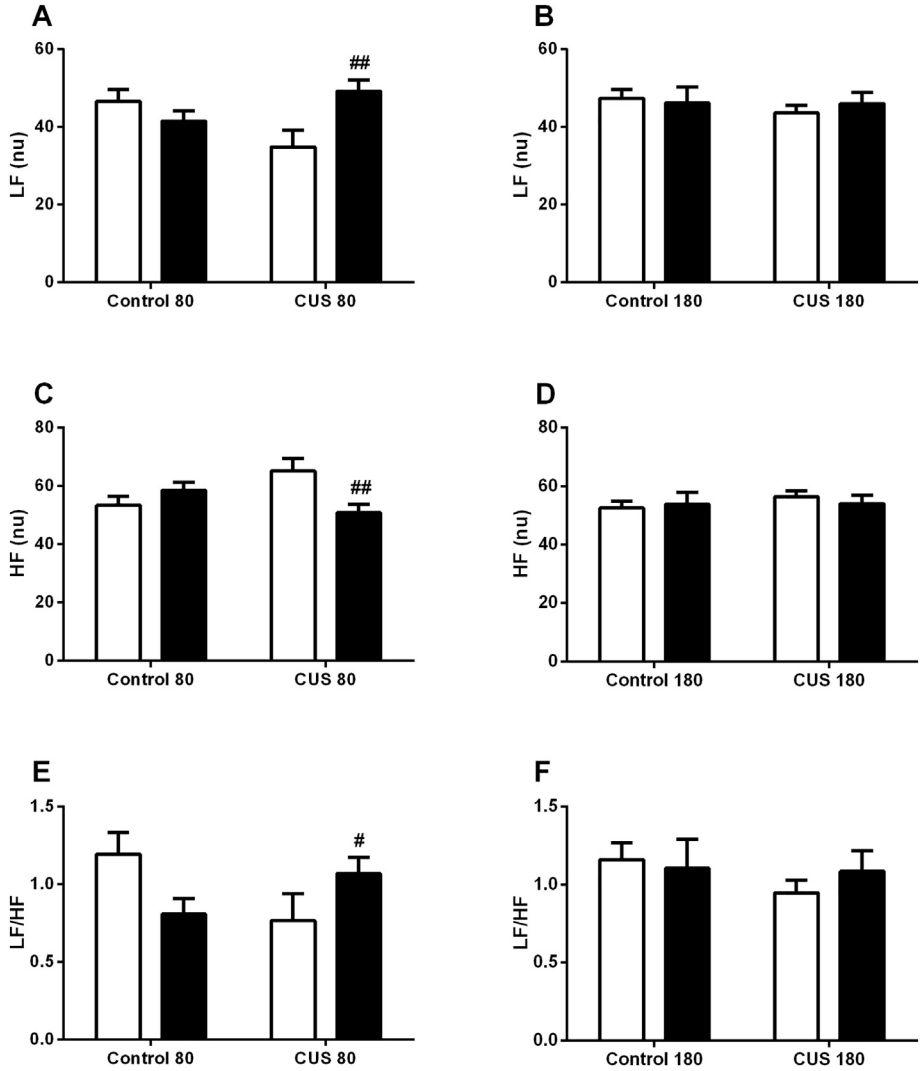

Fig. 1. Power of low (LF- A,B) and high frequency bands (HF- C,D), LF/HF ratio (E,F) of pulse interval spectrum, 80 (A, C and E) and 180 (B, D and F) days after the initiation of treatment with VCD in rats (VCD, black bars) or vehicle (Vhc, white bars), exposed to chronic unpredictable stress (CUS) or not (Control). Data are means ± SE. $\#P < 0.05$ compared with CUS-Vhc group.

F1,48 = 4.20; η^2 = 7.87%; Fig. 4B). Also, VCD treatment led to a larger fall in MAP after administration of hexamethonium ($p = 0.01$; F1,42 = 6.06; η^2 = 11.94%; Fig. 4D), regardless the stress exposure. The α1-adrenergic receptor responsiveness to vessels was similar among experimental groups and their respective controls (Fig. 4F).

