

## Ovariectomy provokes inflammatory and cardiovascular effects of endotoxemia in rats: Dissimilar benefits of hormonal supplements

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### ABSTRACT

The female gender is protected against immunological complications of endotoxemia. Here we investigated whether gonadal hormone depletion by ovariectomy (OVX) uncovers inflammatory and cardiovascular effects of endotoxemia and whether these effects are reversed by hormone replacement therapies. Changes in inflammatory cytokines, blood pressure (BP), left ventricular (LV) function, and cardiac autonomic activity caused by lipopolysaccharide (LPS) in conscious female rats with different hormonal states were determined. In contrast to no effects in sham-operated females, treatment of OVX rats with LPS (i) decreased BP, (ii) increased spectral low-frequency/high-frequency ratio of HRV, denoting enhanced cardiac sympathetic dominance, (iii) attenuated reflex tachycardic responses to sodium nitroprusside, and (iv) increased systolic contractility ( $dP/dt_{max}$ ). The developed hypotension was (i) fully eliminated in estrogen ( $E_2$ )-pretreated OVX rats, (ii) partially counteracted after selective activation of estrogen receptor- $\alpha$  (PPT) or  $\beta$  (DPN). All estrogenic compounds abrogated LPS enhancement of cardiac sympathetic drive. However, PPT was more successful than  $E_2$  or DPN in compromising LPS depression in baroreflex activity and elevation in  $dP/dt_{max}$ . Molecular studies showed that PPT was most effective in attenuating the upregulated myocardial expressions of NF- $\kappa$ B and iNOS in endotoxemic OVX rats. Myocardial expression of the defensive HSP70 was comparably increased by all estrogenic products. Except for improved cardiac spectral activity, none of these functional or molecular entities was affected by medroxyprogesterone acetate (MPA). Overall, our data suggest diverse therapeutic advantages for gonadal hormones in the worsened endotoxemic complications in rats with surgical menopause, with probably more favorable role for ER $\alpha$  agonism within this context.

### 1. Introduction

Clinical studies demonstrate that the immune and inflammatory reactions induced by endotoxemia are sexually dimorphic, with females exhibiting better clinical outcomes than age-matched males (Klein and Flanagan, 2016; Santos-Marcos et al., 2018). Experimental studies also support the notion the animal sex is an important determinant of the production of pro- and antiinflammatory cytokines in response to endotoxemia (Chen et al., 2014; Nasir et al., 2015; Kuo, 2016; El-Lakany et al., 2018). Male mice challenged with LPS consistently exhibit higher plasma levels of IL-6 and TNF- $\alpha$  than their female counterparts (Kuo, 2016). Losonczy et al. (2000) reported that LPS lowers BP in anesthetized male, but not female, rats. Moreover, echocardiographic studies revealed more serious signs of cardiac dysfunction, e.g. reduced ejection fraction and fractional shortening, in male compared with female

mice (Chen et al., 2014).

Gonadal hormones play fundamental roles in the diminished immune and inflammatory responses to endotoxemia in females.  $E_2$  acts via genomic and non-genomic mechanisms to inhibit NF- $\kappa$ B cascade (Ghisletti et al., 2005; Murphy et al., 2010) and reduce synthesis of inflammatory mediators during the endotoxemic insult (Lewis et al., 2008; Saia et al., 2015). Both ER $\alpha$  and ER $\beta$  have been implicated in the antiinflammatory action of  $E_2$  in endotoxemia (Ghisletti et al., 2005; Xing et al., 2007). Clinically, higher blood levels of TNF- $\alpha$  are observed in endotoxemic postmenopausal females compared with premenopausal ones (Moxley et al., 2004). Ovariectomy augments lung damage and inflammation in endotoxemic mice and this effect is suppressed after  $E_2$  repletion (Speyer et al., 2005). The hypotensive response to endotoxemia is also potentiated in OVX rats and is reversed by raloxifene, a selective estrogen receptor modulator (Shen et al., 2017).

**Abbreviations:** OVX, ovariectomy; BP, blood pressure; LV, left ventricle; LPS, lipopolysaccharide; HRV, heart rate variability; Estrogen,  $E_2$ ; inducible nitric oxide synthase, iNOS; NF- $\kappa$ B, nuclear factor kappa B; MPA, medroxyprogesterone acetate; PE, phenylephrine; SNP, sodium nitroprusside

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Alternatively, little or no information is available regarding the interaction of progesterone replacement therapy with end organ damage induced by endotoxemia (Zöllner et al., 2020).

