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TREATMENT WITH STANDARD AND LOW DOSE OF CONJUGATED EQUINE ESTROGEN DIFFERENTIALLY MODULATES ESTROGEN RECEPTOR EXPRESSION AND RESPONSE TO ANGIOTENSIN II IN MESENTERIC VENULAR BED OF SURGICALLY POSTMENOPAUSAL HYPERTENSIVE RATS.

Priscila Xavier Araujo; Tiago Januário Costa; Cinthya Echem; Maria Aparecida de Oliveira; Rosangela Aparecida Santos-Eichler; Lucas Giglio Colli, Francesc Jiménez-Altayó, Elisabet Vila, Eliana Hiromi Akamine; Ana Paula Dantas; Graziela Scalianti Ceravolo, Maria Helena Catelli de Carvalho.

Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, Brazil. (PXA, TJC, CE, MAO, RASE, LGC, EHA, MHCC)

Department of Physiological Sciences, State University of Londrina, Londrina, Brazil. (GSC)

Facultat de Medicina, Departament de Farmacologia, Terapèutica i Toxicologia, Institut de Neurociències, Universitat Autònoma de Barcelona, Bellaterra, Spain. (FGA, EV)

Group of Atherosclerosis and Coronary disease, Institut Clinic del Torax, Institut d'Investigaciones Biomédiques August Pi I Sunyer (IDIBAPS), Barcelona, Spain. (APD)

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Running title: Dose-dependent regulation of venular function by CEE

Corresponding authors:

Maria Helena Catelli de Carvalho. Department of Pharmacology, Institute of Biomedical Sciences I, University of Sao Paulo. Av. Prof. Lineu Prestes, 1524. São Paulo, SP. 05508-900, Brazil. Tel/Fax: +551130917433 mhcarval@icb.usp.br

Graziela Scalianti Ceravolo. Department of Physiological Sciences, Department of Physiological Sciences, Biological Sciences Center, State University of Londrina, 86057-970, Brazil. Tel/Fax: +55 43 33715930 gsceravolo@uel.br

Ana Paula Dantas. Group of Atherosclerosis and Coronary Disease, Institut Clinic Cardiovascular, Institut d'Investigaciones Biomédiques August Pi I Sunyer (IDIBAPS). C. Casanova, 143, Cellex-2A. Barcelona, 08036, Spain. Tel/Fax: +34934516905 adantas@clinic.ub.es

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List of non-standard abbreviations

Akt - protein kinase B

Ang-II - angiotensin II

AT_1R - angiotensin II receptor type 1

AT_2R - angiotensin II receptor type 2

CEE - conjugated equine estrogen

CEE-LD - standard dose of conjugated equine estrogen

CEE-SD – standard dose of conjugated equine estrogen

DAF-2 - 4.5-diaminofluorescein diacetate

DHE - dihydroethidium

eNOS – endothelial nitric oxide synthase

ER-α – estrogen receptor alpha

ER-β - estrogen receptor beta

GPER - G protein-coupled estrogen receptor

HT - Hormone therapy

L-NAME - NG-nitro-L-arginine-methyl ester

NADPH - nicotinamide adenine dinucleotide phosphate

NOS – nitric oxide synthase

OVX – ovariectomy

PEG-SOD - polyethylene glycol superoxide dismutase

ROS - reactive oxygen species

SHR – spontaneously hypertensive rats

WHI - Women's Health Initiative

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ABSTRACT

Standard hormone therapy for menopausal women [conjugated equine estrogen (CEE) 0.625 mg] has been associated with increased risk of venous thrombosis. Regimens containing lower CEE dose (0.30 mg) have been clinically used to decrease side effects of supra-physiological doses of estrogen. In this study, we determined the effects of standard (SD) and low (LD) dose of CEE on venular function in ovariectomized (OVX) spontaneously hypertensive rats (SHR). Contractions to Angiotensin-II (Ang-II 10μM) in perfused mesenteric venular bed were markedly increased in OVX (21.5±1.3 mmHg) compared to Sham (14.7±1.1 mmHg, $p<0.05$). CEE-SD did not modify Ang-II responses in OVX, while CEE-LD restored Ang-II contraction to Sham levels. eNOS inhibition by L-NAME increased Ang-II contractions in Sham and CEE-LD, and was without effect in venules of OVX and CEE-SD. OVX decreased NO generation in association with diminished eNOS phosphorylation and increased O_2^- generation in the venular wall. CEE-LD reverted the deleterious effects of OVX. Although CEE-SD augmented eNOS phosphorylation in OVX, it was unable to increase NO levels, likely due to its inability to reduce O_2^-. Distinct effects by CEE-SD and CEE-LD are in parallel with the differential modulation of Ang-II (ATR) and estrogen (ER) receptors. Compared to Sham, CEE-LD increases AT_2R while CEE-SD modifies ERβ expression in the venous bed. Interestingly, both CEE doses increased GPER in OVX. Our data suggest that estrogen dose is an important factor for venous function. While CEE-LD reverted deleterious effects of OVX, CEE-SD showed null effects despite its ability to increase eNOS activity.

INTRODUCTION

For decades, postmenopausal women have been using hormone therapy (HT) worldwide for the relief of menopausal symptoms. The beneficial effects of HT in urogenital atrophy and to prevent osteoporosis have been well documented by randomized clinical trials (Christiansen et al., 1980; Horsman et al., 1983; Lindsay et al., 1984; Lindsay, 1993; The Writing Group for the et al., 1995). In Western countries, oral formulations of conjugated equine estrogen (CEE) represent the most commonly prescribed HT (Steinkellner et al., 2012). However, serious concerns have been raised over the effects of CEE on the cardiovascular health of menopausal women following the release of data from the Women's Health Initiative (WHI) randomized trial (Rossouw et al., 2002) and Million Women Study (Beral and Collaborators, 2003). Results from these studies prompted many women to discontinue HT with CEE in the United States, and to practically abandon its use in Europe, and to seek safer alternatives for treatment of menopausal symptoms (Files et al., 2011; Steinkellner et al., 2012).