Plasma progesterone and estradiol levels were similar among the studied groups 80 days after VCD or vehicle treatment (Fig. 5A and C, respectively). There was a significant effect of VCD treatment reducing corticosterone plasma levels ($p = 0.04$; F1,33 = 4.2; η^2 = 10.31%; Fig. 5E). However, after stress, plasma corticosterone levels were

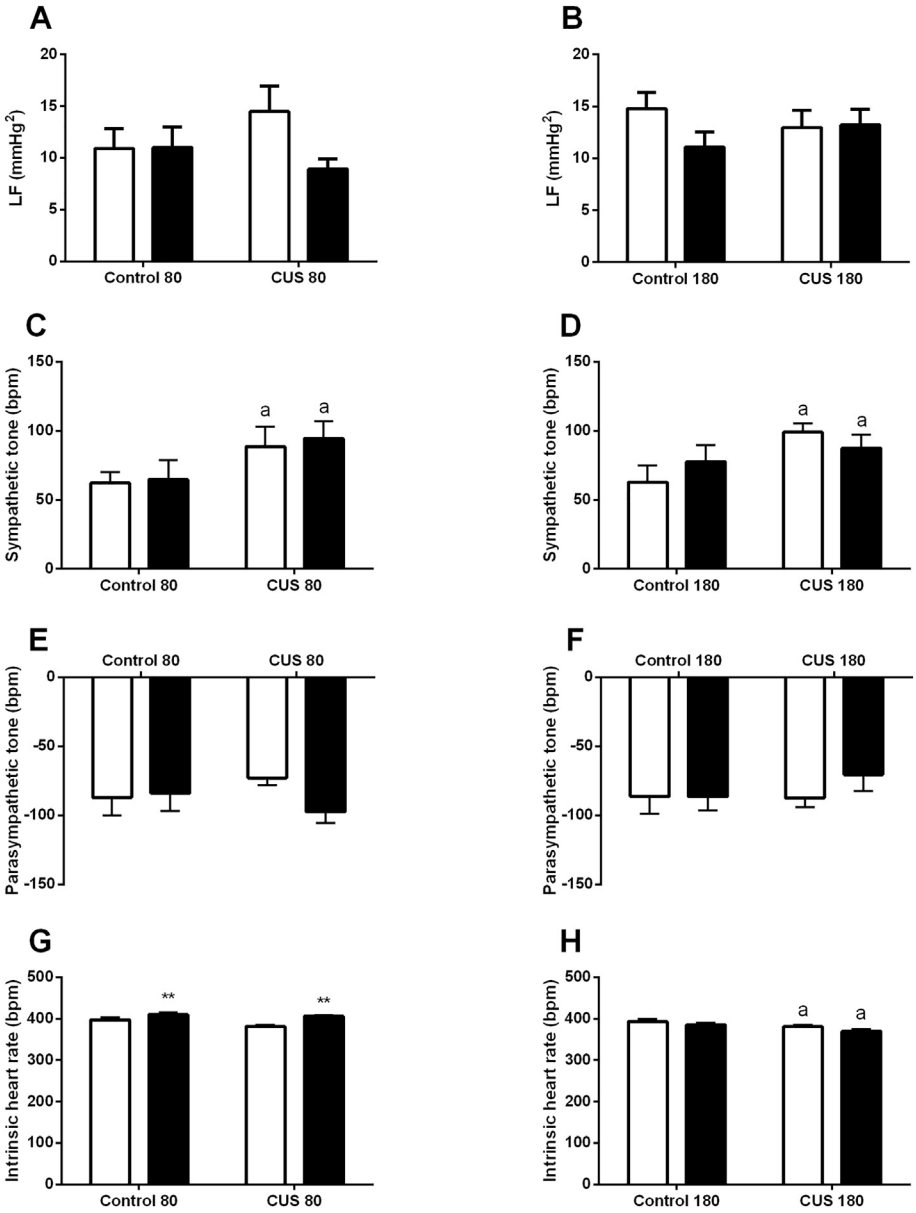

Fig. 2. Power of SAP spectra at low frequency (LF, A,B), sympathetic tone (C,D), parasympathetic tone (E,F) and intrinsic heart rate (G,H), 80 (A, C, E e G) and 180 (B, D, F e H) days after the initiation of treatment with VCD in rats (VCD, black bars) or vehicle (Vhc, white bars), exposed to chronic unpredictable stress (CUS) or not (Control). Data are means ± SE. [a] $p < 0.05$ compared to respective control groups; * $p < 0.05$ compared to respective vehicle groups.