The current investigation employs integrative and molecular studies to address three main questions regarding the effects of gonadal hormone depletion and repletion on endotoxic inflammation and associated cardiovascular manifestations of hypotension, left ventricular dysfunction, and cardiac autonomic and baroreflex impairment. First, what is the effect of surgical menopause induced by bilateral OVX on inflammatory and cardiovascular effects of endotoxemia? Second, does estrogen or progesterone supplementation blunt endotoxic responses revealed by OVX? Third, do these effects involve distinct contributions of estrogen receptors of the  $\alpha$  and  $\beta$  types? Experiments were undertaken in conscious OVX or sham-operated rats pre-instrumented with indwelling femoral catheters. The current data identified potential molecular therapeutics targets for alleviating the deleterious inflammatory and cardiovascular consequences of endotoxemia in female subjects.

## 2. Materials and methods

### 2.1. Animals

Adult female Wistar rats (180–220 g; animal facility at the Faculty of Pharmacy, Alexandria University, Alexandria, Egypt) were used. All experiments were approved by Institutional Animal Care and Use Committee of Alexandria University, Egypt (IACUC project # 28/2014) and according to guidelines of the American Veterinary Medical Association. As indicated below, surgery was performed under ketamine/xylazine anesthesia and all efforts were made to minimize the animal suffering.

### 2.2. Ovariectomy

The procedure described in our previous studies was followed (El-Mas and Abdel-Rahman, 2001; Fouda et al., 2018) and performed 2 weeks before cardiovascular measurements. Briefly, rats were anesthetized with a mixture of i.p. ketamine (70 mg/kg) and xylazine (7 mg/kg) and two 1-cm incisions were made above the ovaries in the back skin and the underlying dorsolateral muscles. On each side, periovarian fat was pulled, the distal uterine horn was ligated, and the ovary was cut. In sham surgery, the ovaries were exposed without isolation.

### 2.3. Intravascular cannulation

Twelve days after OVX or sham operation (i.e. 2 days before cardiovascular measurements), rats were anesthetized with i.p. ketamine (70 mg/kg) and xylazine (7 mg/kg) and femoral artery and veins were cannulated for the measurement of blood pressure and i.v. drug administration, respectively, as described in our previous studies (El-Mas and Abdel-Rahman, 1993; El-Mas and Abdel-Rahman, 1997; El-Mas et al., 2011).

### 2.4. Frequency domain analysis of heart rate variability

Power spectral analysis of inter-beat variation provides quantitative assessment of cardiac autonomic control and spectral hemodynamic fluctuations were employed to reflect changes in cardiac sympathovagal outflow (El-Mas and Abdel-Rahman, 2007; El-Mas and Abdel-Rahman, 2013). R-R intervals were calculated in LabChart® Pro software as the time intervals between the maxima of the first derivative of the blood pressure trace. HRV was analyzed in the frequency domain using FFT algorithms of the R-R data series. Spectra were integrated into 2 specific frequency bands, LF (0.25–0.75 Hz) and HF (0.75–3 Hz) bands and expressed in normalized units (LF<sub>nu</sub> and HF<sub>nu</sub>). The LF/HF

ratio was used as an index of the cardiac sympathovagal balance. Spectral data were estimated before (baseline) and at 15-min intervals after LPS treatments. For each time point, 5-min values of each variable were averaged. Segments analyzed were visually checked for erroneous beats. Ectopic beats and artifacts were excluded from the analysis.

### 2.5. Baroreflex testing

At the end of the hemodynamic monitoring window, BRS was determined using the vasoactive method (El-Mas and Abdel-Rahman, 1993; Kumagai et al., 1993; El-Mas et al., 2012), which measures negative and positive heart rate changes in response to blood pressure rises and falls evoked by bolus intravenous injections of randomized doses of phenylephrine (PE) or sodium nitroprusside (SNP), respectively (1–16 g/kg each, every 5 min). For each dose, an injection volume of 0.05 ml/100 g body weight was flushed with approximately 0.1 ml of saline. After injection, peak changes in mean arterial pressure (MAP) and heart rate (HR), from baseline before injection, were determined for each dose. The corresponding changes were used to construct baroreflex curves for PE and SNP. Slopes of the regression lines (BRS<sub>PE</sub> and BRS<sub>SNP</sub>) were recruited as indicators of BRS.