CEE is comprised of more than 10 different forms of estrogens derived from the urine of pregnant mares. The main components are estrone and equilin, with smaller amounts of 17α-estradiol, equilenin, 17α-dihydroequilin, 17β-dihydroequilin, 17α-dihydroequilenin, and 17β-dihydroequilenin, and only a minimal level of 17β-estradiol (see Table 1). Besides, CEE mixture contains not only estrogens, but also traces of progestins, androgens and substances of unknown activity/modes of action (Barton et al., 2007; Bhavnani et al., 2008). As such, CEE mixture is not considered bioidentical to naturally occurring estrogens in women (i.e., 17β-estardiol, estrone and estriol) and therefore may not provide comparable effects. In fact, studies have shown that CEE effects are markedly lower than 17β-estradiol depending on cell type or tissue studied (Bhavnani et al., 2008; Novensa et al., 2011).

Besides the type of estrogens, other concerns have been raised as probable causes of cardiovascular risk by CEE, including its association with

medroxyprogesterone, the administration route, duration of treatment, and the administration of supra-physiological doses of estrogen. With time, dosage of HT regimens in postmenopausal women has declined progressively and in the past 10 years, the use of lower dose of HT has grown in popularity. At present, the regimens containing 0.30 mg of CEE are considered as low-dose HT (Grodstein et al., 2000; Peeyananjarassri and Baber, 2005). Although the effects of lower doses of HT have been questioned (Gambacciani et al., 2008), clinical studies have shown comparable beneficial effects as the standard-dose of HT with less side-effects for postmenopausal women (Peeyananjarassri and Baber, 2005). These studies showed the efficacy of low-dose HT to prevent osteoporosis (Gambacciani et al., 2001; Lindsay et al., 2002; Gambacciani et al., 2003b) and relief of other symptoms associated with menopause (Gambacciani et al., 2003a). However, there is still little and controversial information on the cardiovascular risk/benefits of low-dose HT (Hale and Shufelt, 2015), and there is even less data on the effects of low-dose HT in venules.

Studies using animal models have demonstrated that treatment with oral CEE improves arterial function (Ceravolo et al., 2013), and may be, therefore, beneficial to the cardiovascular system. However, transdermal estrogen (Speroff, 2010) and oral CEE (Peeyananjarassri and Baber, 2005) have also been associated with increased risk of venous thrombosis (Hu and Grodstein, 2002; Blondon et al., 2014; Smith et al., 2014), suggesting that CEE may display opposing effects in arteries and veins.

The link between estrogen use and venous thromboembolism was identified more than 20 years ago, and there has been extensive literature that describes this risk. It has been demonstrated that the risk of venous thromboembolism increases as estrogen dose increases. The most prescribed oral dose of CEE (0.625 mg) leads to a supra-physiological plasma levels of estrogen in both postmenopausal women (Sarrel et al., 1998) and animal models (Costa et al., 2015), which may increase estrogen-associated side effects and contribute to the pro-thrombotic effects associated with HT.

For that reason, there is an increasing interest in applying lower-doses of HT in postmenopausal women (Gambacciani et al., 2003a; Peeyananjarassri and Baber, 2005; Zang et al., 2010).

There is an increased number of *in vitro* evidence that Ang-II plays a key role in the formation of thrombosis (González Ordóñez et al., 2000; Mogielnicki et al., 2005). Although Ang-II has no direct effect on platelet aggregation, it significantly potentiates platelet-endothelial cell adhesion (Senchenkova et al., 2014), therefore, Ang-II-induced changes in venular responses may be an indicative of higher risk of vein thrombosis. In this regard, we sought to determine the effects of estrogen withdrawal (ovariectomy) and different doses of CEE regimen on mesenteric venular responses to Ang-II and the intrinsic mechanisms involved. We used ovariectomized hypertensive female rats (SHR) as our established experimental model of surgical menopause resembling postmenopausal hypertension in women.

METHODS

Animals

We used ovariectomized (OVX) hypertensive female rats (SHR) as an experimental comorbidity model of post menopause and hypertension in women (Dantas et al., 1999; Fortepiani et al., 2003), considering that estrogen deprivation by ovariectomy and post-menopause induces endothelial dysfunction (Pinto et al., 1997; Virdis et al., 2000) and increases blood pressure when compared to healthy and younger pre-menopausal women (Barton and Meyer, 2009). Twelve-week-old female spontaneously hypertensive rats (SHR) were obtained from breeding stock of the Institute of Biomedical Sciences of University of São Paulo (ICB-USP). The study was approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences, University of São Paulo (Protocol number 145, page 95, book 2, December 06, 2010). Rats were ovariectomized (OVX) as previously described (Dantas et al., 1999). Thirty days after ovariectomy, a group of OVX was treated by gavage for 15 days with either a standard dose (SD) of CEE (Premarin®, Pfizer; 9.6µg/Kg/day, CEE-SD) or a low dose (LD) of CEE (Premarin®, Pfizer; 0.96µg/Kg/day, CEE-LD). To extrapolate standard and low dose of CEE from women to rats, we considered BMI of postmenopausal women described in the WHI (approximately 65Kg) and in accordance with the standard dose of Premarin® (0.625mg/day) used to treat postmenopausal women (Rossouw et al., 2002). Age-matched female SHR in physiological estrous was used as control (Sham). Physiological estrus was determined by microscopic evaluation of vaginal smears, as described (Cora et al., 2015).

Tissue preparation for Immunofluorescence and measurements of reactive oxygen species (ROS) and NO production

Segments of second and third-order branches of the venular mesenteric bed were dissected free of fat, connective tissues and mounted in OCT® freezing medium. Cross sections (10μm) were obtained using a cryostat (Leica, Germany).

Blood Pressure Measurement

Arterial blood pressure was determined in conscious rats by indirect tail-cuff plethysmography (pneumatic transducer, Power Lab 4/S, AD Instruments) as previously described (Ceravolo et al., 2007). Systolic blood pressure was defined as the moment a definitive pulse could be detected. Results were calculated as an average of three consecutives measurements and expressed as millimeters of mercury (mmHg).

Effectiveness of Ovariectomy and Estrogen Therapy

At the day of sacrifice, rats were anesthetized with sodium thiopental (50mg/kg, intraperitoneal), a laparotomy was performed, the uterus removed and dried for 24 hours in incubator at 37°C (dry weight). The results were expressed as milligrams of tissue and normalized by tibia length (mg/cm). Peripheral blood was collected from abdominal aorta in a glass tube and centrifuged at 3000g, 15°C for 15 minutes to obtain the serum. Estrogen concentration was determined using the radioimmunoassay (Siemens Coat-A-Count® Estradiol, Siemens Healthcare Diagnostics, USA) according to manufacturer protocol.