similar in control and VCD-treated rats and stress alone did not significantly affect plasma corticosterone levels. (Fig. 5E). Comparatively, 180 days later, VCD treatment caused a reduction of progesterone plasma levels independently of stress exposure ($p = 0.02$; F1,46 = 9.97; $\eta^2 = 17.59\%$; Fig. 5B). We found a significant interaction of the factors "VCD" and "stress" ($p = 0.03$; F1,27 = 5.11; $\eta^2 = 13.64\%$; $\eta_p{}^2 = 0.159$), reducing estradiol levels only in rats stressed and treated with VCD (Fig. 5D). Even though there was no significant effect of stress by itself, the *p*-value was 0.07, showing a tendency of stress to increase estradiol levels only in rats not treated with VCD (Fig. 5D). Results indicated a significant interaction of the factors "VCD" and "stress" reducing corticosterone plasma levels only in non-stressed animals ($p = 0.05$; F1,45 = 9.69; $\eta^2 = 14.06\%$; $\eta_p{}^2 = 0.162$; Fig. 5F), similarly to the results found in rats evaluated 80 days after VCD treatment. Stressed groups had, likewise, shown no difference in plasma corticosterone levels when compared to their respective control groups (Fig. 5F).

Regarding the ovarian morphology, results from rats evaluated 80 days after VCD treatment and controls showed a significant interaction between the factors "VCD" and "stress" ($p = 0.01$; F1,25 = 6.25; $\eta^2 = 17.03\%$; $\eta_p{}^2 = 0.20$) indicating that stress led to an increased number of antral follicles in animals treated with vehicle, but not in those treated with VCD (Fig. 6A). There was a significant effect of both factors, stress ($p = 0.03$; F1,25 = 5.07; $\eta^2 = 6.76\%$) and VCD ($p < 0.0001$; F1,33 = 39.95; $\eta^2 = 53.18\%$) increasing the number of ovarian cysts (Fig. 6C). With reference to the corpora lutea, there was a significant interaction between the factors "VCD" and "stress" (p = 0.01; F1,25 = 7.25; $\eta^2 = 19.35\%$; $\eta_p{}^2 = 0.225$) showing that VCD-treatment led to an increased number of corpora lutea, but only in rats exposed to stress (Fig. 6E). Comparatively, in rats studied 180 days after VCD treatment and controls, the number of antral follicles and cysts did not vary among the studied groups (Fig. 6B and D, respectively). Instead, there was a significant effect of VCD treatment on reducing the number of corpora lutea in stressed and non-stressed animals (p = 0.03; F1,24 = 4.87, $\eta^2 = 16.66\%$; Fig. 6F).