### 2.6. Immunohistochemistry

The technique described in our previous studies was adopted (Wedn et al., 2019). The anterior part of the heart apex was excised transversally. A 3–4 mm thick portion of the aortic arch was cut to allow the visualization of the whole aortic perimeter. Tissues were washed with saline, fixed in 10% formaldehyde solution in phosphate buffered saline, and transferred to labeled cassettes for tissue processing. Tissue processing was carried out by immersing the cassettes in ascending concentrations of ethanol, followed by clearing with xylene and embedding in paraffin wax. Transverse 5- $\mu$ m sections (2 from each rat) were cut and mounted on positively charged slides (Thermo Scientific®, Berlin, Germany). Following deparaffinization in xylene and rehydration, antigen retrieval was carried out by immersing the slides in citrate buffer (pH 6.0) and heating in a microwave for 10 min. The sections were then incubated with the primary antibodies against the target proteins (NF- $\kappa$ B p65, iNOS, or HSP70; 1  $\mu$ g/ $\mu$ l, Bioss® Inc., Massachusetts, USA) for 24 h at 4 °C. All antibodies were diluted with substrate buffer (Dako Agilent®, Santa Clara, CA, USA) to a dilution of 1:200 except for HSP70 antibody in aortic sections which was diluted to 1:100. Afterwards, sections were incubated with the secondary horseradish peroxidase (HRP)-conjugated antibodies (Dako Agilent®, Santa Clara, CA, USA) for 30 min at room temperature. All sections were counterstained with hematoxylin. Then, the sections were examined microscopically for the dark granular brown staining indicative of a 3,3-Diaminobenzidine (DAB) reaction product. The resultant signal was quantified using ImageJ software. Controls were included to support the validity of the staining pattern and to exclude experimental artifacts or nonspecific binding. A negative control was done by examining myocardial sections treated with secondary, but not primary, antibody. In this case, tissues showed no or minimal staining that did not exceed 0.3% of total area.

For imaging and image analysis, at least 20 images were taken from each anatomical area of individual rats using Optika® Optikam B9 digital camera mounted on Optika® B-193 microscope and the company's Vision Lite software version 2.13. Immunohistochemical signals of NF- $\kappa$ B p65, iNOS and HSP70 were quantified by the Fiji ImageJ software version 1.51n (National Institutes of Health, USA) using the color deconvolution plugin. This plugin splits the stained image into three separate color channels: hematoxylin (blue, color 1), DAB (brown, color 2) and background (color 3). The intensity of the brown DAB color was then measured as the percentage of the area above a cut-off threshold.

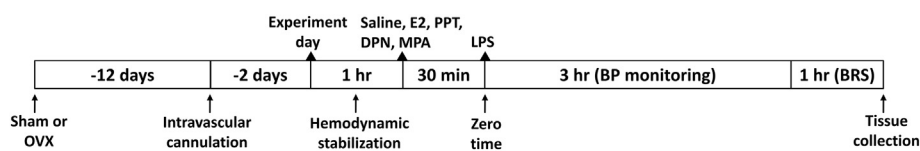


Fig. 1. Time schedules for surgical procedures and drug regimens. OVX, bilateral ovariectomy; LPS, lipopolysaccharide; PPT, 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol; DPN, diarylpropionitrile; MPA, medroxyprogesterone acetate; BP, blood pressure; BRS, baroreflex sensitivity.