Mesenteric Venular Bed Perfusion

Isolated perfused mesenteric venular bed preparations were performed as previously described (Warner, 1990; Loiola et al., 2011). Rats were anesthetized with sodium thiopental (50mg/kg, intraperitoneal), the mesenteric bed was exposed, a cannula was inserted retrogradely into portal vein and the vascular mesenteric bed was dissected out. The mesenteric venular bed was perfused using a peristaltic pump

(Miniplus 3, Gilson, France) with Krebs-Henseleit solution at 37 °C in the presence of 95% O_2 and 5% CO_2 for 40 minutes (the first 20 minutes at a constant rate of 2mL/minute and the remaining time at 4mL/minute).

After the equilibration period, 10μM phenylephrine was perfused for 10 minutes (to avoid desensitization of Angiotensin II receptor type 1 - AT_1R) followed by contractile responses to Angiotensin II (Ang-II) at 10nM-10μM (bolus injection) were performed. Because significant effects by Ang-II were seen only at 10μM, only results at this concentration were considered in this study. Ang-II responses were obtained in the absence or presence of L-NAME (NG-nitro-L-arginine-methyl ester; 100μM), a nitric oxide synthase (NOS) inhibitor. Effects of NOS inhibition were calculated as delta (Δ) of changes between basal (untreated) and stimulated (L-NAME) perfusion pressure (mmHg).

Nitric Oxide Production

Basal NO production was measured using 4.5-diaminofluorescein diacetate (DAF-2), a fluorescent marker sensitive to NO. Sections (10μm) were placed on glass slides and incubated at 37°C for 30 minutes with DAF-2 (12.5μM) in phosphate buffer (0.01M, pH 7.4) containing $CaCl_2$ (0.45mM). Digital images were captured in a microscope (Nikon E 1000, Japan) equipped with epifluorescence. Mean optical density of fluorescence was analyzed using the Image J program (NIH, USA) in three different locations of each image.

Reactive Oxygen Species (ROS) Production

ROS production was measured by dihydroethidium (DHE) fluorescence. The 10μm sections were placed on glass slides and incubated for 30 minutes at 37ºC in a light-protected and humidified chamber with DHE solution (5μM). Digital images were captured in a microscope (Nikon E 1000, Japan) equipped with epifluorescence. Mean

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optical density of fluorescence was analyzed by a computer system (KS-300 Software, Zeiss) in three different locations of each image and normalized by the area. Specific signal for O_2^- was determined by treatment with permeable superoxide dismutase (PEG-SOD, 150U/ml - 30 minutes). The mechanisms influencing ROS production in the venular wall were determined by pre-treatment (30 minutes) with an Ang-II type 1 receptor (AT_1R) blocker (losartan, 1µM), a NADPH-oxidase inhibitor (Apocynin, 10 µM), and a NOS inhibitor (L-NAME, 10µM).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA (tRNA) was isolated from mesenteric venular bed using TRIzol® Reagent (Thermo Fisher, USA) per the manufacturer's protocol. RNA quantification was done by spectrophotometry (260nm) in Nanodrop (Thermo Fischer, USA). Transcriptase reverse (RT) reaction was performed in a final volume of 20µL using 2µg tRNA mixed with 0.5µg of oligo dT primer, 0.5nM of dNTP, 1x MMLV buffer, 10mM of DTT, 40U of RNAse inhibitor and 200U of MMLV enzyme. Samples were heated at 42°C for 50 minutes, 70°C for 15 minutes and kept on ice. Real-time polymerase chain reaction (PCR) was performed in a final volume of 12.5µL containing 0.5uM of each oligonucleotide **(Table 2)**, and fifty percent of total volume of GoTaq® qPCR Master Mix (Promega). Real-time PCR reactions were performed using Corbett Research system (Corbett Life Sciences, Sydney, Australia). The conditions for PCR were as follows: 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Expression data were calculated from cycle threshold (Ct) value using ΔΔCt method for quantification (Pfaffl, 2001). The β-actin mRNA was used for normalization and results were analyzed by fold increases relative to the values obtained in Sham group.

Immunofluorescence

Second-order branches of the superior mesenteric vein were fixed with 4% phosphate buffered paraformaldehyde (pH = 7.4) for 1 h and washed in three changes of phosphate buffered solution (PBS; pH = 7.4).) Frozen sections (20 μm) were incubated with a rabbit primary polyclonal antibody against ER-α, ER-β and GPER (1:50; Santa Cruz Biotechnology Inc., Dallas, TX, USA). After washing, rings were incubated with the secondary donkey anti-rabbit IgG antibody conjugated to CyTM3 (1:200; Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). Sections were processed for immunofluorescence staining essentially as previously described (Jiménez-Altayó et al., 2009). Immunofluorescent signals were viewed using an inverted Zeiss LSM 780-NLO (São Paulo, Brazil) confocal laser scanning microscope. Quantitative analysis of fluorescence was performed with ImageJ (NIH Image). The average intensity was measured in at least two rings of each animal, and the results were expressed as arbitrary units.

Western Blotting

Frozen mesenteric venules were homogenized and protein extract obtained with lysis buffer containing 1% of Triton-X, 100mM Tris pH 7.4, 100mM sodium pyrophosphate, 100mM sodium fluoride, 10mM EDTA, 10mM sodium orthovanadate, 2mM PMSF and 0.01mg/mL of protease inhibitor. Samples were centrifuged at 10.000g for 30 minutes at 4°C and protein content quantified by BCA protein assay (23227, Pierce Protein Research), according to manufacturer's protocol. Equal amount (70μg) of total protein was separated by electrophoresis on a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with blocking solution (5% bovine serum albumin in TTBS-Tris buffer pH 7.4 containing 10mM Tris-HCl, 100mM NaCl and 0.1% Tween 20), for 2 hours at room temperature. Membranes were incubated with specific antibodies, overnight at 4°C. Primary antibodies: mouse monoclonal anti-eNOS (1:1000, 610297, BD Biosciences) and rabbit polyclonal anti-

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phospho-eNOS$^{Ser-1177}$ (1:1000, 95715, Cell Signaling). Membranes were incubated with secondary antibodies, goat-anti-Mouse (1:10000, 115-035-166, Jackson ImmunoResearch) and goat-anti-Rabbit (1:1500, 111-035-144, Jackson ImmunoResearch) for 2 hours at room temperature. Membranes were revealed with chemiluminescence (Signal West Pico Chemiluminescent, Thermo Scientific), visualized by autoradiography film, quantified by densitometry using ImageJ program (Wayne Rasband, National Institute of Health). All membranes were reblotted using a monoclonal antibody anti αActin (1:2000, Sigma-Aldrich) as a loading control. Data were normalized to corresponding values of αActin densitometry. Phosphorylation of eNOS at Ser-1177 was expressed as the ratio of p-eNOS$^{Ser-1177}$ and total eNOS densitometry.