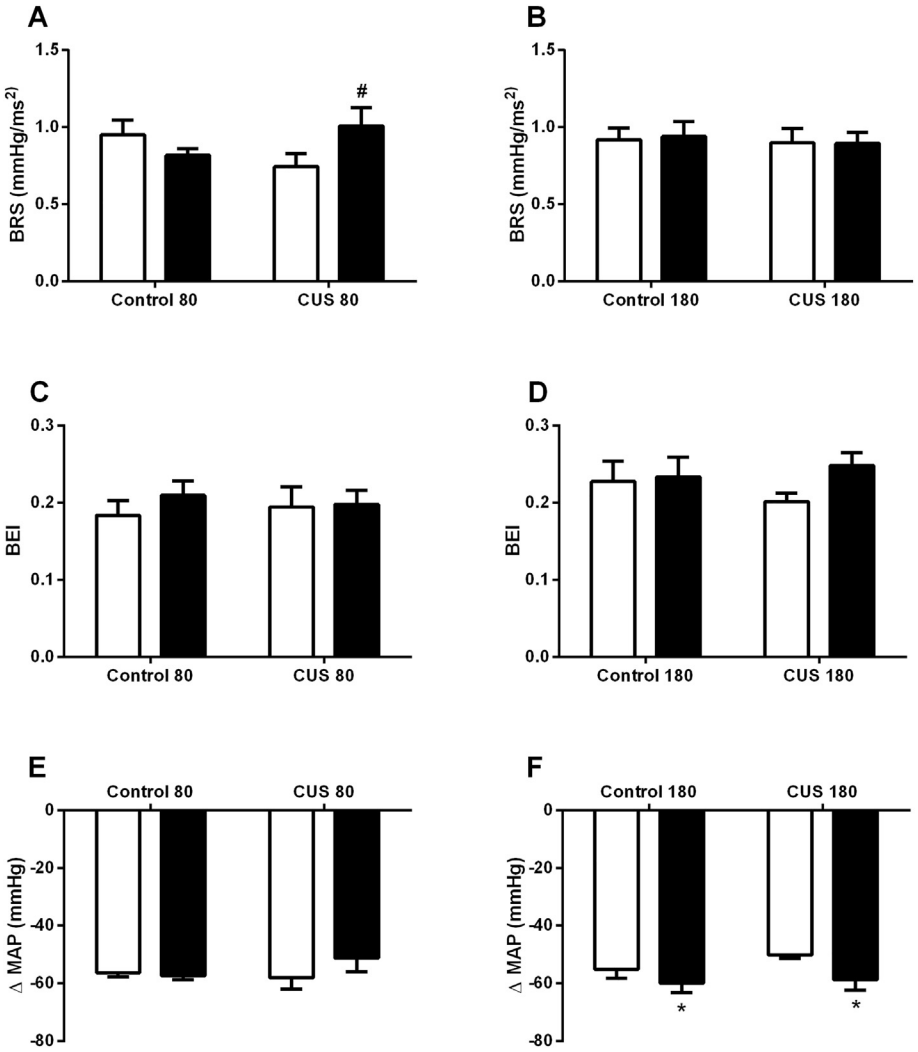

Fig. 3. Spontaneous baroreflex sensitivity (BRS-A,B), baroreflex effectiveness index (C,D), changes in mean arterial pressure (ΔMAP) in response to ganglionic blockade (Hexamethonium, 25 μg/kg; i.v., panels E,F), 80 (A, B and C) and 180 (B, D and F) days after the initiation of treatment with VCD in rats (VCD, black bars) or vehicle (Vhc, white bars), exposed to chronic unpredictable stress (CUS) or not (Control). Data are means ± SE. * $p < 0.05$ compared with their respective Vhc groups. # $p < 0.05$ compared with CUS-Vhc group.

4. Discussion

The chemical VCD causes progressive OF in rodents by accelerating the natural process of atresia. Since most women enter menopause by a gradual OF, maintaining intact ovaries, the transitional OF found in the VCD model closely approximates to the natural human progression through perimenopause and post-menopause (for review see [36]). For this reason, the present study aimed to study the cardiovascular responses to the OF induced by VCD in rats exposed to stress.

4.1. 80 days after VCD treatment

Neither the body weight nor the relative weight of adrenals was affected by the association of VCD and CUS. Male rats present adrenal hypertrophy and hyperplasia induced by CUS [37]; however, the use of young females and different experimental protocols could explain our different results.

Plasma progesterone and estradiol were similar among the studied groups, which corroborates our previous findings [22] and supports the hypothesis that fluctuating levels of estradiol might reflect disruptions in the cycle length rather than impairment of the ovaries steroidogenic capacity [38]. In contrast, we expected to find reduced progesterone levels in VCD-treated rats [22], but progesterone might have been released by adrenals in response to the surgical stress [39], masking the reduction expected on VCD-treated groups. Progesterone released in response to stress can also be observed in women [40].

VCD treatment reduced corticosterone plasma levels in non-stressed rats. Premenopausal women present reduced HPAA activity [41], and the acute estrogen deficit following ovariectomy does not impair cortisol production [42], suggesting that changes in the ovarian function can impact on the HPAA activity. Whether the VCD model is representative of modifications that could lead to this effect on the HPAA cannot be assessed in this study. Contrary to the expected, stress alone did not increase plasma corticosterone levels. As it might have occurred with progesterone levels, a stress-sensitive hormone as well, the surgical stress could have masked the effects of CUS. Also, high levels of corticosterone are found in the dark phase of the circadian cycle [43]. Since here rats were euthanized at different times along the day, our data might present a high variability that could hinder any differences between the groups.

Rats evaluated 80 days after VCD treatment showed a decreased number of healthy antral follicles, corroborating our previous study, increased the number of ovarian cysts and decreased primordial follicles and the ovarian mass, confirming the VCD-induced OF [19,22]. Also, stress increased the number of antral follicles in the controls, but not in VCD-treated rats. Activation of HPAA is one of the major factors