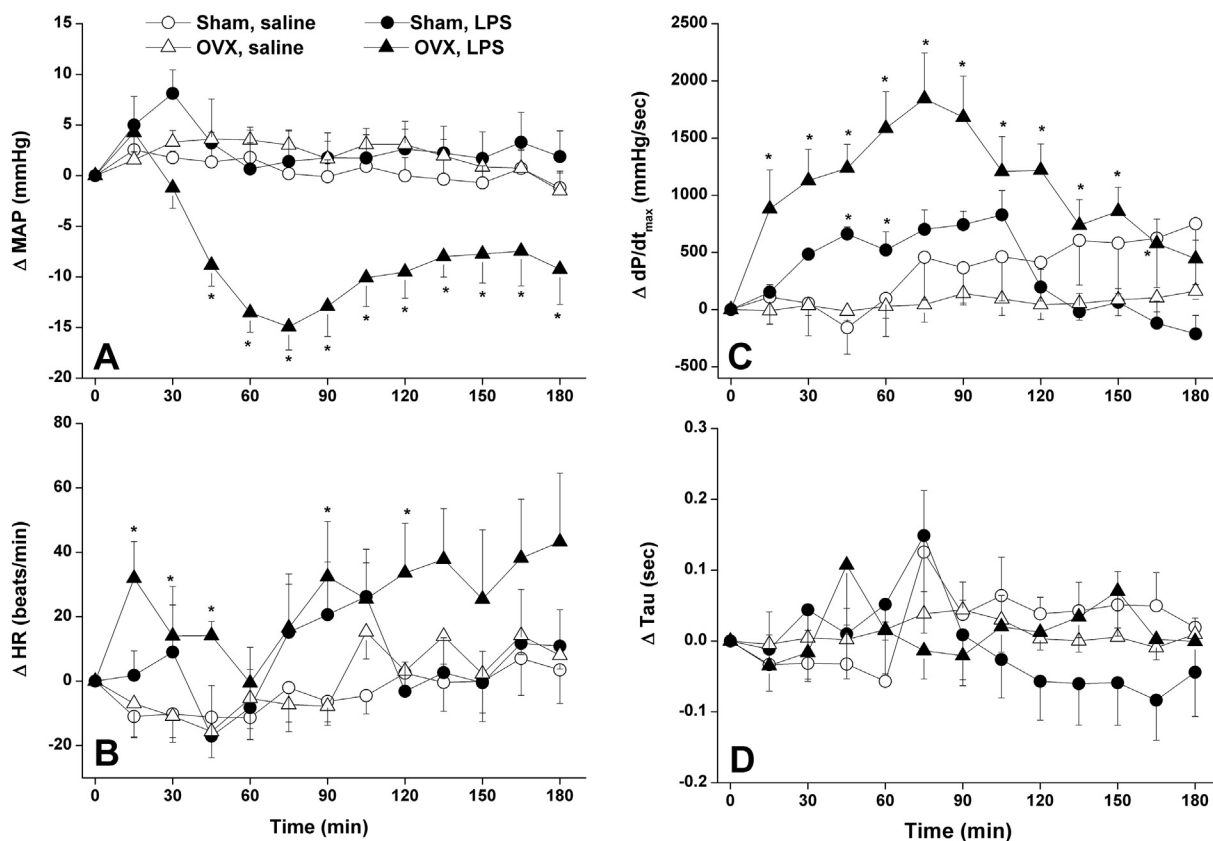


Fig. 2. Effects of LPS (10 mg/kg, i.v.) on changes in mean arterial pressure (MAP, panel A), heart rate (HR, panel B), maximum rate of rise in left ventricular pressure ( $dP/dt_{max}$ , panel C) and left ventricular relaxation time constant ( $\tau$ , panel D) in conscious sham and OVX rats. Values are means  $\pm$  S.E.M. of 6–8 observations. \* $P < .05$  versus sham/saline values.

## 2.7. Protocols and experimental groups

### 2.7.1. The effect of OVX on LPS-induced cardiovascular anomalies in rats

Four groups of conscious rats (2 OVX and 2 sham,  $n = 6-8$  each) were employed in this experiment to test the cardiovascular consequences that follow i.v. injection of LPS (10 mg/kg) or an equivalent volume of its vehicle (saline). On the experiment day, the arterial catheter was connected to a pressure transducer (model P23XL; Astro-Med, West Warwick, RI) attached to Power Lab (4/35, model ML866/P; AD Instruments Pty Ltd., Castle Hill, Australia) using LabChart-7 Pro software for data acquisition. A period of at least 1 h was allowed at the beginning of the experiment for hemodynamic stabilization. Hemodynamic monitoring then continued for 3 more hr to determine changes caused by LPS or saline in BP, HRV, and LVF. Subsequently, the arterial baroreflex function was assessed by the vasoactive method as described earlier. Rats were then euthanized by i.p. administration of an overdose of thiopental (100 mg/kg), and ventricular and aortic tissues were paraffin molded for subsequent immunohistochemical evaluation of the protein expression of NF- $\kappa$ B p65, iNOS, and HSP70. Time schedules for surgical procedures and drug regimens are shown in Fig. 1.