Statistical analysis

Data are expressed as mean ± SEM and *n* represents the number of animals used in each experiment. Differences between the groups were analyzed by one-way ANOVA with Tukey-Kramer post hoc test. Values of $p<0.05$ were considered statistically significant.

RESULTS

Systolic blood pressure was not modified by either ovariectomy or CEE treatments **(Table 3)**. Uterine weight and serum levels of estrogen were reduced in OVX rats when compared with Sham. Both CEE-SD and CEE-LD treatments restored the uterine weight to similar levels found in Sham. CEE-LD treatment increased estrogen to levels comparable with Sham, groups, while the CEE-SD induced a 3.5-fold increase in serum estrogen levels **(Table 3)**.

In venular bed, 10μM of Ang-II induced contractions in all groups **(Figure 1A)**. Contractile response to 10μM Ang-II was higher in OVX when compared to Sham. CEE-

SD treatment did not modify Ang-II contraction when compared to OVX. In contrast, the response induced by 10μM of Ang-II in CEE-LD was reduced when compared to OVX, and similar to the response observed in Sham **(Figure 1B).** In the presence of L-NAME, Ang-II contractions were markedly increased only in Sham and CEE-LD groups **(Figure 1C)**. L-NAME did not modify Ang-II responses in OVX and CEE-SD, suggesting an attenuation of NO release by Ang-II when estrogen is absent (OVX) or at supra-physiological concentrations (CEE-SD). The increase in Ang-II responses in OVX was paralleled by a 2-fold increase in the ratio of type 1 Ang-II receptor (AT_1R) / type 2 Ang-II receptor (AT_2R) **(Figure 2C)**, because of up-regulation of AT_1R **(Figure 2A)** and unchanged AT_2R expression **(Figure 2B)**. Treatment with CEE-SD restored the ratio of AT_1R/AT_2R to levels comparable to Sham, by decreasing AT_1R expression. On the other hand, LD of CEE treatment led a 2-fold decrease AT_1R/AT_2R in OVX by restoring AT_1R and inducing a two-fold increase of AT_2R.

To determine the intrinsic mechanisms that contribute to the altered Ang-II responses observed, we first determined NO production in venular sections by DAF-2 fluorescence. Our results reveal a diminished basal NO production in mesenteric venues by OVX, when compared to Sham **(Figure 3A and 3B)**. Treatment of OVX with CEE-SD did not modify venous production of NO when compared to OVX, however CEE-LD treatment increased this parameter to the values observed in Sham **(Figure 3A and 3B)**. We next sought to determine whether a difference in the modulation of eNOS expression/activity could account for the disparity in NO production induced by SD and LD of CEE. We found that although the levels of eNOS protein and gene expression were similar in venues of all groups **(Figure 3C)**, the degree of eNOS phosphorylation at Serine-1177 was markedly reduced by OVX. Both CEE (SD and LD) equally increased eNOS phosphorylation in OVX females to the levels observed in Sham **(Figure 3C)**. Therefore, the inability of CEE-SD treatment to increase NO levels in venues of OVX

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SHR may not be dependent on changes of eNOS expression/activity, but rather a consequence of increased NO degradation by O_2^-.

In this regard, we next determined the effects of SD and LD of CEE on venular ROS generation. Measurements of ROS generation by DHE fluorescence showed an increase of oxidative stress in mesenteric venues by OVX when compared to Sham **(Figure 4A and 4B)**. Treatment with CEE-LD decreased DHE fluorescence in OVX to a similar degree observed in Sham. In contrast, CEE-SD did not modify DHE fluorescence in OVX **(Figure 4A and 4B)**, suggesting dual dose-dependent effect of estrogen on ROS generation. Incubation of venular sections with polyethylene glycol SOD (PEG-SOD) markedly decreased DHE signal in OVX and CEE-SD, revealing higher O_2^- levels in these groups. In OVX group, DHE fluorescence was diminished by apocynin or losartan treatment, an indicative of the contribution of NADPH oxidase and AT_1R in O_2^- generation, respectively **(Figure 4D)**. Interestingly in CEE-SD, ROS generation was also inhibited by L-NAME **(Figure 4E),** suggesting O_2^- release by NOS when estrogen is at supra-physiological levels. In Sham **(Figure 4C)** and CEE-LD **(Figure 4F)** all treatments were without effect on DHE signal.

We next explored a potential role of estrogen treatments on ERs expression that could correlate with the altered responses on NO/O_2^- production On **Figure 5** we show the results of immunofluorescence analysis that determined the expression of estrogen receptors ERα, ERβ and GPER along the venular wall of female SHR. In these studies, we observed that estrogen withdrawal by OVX was associated to an increased expression of ERβ and decreased expression of GPER. CEE-LD restored ERβ and GPER expression in OVX to levels observed in Sham. Although CEE-SD restored GPER, it further increased ERβ in venues of OVX SHR. No changes in ERα expression were observed.

DISCUSSION

This is the first study describing the effects of standard and low dose estrogen treatment in venular function. The rational of our study was based on evidence that standard dose of CEE has been associated with increased risk of venous thrombosis in menopausal women. Although regimens using lower doses of estrogen have been recommended to improve risk/benefits of hormone therapy in the post-menopause (Santen, 2015), the dose-effect relationship in venous function is largely unknown. In this study, CEE-SD treatment in OVX led to supra-physiological estradiol concentrations, while CEE-LD treatment resulted in similar serum estradiol concentrations than Sham. The different levels of circulating estrogens were associated with opposing effects of CEE-SD and CEE-LD on venular responses to Ang-II, and were in correlation with differential regulation of NO/O_2^- systems.

In the present study, we found that ovariectomy markedly increased venular contractions to Ang-II, an effect that was improved by treatment with low doses of CEE, but was not modified when CEE was administrated at standard doses. Inhibition of NOS inhibition by L-NAME led to an increase in Ang-II-induced contraction in venular bed from Sham (~32% increase in the maximal contraction) and CEE-LD (~68%). On the other hand, L-NAME induced no changes in the contractile responses to Ang-II when estrogen levels were low (i.e. OVX) or at supra-physiological levels (i.e. CEE-SD). These data clearly establish a difference in the contribution of NO to attenuate Ang-II contractile responses in the venular bed that prevails when estrogen is at physiological concentration.