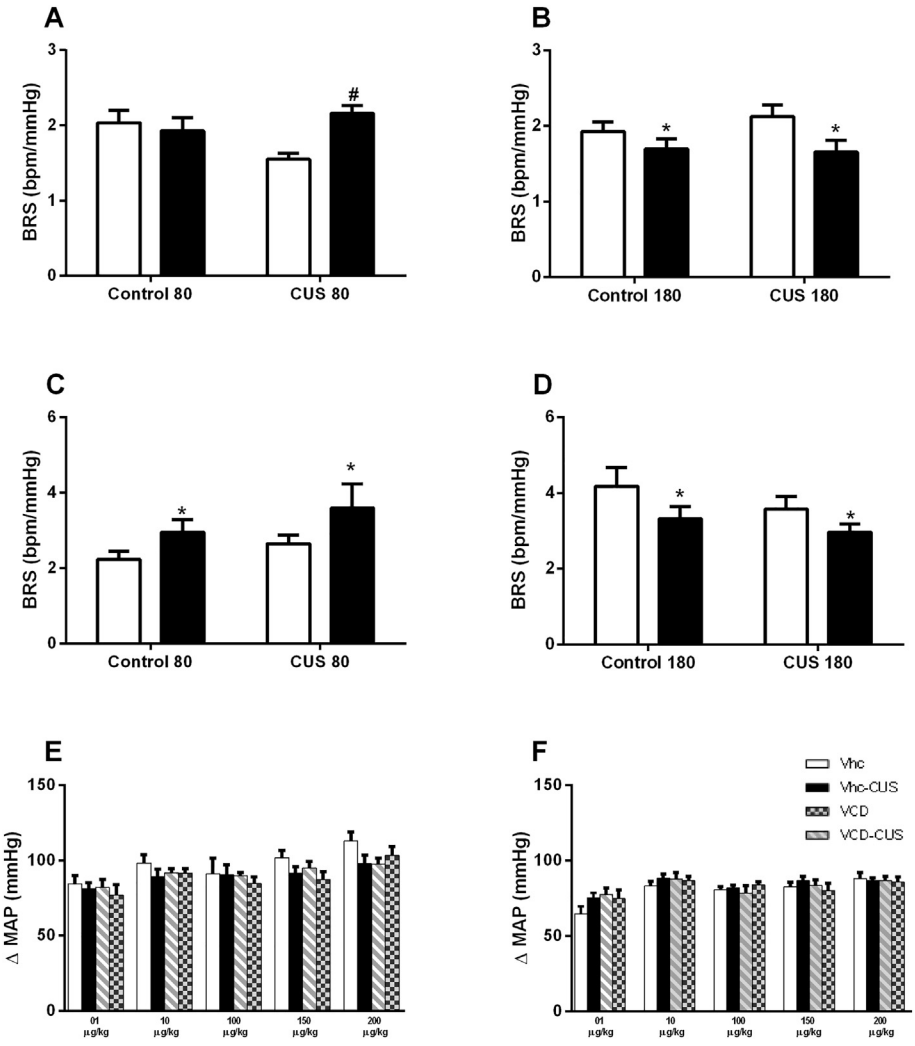

Fig. 4. Baroreflex sensitivity (BRS, ΔHR/ΔMAP) to bradycardia reflex induced by administration of phenylephrine 8 µg/kg (A,B) and to tachycardia reflex induced by administration of sodium nitroprusside 32 µg/kg (C,D), and response to vascular α1-adrenergic vascular receptor stimulation induced by administration of increasing doses of phenylephrine (01, 10, 100,150 and 200 µg/kg, panels E,F) 80 (A, C and E) and 180 (B, D and F) days after the initiation of treatment with VCD in rats (VCD, black bars) or vehicle (Vhc, white bars), exposed to chronic unpredictable stress (CUS) or not (Control). Data are means ± SE. * $p < 0.05$ compared with their respective Vhc groups. # $p < 0.05$ compared with CUS-Vhc group.

interfering on the ovarian functions [44], including the increase of antral follicles in rats [45]. This increase was not seen in VCD rats probably due to their lower number of primary follicles, which impairs the development to antral follicles. Interestingly, the association of stress and OF led to an increase of corpora lutea, suggesting that the follicular development, which is already changed by VCD, might be accelerated by stress exposure. The number of ovarian cysts was increased by VCD-treatment and this effect was aggravated by stress, corroborating the well-known effects of stress (for review see [46]).