Two major cell surface G-protein-coupled receptor subtypes trigger vascular actions of Ang-II. Most of the detrimental effects of Ang-II are mediated by stimulation of Ang-II type 1 receptor (AT_1R), while actions by Ang-II type 2 receptor (AT_2R) are known to compensate vascular effects by AT_1R (Touyz and Schiffrin, 2000). We have previously demonstrated that estrogen modulates Ang-II receptors subtype in arterial bed of female

SHR (Silva-Antonialli et al., 2004). Interestingly, we observed differences in the regulation of these receptors depending on the type of estrogen used and the serum estradiol concentration reached by each therapeutic regimen. When OVX SHR were treated with a regimen that lead to a more physiological concentration of estradiol (i.e. subcutaneous pellet of 50µg 17β-estradiol), we observed a lower ratio of AT_1R/AT_2R in aortas and mesenteric arteries, than those arteries from untreated OVX SHR (Silva-Antonialli et al., 2004). In opposition, when OVX SHR were treated with standard doses of CEE, reaching supra-physiological levels of estradiol in the serum, we saw no differences in arterial expression of both AT_1R and AT_2R (Costa et al., 2015). In the present study, the greater venous constriction by Ang-II in OVX was paralleled by an increase in AT_1R expression. Treatment with low dose of CEE diminished AT_1R expression and concomitantly increased AT_2R in venues of OVX rats, an effect that was associated with a marked decrease in Ang-II contractions. Nevertheless, even though treatment with standard doses of CEE had decreased AT_1R expression in the mesenteric venular bed of OVX, we did not see any change in Ang-II-induced vasoconstriction, suggesting that treatment with CEE-SD modulates intrinsic molecular mechanisms that contribute to maintain Ang-II hypercontractility.

The favorable effects of estrogen on vascular function have been largely associated with regulation of NO production by mechanisms that involve genomic up-regulation of endothelial isoform of NOS (eNOS) expression (Goetz et al., 1994; Weiner et al., 1994). Nevertheless, most of the benefits of estrogen therapy on eNOS were described in the arterial bed (Chambliss and Shaul, 2002), while little is known on how estrogen therapies affect venular NO generation. In OVX-SHR, we found an important disparity in the modulation of venular NO bioavailability by standard and low dose of CEE. We observed that NO production and eNOS phosphorylation was decrease in OVX, and that low dose of CEE restored this parameter. However, standard dose of CEE did not modify NO bioavailability when compared to OVX, despite its ability to promote

eNOS phosphorylation. We, therefore, we hypothesized that CEE-SD is ineffective to modify NO availability in OVX, not because it lacks the ability to modulate NO release, but above all because it increases NO degradation after synthesis.

It is well established that the biological activity of NO is modified by ROS, such as O_2^-. Increased O_2^- concentration in the vasculature results in rapid scavenging of NO and decrease of its bioavailability (Touyz, 2003). In previous studies, we have shown that CEE-SD treatment reduces ROS production in the arterial bed of OVX SHR by down-regulating pro-oxidant and increasing antioxidant enzyme expression, which improves endothelial function (Ceravolo et al., 2013; Costa et al., 2015). Contrary to that, CEE-SD does not decrease O_2^- generation in the venular bed of the same menopause model, revealing a differential regulation of NO/O_2^- systems by estrogens in arteries and veins.

It has been shown that Ang-II increases O_2^- production in different arterial beds via AT_1R-dependent and NADPH-oxidase-mediated signaling pathways (Griendling et al., 1994; Schramm et al., 2012). Angiotensin-II regulates the expression and activity subunit of NADPH (Nox) via AT_1R *in vivo.* Infusion of Ang-II induced a significant increase in blood pressure, accompanied by augmented expression of Nox1 mRNA and O_2^- production in aortas of Nox1 wild-type mice, whereas the elevation in blood pressure and production of O_2^- were significantly blunted in Nox1 knockout (Matsuno et al., 2005). In agreement with our previous studies in the mesenteric arterial bed (Dantas et al., 2002), our present data show that O_2^- overproduction in mesenteric venules of OVX SHR is lessened by the blockage of AT_1R with losartan and by inhibition of NADPH-oxidase activity with apocynin. However, contrary to what was seen in the arterial bed (Ceravolo et al., 2013), treatment with CEE-SD did not decrease AT_1R and NADPH-derived O_2^- production in venules, and moreover, facilitates O_2^- generation via eNOS, as evidenced by decrease DHE fluorescence after L-NAME inhibition. Under normal conditions, the primary product of eNOS activation is NO, which produces beneficial effects on vascular

biology. There is evidence, however, that in certain pathophysiological conditions when there is a depletion of important molecules essential for NO production (e.g., tetrahydrobiopterin, l-arginine) eNOS becomes increasingly "uncoupled" from NO production and produces O_2^- (White et al., 2010). Although studies suggest that conditions, such as aging and cardiovascular risk factors, can modify the effects of estrogen on NO production due to disarrangement of eNOS (White et al., 2010; Murphy, 2011), there is no data describing the direct effect of estrogens regimens and doses on eNOS uncoupling. Unfortunately, in our studies we had few limitations (amount of tissue, assay sensitivity) that did not allow us to better characterize the role of estrogens on eNOS uncoupling in veins. Nevertheless, our results on L-NAME inhibition of O_2^- production bring new insights on the potential detrimental mechanisms of supra-physiological doses of CEE.

Vascular estrogen actions are mediated by two members of the nuclear receptor superfamily (ERα and ERβ) and by a G protein-coupled membrane-bound receptor (GPER). ERα has been described to be the key player on cardiovascular protection by estrogen, although insufficient and controversial information is available on the contribution of ERβ and GPER (Meyer and Barton, 2009). ERα is known to up-regulate eNOS expression (Sumi and Ignarro, 2003) and activates the enzyme via Ser-1177 phosphorylation (Tarhouni et al., 2013). However, recent evidence has recognized GPER activation results in the rapid mobilization of intracellular calcium and activation of eNOS, via Akt-mediated signaling pathway (Meyer et al., 2011). In our studies, we saw no changes in ERα expression in veins of OVX or CEE-treated rats. Instead, we found a major regulation of GPER expression by OVX and estrogen treatments. Our study is the first to describe that GPER expression in veins is decreases by estrogen withdrawn, and that chronic treatment with CEE restores GPER expression. Interestingly, changes of eNOS phosphorylation by OVX and CEE treatments were paralleled with variations in GPER expression in the venular wall.

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Although this parallelism with GPER is a plausible explanation for the effects of CEE on eNOS phosphorylation, it does not correlate with the differences in NO bioavailability and O_2^- production observed after treatment with low and standard doses of CEE. Interestingly, previous studies have shown that GPER can be associated with cardiovascular damage and increased O_2^- production in aged females (Meyer et al., 2016). Moreover, in primary vascular smooth muscle cells from GPER knockout mice, the Ang-II–stimulating effect on $\cdot O2-$ generation was completely absent (Meyer et al., 2016). In contrast, we found a marked difference in ERβ expression in the venular wall could better correlate with O_2^- production. OVX induces an increase of ERβ expression that was corrected by CEE-LD, but not CEE-SD. In fact, the degree of ERβ expression after CEE-SD treatment was considerably higher than the levels observed in OVX. The mechanisms to explain how the imbalance of ER ratio can modify estrogen effects are largely unknown, but recent studies have interestingly revealed that ERβ exhibits an inhibitory action on ERα-dependent gene expression and may oppose the actions of ERα (Matthews and Gustafsson, 2003; Bhavnani et al., 2008)

Taken together our data have established that CEE may display opposite effects in the venular function when administrated at standard (SD) or lower (LD) dose. While treatment of OVX SHR with CEE-LD has a beneficial venular effect by increasing NO bioavailability and improving vascular responses to Ang-II, CEE-SD does not modify the detriment induced by OVX. Although CEE-SD is as effective to activate eNOS phosphorylation as CEE-LD, this treatment does not improve NO bioavailability, because it maintains higher O_2^- production. Increased risk of vein thrombosis is the major vascular complication during HT and has been largely associated with increased risk of cardiovascular disease in postmenopausal women. These results improve our knowledge into the field of cardiovascular modulation by estrogen, and establish that dose regimen significantly affects the direct estrogen-mediated effects in vascular

function, which may influence the risk/benefits of HT on cardiovascular events and other outcomes through multiple pathways.

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Authorship Contributions

1. Participated in research design: Araujo, Akamine, Vila, Dantas, Ceravolo and Carvalho.
2. Conducted experiments: Araujo, Costa, Echem, Oliveira, Santos-Eichler, Colli, Jimenéz-Altayó.
3. Contributed new reagents or analytic tools: Araujo, Carvalho, Ceravolo, Dantas.
4. *Performed data analysis*: Araujo, Costa, Echem, Oliveira, Santos-Eicheler, Jiménez-Altayó, Vila, Akamine, Dantas, Ceravolo and Carvalho.
5. Wrote or contributed to the writing of the manuscript: Araujo, Costa, Echem, Oliveira, Santos-Eichler, Colli, Jiménez-Altayó, Vila, Akamine, Dantas, Ceravolo and Carvalho.

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Footnotes

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MHCC, GSC, APD contribute equally to this work.

Legends for Figures

Figure 1. Angiotensin II (Ang-II)-induced contractions. Shown are representative image of contraction by 10µM of Ang-II in mesenteric venular bed **(A)** and mean ± SEM of maximal response obtained in venular bed of female SHR **(B)**. The role of NO on Ang-II-induced contraction was assessed with L-NAME (100 µM) **(C)**. Contractile responses were obtained in mesenteric venular bed from sham-operated (Sham), ovariectomized (OVX), and OVX rats treated with conjugated equine estrogens (CEE) at standard (CEE-SD) and low (CEE-LD) dosages. Each point represents mean ± SEM from 6-8 independent experiment. * $p < 0.05$, ***$P< 0.001$ by ANOVA with Tukey-Kramer post hoc test.

Figure 2. Role of ovariectomy and CEE treatments on Angiotensin II (Ang-II) receptor expression. Bar graphs show the results of comparative analysis of mRNA levels of Ang-II receptor type 1 (AT_1R) **(A)** and Ang-II receptor type 2 (AT_2R) **(B)** in mesenteric venular bed from sham-operated (Sham), ovariectomized (OVX), and OVX rats treated with conjugated equine estrogens (CEE) at standard (CEE-SD) and low (CEE-LD) dosages. mRNA levels are expressed as $2^{-\Delta\Delta Ct}$ using β-actin mRNA as internal control and Sham as reference group. The ratios of AT_1R / AT_2R mRNA expression in each animal are expressed as Log2. Data represent the mean ± SEM derived from 6 independent experiments. *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$ by ANOVA with Tukey-Kramer post hoc test.

Figure 3. NO production and eNOS expression and phosphorylation in mesenteric venular bed. Representative images of DAF-2 fluorescence in venular sections (10µm) **(A)** and mean ± SEM of densitometric analysis of DAF-2 fluorescence (arbitrary units) obtained in venular bed of female SHR **(B)**. **(C)** shows the results of immunoblots

analyzed in mesenteric venular bed and probed with antibodies against phosphorylated eNOS at serine 1177 [(p)eNOS^{Ser1177}] or total eNOS [(t) eNOS], as indicated, and plotted as the ratio of (p)eNOS^{Ser1177} / (t) eNOS. Analysis was performed in mesenteric venular bed from sham-operated (Sham), ovariectomized (OVX), and OVX rats treated with conjugated equine estrogens (CEE) at standard (CEE-SD) and low (CEE-LD) dosages. Data represent the mean ± SEM derived from 6 independent experiments. *P< 0.05, ***P< 0.001 by ANOVA with Tukey-Kramer post hoc test.

Figure 4. Reactive oxygen species (ROS) generation. Shown are **r**epresentative fluorescent images of nuclei labeled with ethidium bromide produced by oxidation of dihydroethidium (DHE) by ROS **(A)**. Bar graphs show the results of densitometric analyses of fluorescence intensity expressed as arbitrary units of vehicle-treated venular sections **(B)** from sham-operated (Sham), ovariectomized (OVX), and OVX rats treated with conjugated equine estrogens (CEE) at standard (CEE-SD) and low (CEE-LD) dosages; as well as in venular sections of Sham **(C)**, OVX **(D)**, CEE-SD **(E)** and CEE-LD **(F)** following 30-minut treatment with permeable superoxide dismutase (PEG-SOD, 150U/ml); with NOS inhibitor (L-NAME, 100µM), NADPH-oxidase inhibitor (Apocynin, 10 mM), and AT_1R blocker (losartan, 1µM). Data represent the mean ± SEM derived from 6 independent experiments. **P< 0.01, ***P< 0.001 by ANOVA with Tukey-Kramer post hoc test.