We showed for the first time that the autonomic activity of the heart is impaired by the interaction of VCD and stress, leading to a worsening of the cardiovascular system function. This result indicates that VCD per se does not impair the cardiovascular system, corroborating our previous study [19] but when this system is challenged by stress, the impairment becomes evident. The autonomic tone was also measured after a pharmacological challenge with atropine and propranolol, resulting in an increased sympathetic tone in all stressed animals. Conversely, our data from the spectral analysis showed an increased sympathetic tone only in VCD-treated animals. The spectral analysis evaluates physiological conditions while the total blockage of the receptors causes a stronger response of the system. Only after this blockage, we were able to see an effect on control stressed animals, while spectral analysis was more sensitive to catch an interactive effect of stress and VCD-treatment.

The IHR was higher in VCD-treated groups, independently of stress exposure, suggesting cardiac pacemaker compensation to an increased vagal tone or a decreased sympathetic tone to the heart, once the basal HR was normal [47]. However, we did not find an alteration of the autonomic tone to the heart. Surprisingly, the interaction of VCD and stress improved the baroreflex, suggesting that rats studied 80 days after VCD treatment might exhibit a compensatory response to the increased sympathetic tonus. It is known that increased sympathetic tonus and/or decreased parasympathetic tonus, overcharges the cardiovascular system, which can lead to its long-term malfunctioning [48,49]. Our data also indicate that the sympathetic nerve activity to vessels and the α-adrenergic pressor reactivity were not affected by VCD or worsened by stress exposure.

Altogether, these data not only support our hypothesis that the association of stress and VCD-induced OF leads to an imbalance of the autonomic control of heart rate, but also indicates that under this situation, VCD-treated rats can show a compensatory improvement of the baroreflex sensitivity. Although these results cannot be directly extrapolated to human menopause, it contributes to the understanding of part of the cardiovascular mechanisms that became more vulnerable after the reproductive age.

4.2. 180 days after VCD treatment

We had previously shown that VCD alone does not reduce body weight [19], but the present data showed that when a challenge is imposed (CUS), rats evaluated 180 days after VCD treatment were more vulnerable to weight loss. Differently from data observed after 80 days, stress led to an increase of adrenal gland, despite the VCD-treatment. It might be due to ovarian aging since a reduced tolerance to stress can be