Figure 5. Role of ovariectomy and CEE treatments on estrogen receptors expression. Representative photomicrographs of estrogen receptors subtype (ERα, ERβ and GPER) immunofluorescence (red) of confocal microscopic mesenteric venular sections of females SHR rats. Natural auto fluorescence of elastin (green) is also shown. Bar graphs show the results of densitometric analyses of fluorescence intensity (arbitrary units) venular sections from sham-operated (Sham), ovariectomized (OVX), and OVX

rats treated with conjugated equine estrogens (CEE) at standard (CEE-SD) and low (CEE-LD) dosages. Data represent the mean ± SEM derived from 6 independent experiments. *P< 0.05, ***P< 0.001 by ANOVA with Tukey-Kramer post hoc test.

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Table 1. Constituents of Conjugated Equine Estrogens (Barton et al., 2007; Bhavnani et al., 2008)

Estrogens	Estrone sulfate
	Equilinsulfate
	17α-dihydroequilinsulfate
	17α-estradiolsulfate
	17β-dihydroequilinsulfate
	17α-dihydroequileninsulfate
	17β-hydroequileninsulfate
	Equileninsulfate
	17β-estradiolsulfate
	∆8,9-dehydroestronsulfate
Progestins	5α-Pregnane-3β, 20β-diol
	5α-Pregnane-3β, 16α, 20β-triol
	5α-Preg-16-en-3β-ol-20-one
	5α-Pregnane-3β-ol-20-one
	4-pregene-20-ol-3-one-sulfate
	3β-Hydroxy-5(10), 7-estradiene 17-one-3-sulfate
Androgens	5α-Androstane-3β, 17α-diol
	5α-Androstane-3β, 16η-diol
	5α-Androstane-3β, 16α-diol
	5α-Androstane-3β-ol, 16-one
Other substances	5,7,9 (10) Estratriene-3β, 17β-diol
	17α-Dihydro-delta 8,9-dehydroestrone
	17β-Dihydro-delta 8,9-dehydroestrone
	5,7,9,(10) Estratriene-3β-ol-17-one
	2-Hydroxyestrone
	2-Methoxyestrone

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Table 2. Oligonucleotides for real-time polymerase chain reaction

Gene	Sequence (5' → 3')
AT_1R (NM_030985.4)	F: CACTTTCCTGGATGTGCTGA R: CCCAGAAAGCCGTAGAACAG
AT_2R (NM_007429.5)	F: CTGCTGGGATTGCCTTAATGAA R: AGCAGATGTTTTCTGATTCCAAAGT
eNOS (NM_021838.2)	F: TTCTGGCAAGACCGATTACACGACAT R: AAAGGCGGAGAGGACTTGTCCAAA

AT_1R: angiotensin II receptor type 1; AT_2R: angiotensin II receptor type 2; eNOS: endothelial nitric oxide synthase.

Table 3. Systolic blood pressure (SBP), uterine weight and serum estrogen levels in female SHR.

	Sham	OVX	CEE-SD	CEE-LD
SBP (mmHg)	171.3±3.6	160.6±3.3	170.3±2.3	167.5±1.8
Uterine weight (mg/cm)	2.65±0.1	0.75± 0.1*	2.32±0.1$^{\#}$	2.08±0.2$^{\#}$
Estrogen (pg/ml)	30.1±1.0	17.52±1.2*	113.1±13.3*$^{\#}$	32.2±6.9$^{\#+}$

Results are expressed as mean±SEM of 5-11 animals. Statistical significance ($p<0.05$) compared to (*) Sham, ($^{\#}$) OVX or ($^{+}$) CEE-SD