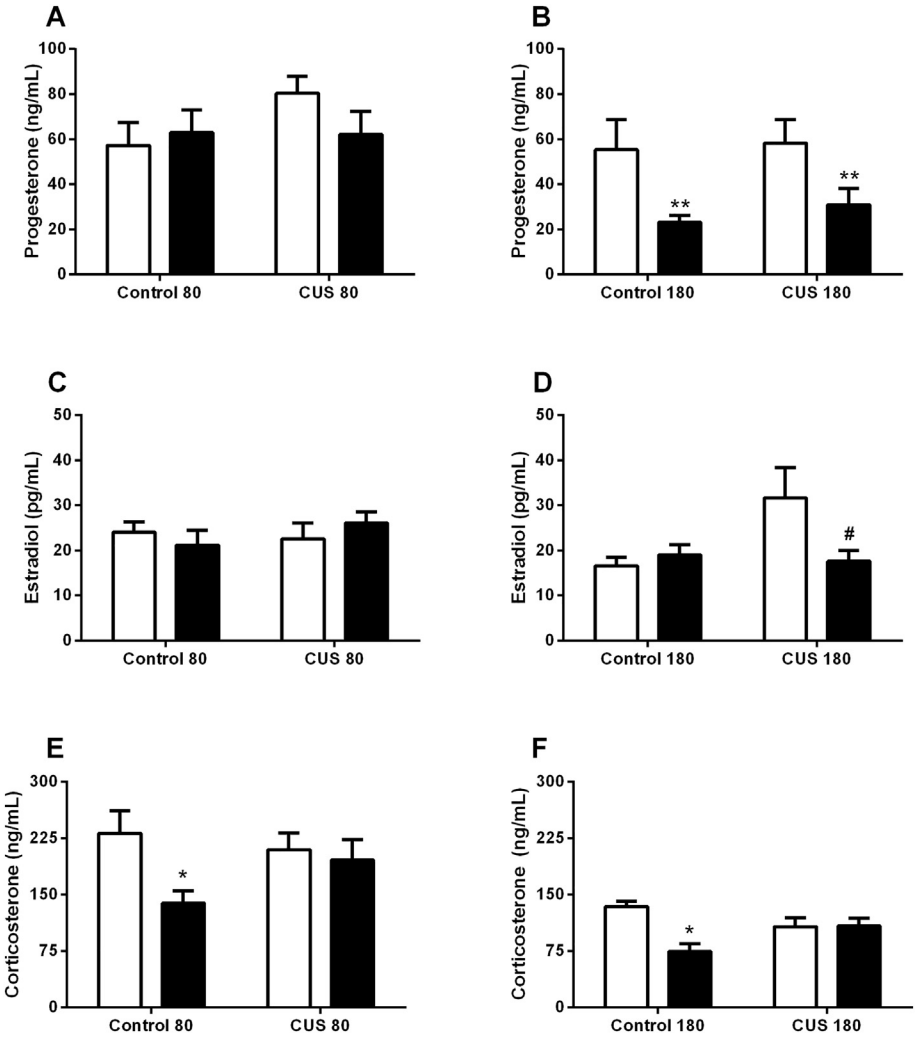

Fig. 5. Progesterone (A,B), estradiol (C,D) and corticosterone (E,F) plasma levels 80 (A, C and E) and 180 (B, D and F) days after the initiation of treatment with VCD in rats (VCD, black bars) or vehicle (Vhc, white bars), exposed to chronic unpredictable stress (CUS) or not (Control). Data are means ± SE. # $p < 0.05$ compared with CUS-Vhc group. * $p < 0.05$ compared with their respective Vhc group.

seen in older individuals [50]. The expected decrease of progesterone levels in VCD-treated rats was observed after 180 days, which was not affected by stress. Lower levels of progesterone are also found in women around menopause [51], probably due to a decrease in the number of corpora lutea [52]. Indeed, our data showed a significant decrease of corpora lutea in VCD-treated groups, independently of stress exposure, opposing the response observed hereafter 80 days. It corroborates the idea that OF occurs as a continuum, reaching a point where stress no longer seems to aggravate the process. Plasma estradiol was not decreased 180 days after VCD treatment, and the reduction of estradiol levels over menopause is not uniform across women either [53]. In the rat VCD model, estradiol levels become erratic and drop only 360 days after the onset of treatment [18]. It seems like 180 days might be too early into the OF process to lead to dropping in estradiol levels. In comparison, the association of OF with stress reduced plasma estradiol levels, corroborating the fact that HPAA activity can suppress the HPG axis, inhibiting estradiol release [54].

Similarly to the results found after 80 days, reduced corticosterone plasma levels in non-stressed VCD-treated animals, indicate that the elevated HPAA activity observed in menopausal women could be a consequence of ovarian aging rather than the fluctuation of estradiol. A longer evaluation of corticosterone levels would be necessary to confirm this idea. The number of antral follicles and cysts was similar in VCD and control rats. Two factors could explain this result: 1) along the ovarian aging process there is a natural increase of ovarian cysts in control rats, independently of stress exposure, minimizing differences among groups; and 2) the stress-induced increase in the number of antral follicles in control rats was abolished at 180 days due to ovarian aging.

The variation on the SAP previously observed in rats evaluated 80 days after VCD treatment did not occur after 180 days. Also, the autonomic activity of the heart was affected by neither VCD nor stress after 180 days. It suggests a compensatory rearrangement of mechanisms controlling the cardiovascular system along the OF progress, normalizing the autonomic imbalance provoked by stress and VCD. Also, there might be an equalization of the autonomic balance between controls and VCD-treated rats due to ovarian aging. Yet, after a pharmacological challenge, there was a higher sympathetic tone in both stressed groups, indicating that, pharmacologically, we were able to see an imbalance in stressed animals at 180 days. These data suggest that ovarian aging might harm the autonomic balance of female rats in response to a pharmacological challenge, in an OF-independent manner. However, other studies would be necessary to confirm the ovarian aging effects in this model. Since the process of ovarian aging is directly related to DNA structure and function, studies propagating cells in culture to stress-induced senescence or the use of ovarian aging markers like telomeric shortening would be helpful.

We have recently shown that VCD-impairs the spontaneous

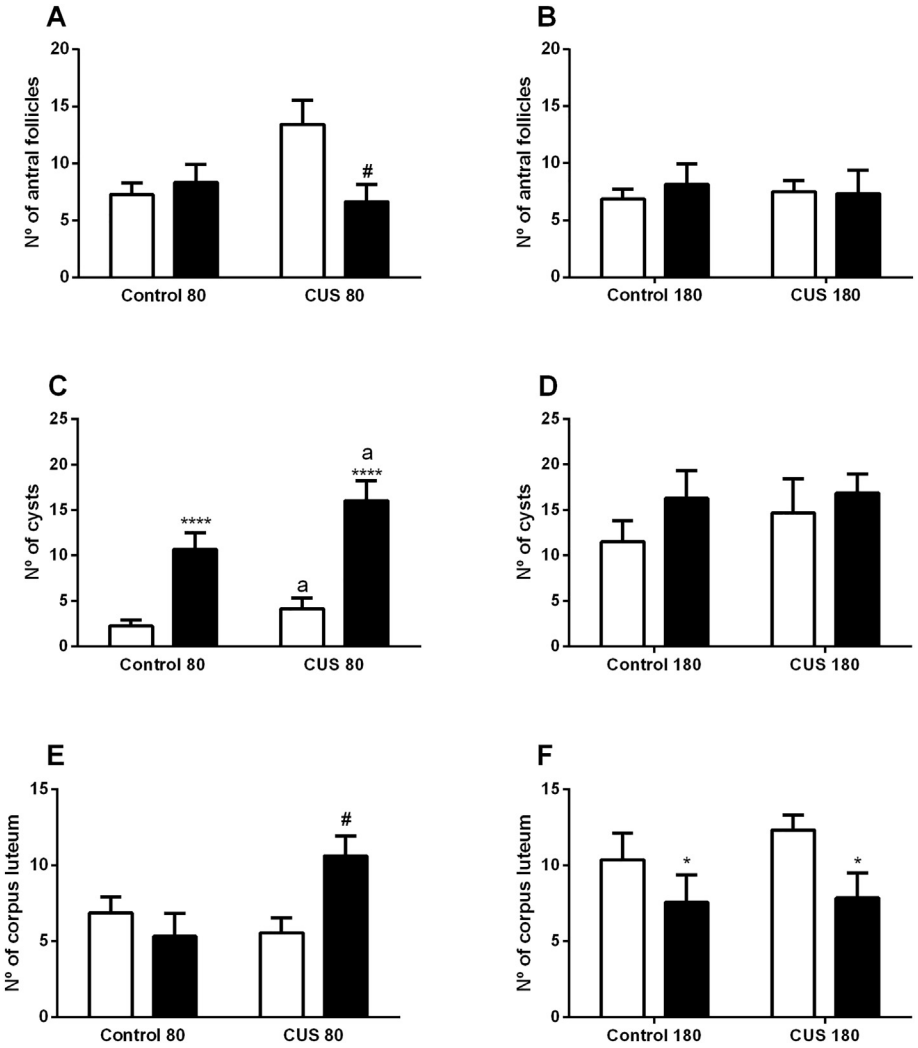

Fig. 6. Number of antral follicles (A,B), number of cysts (C,D) and number of corpus luteum (E,F) 80 (A, C and E) and 180 (B, D and F) days after the initiation of treatment with VCD in rats (VCD, black bars) or vehicle (Vhc, white bars), exposed to chronic unpredictable stress (CUS) or not (Control). Data are means ± SE. # $p < 0.05$ compared with CUS-Vhc group. * $p < 0.05$ compared with their respective Vhc group; [a] $p < 0.05$ compared to control groups.

baroreflex 180 days after treatment [19]. Our present data corroborate these findings and show for the first time that the exposure to stress does not worsen this process. The IHR was reduced in both stressed groups, which is supported by the increased cardiac sympathetic tone, also observed in rats exposed to CUS and evaluated 180 days after VCD treatment. In this case, the decreased IHR might contribute to the basal HR and provide a compensatory mechanism maintaining a normal HR during the altered cardiac autonomic tone.

VCD and/or stress did not affect the BRS, but VCD-treatment increased the sympathetic tonus contribution to the maintenance of MAP in rats after 180 days, independently of the exposure to stress.

5. Conclusion

Altogether, our data indicate that VCD causes an autonomic imbalance, worsening the cardiovascular response to stress 80 days after treatment. However, young rats with OF might compensate this worsening by improving the baroreflex response. VCD does not alter the autonomic balance to the heart after 180 days, but worsens the baroreflex response. Although stress exposure does not aggravate it, an impairment of the baroreflex could lead to cardiovascular complications, mainly when associated with ovarian aging.

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Conflicts of interest

All the authors declare that they have no conflict of interest.

Ethical approval

All procedures were approved by the Ethics Committee on Animal Research of Federal University of Santa Catarina (Florianopolis, SC, Brazil; Protocols PP00832 and PP00842), in accordance with

Guidelines for Ethical Care of Experimental Animals. This article does not contain any studies with human participants performed by any of the authors.

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