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Figure 1

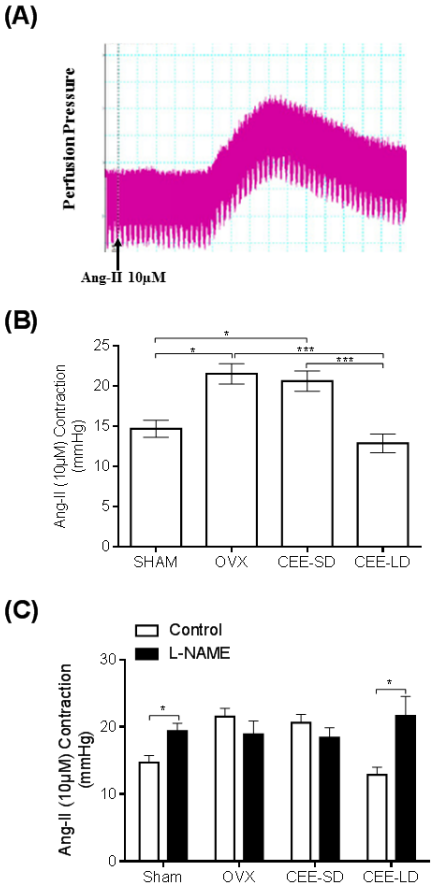

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Figure 2

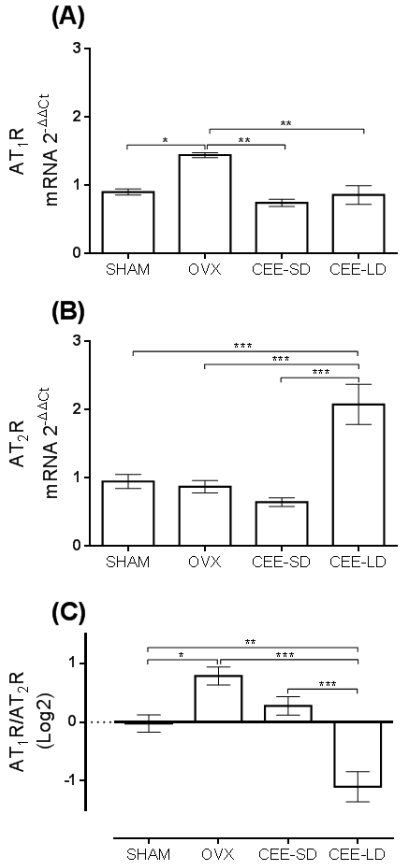

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Figure 3

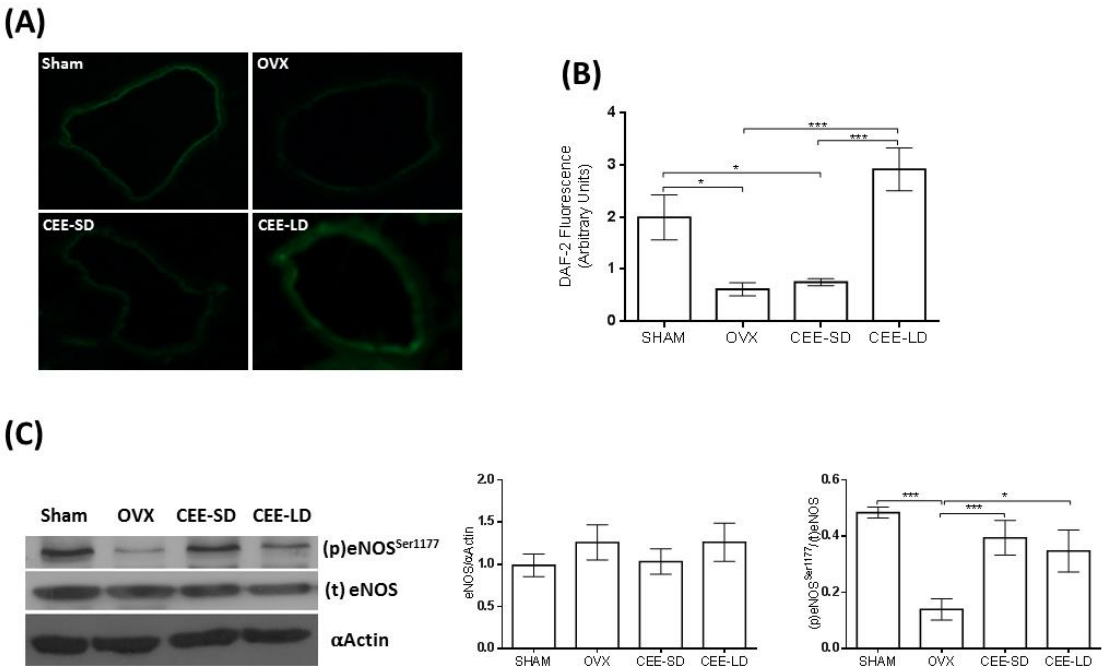

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Figure 4

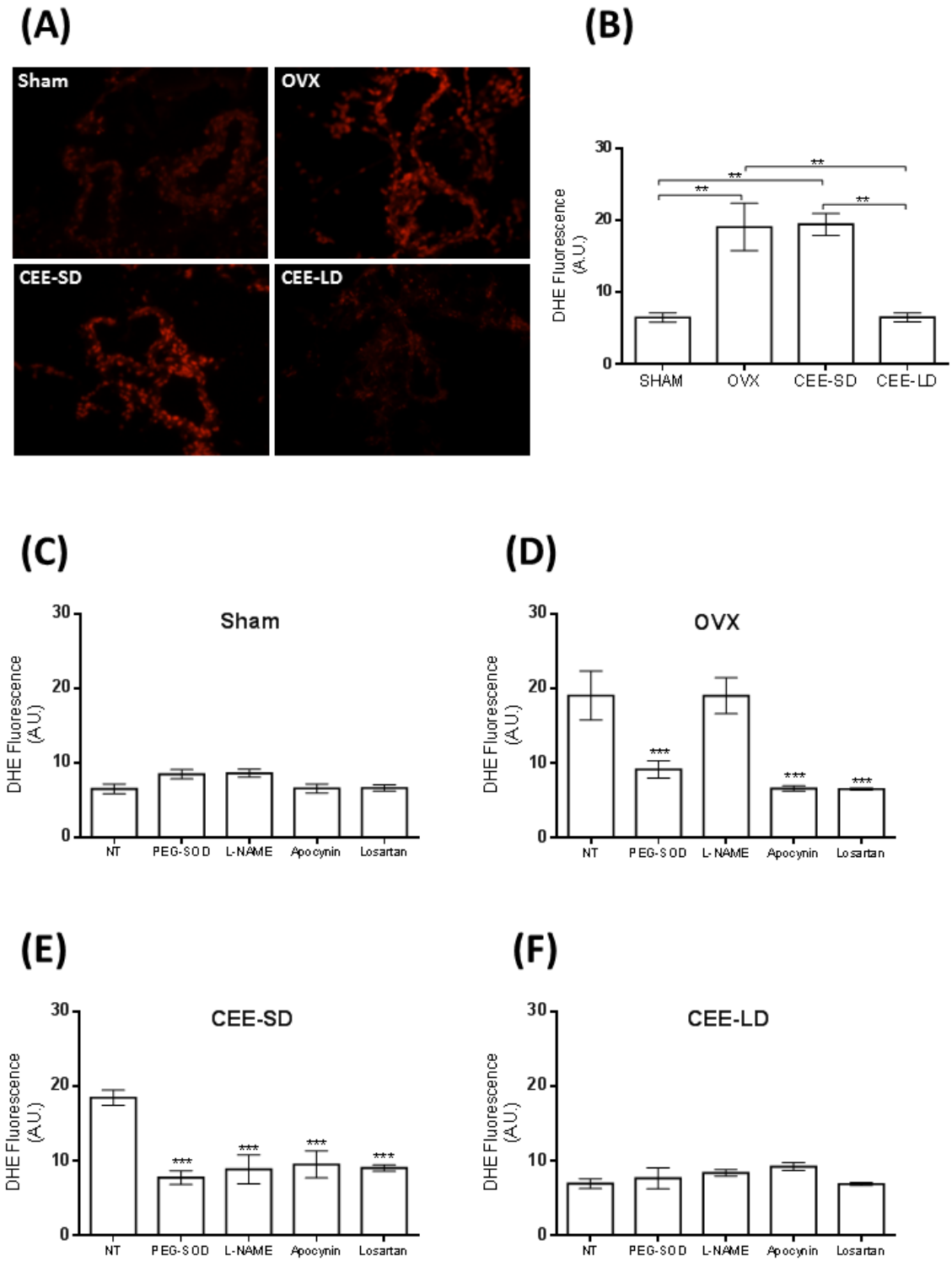

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Figure 5