### 2.7.2. Effects of gonadal hormone supplementation on LPS-evoked cardiovascular alterations in OVX rats

Another four groups of OVX rats ( $n = 6-8$  each) were used to assess the effects of LPS after pretreatment, 30 min earlier, with one of the following i.v. regimens: (i)  $E_2$  (10  $\mu$ g/kg) (Saleh and Connell, 2000), (ii) 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, selective ER $\alpha$  agonist, 10  $\mu$ g/kg) (Yao and Abdel-Rahman, 2017), (iii) Diarylpropionitrile (DPN, selective ER $\beta$  agonist, 10  $\mu$ g/kg) (Yao and Abdel-Rahman, 2017) or (iv) medroxyprogesterone acetate (MPA, progesterone receptor agonist, 10 mg/kg) (Gohar et al., 2014). Hemodynamic monitoring continued for 3 h and followed by the evaluation of arterial baroreflex activity by the vasoactive method. Rats were euthanized, and cardiac and aortic tissues were processed for immunohistochemistry as detailed above and illustrated in Fig. 1.

## 2.8. Drugs

LPS (from *E. coli* 0111:B4), 17 $\beta$ -estradiol sulfate, PE, SNP (Sigma-Aldrich Co. LLC), PPT, DPN (Tocris Bioscience), Ketamine vials (50 mg/ml, Alfasan, Woerden, Holland), thiopental (Sandoz, Basel, Switzerland), xylazine vials (100 mg/ml, ADWIA Co. S.A.E., Egypt), povidone-iodine solution (Betadine; Nile Pharmaceutical Co., Cairo, Egypt), and benzathine penicillin (Penicid; Cid Pharmaceutical Co., Cairo, Egypt) were purchased from commercial vendors. 17 $\beta$ -estradiol

sulfate, LPS, DPN, PPT, PE, SNP and thiopental were dissolved in saline. MPA was dissolved in dimethyl sulfoxide (DMSO). The latter caused no hemodynamic changes when administered parenterally (Harraz et al., 2012; Raji et al., 2013).

For immunohistochemistry, all buffers and other reagents including the secondary anti-rabbit antibody which was conjugated with horse radish peroxidase and 3,3'-diaminobenzidine (DAB) chromogen were purchased from Dako Agilent®, Santa Clara, CA, USA. The rabbit, anti-rat primary antibodies used in this study against NF- $\kappa$ B p65, iNOS and HSP70 were 1 $\mu$ g/ $\mu$ l, Bioss® Inc., Massachusetts, USA., they were diluted as required with substrate buffer (Dako Agilent®, Santa Clara, CA, USA).

### 2.9. Statistical analysis

Values are expressed as means  $\pm$  S.E.M. We employed the one-way or repeated measures analysis of variance (ANOVA) followed by the Tukey's post hoc test for statistical significance. These analyses were performed by GraphPad InStat, software release 3.05. Probability levels less than 0.05 were considered significant.

## 3. Results

### 3.1. OVX reveals endotoxic manifestations of hypotension and cardiac autonomic dysfunction

Figs. 2 and 3 illustrate the cardiovascular and autonomic effects elicited by i.v. administration of LPS (10 mg/kg) in conscious female rats subjected 14 days earlier to bilateral OVX or sham operation. Compared with respective saline values, neither MAP (Fig. 2A) nor HR (Fig. 2B) was affected by LPS over the 3-h period of the experiment. The computed maximum rate of rise of BP waves ( $+dP/dt_{max}$ ), an index of LV contractility, was transiently increased by LPS at 45 and 60 min compared with saline values (Fig. 2C). Diastolic function indexed by the isovolumic relaxation time constant ( $\tau$ ) remained unaffected in LPS-treated sham rats (Fig. 2D). Unlike its insignificant effects in sham rats, the i.v. administration of the same dose of LPS in OVX rats produced significant and maintained falls in MAP (Fig. 2A) together with opposite changes in HR (Fig. 2B). While diastolic function ( $\tau$ ) was not affected (Fig. 2D),  $dP/dt_{max}$  exhibited significant and robust elevations in endotoxic OVX rats with a peak effect observed at 75 min (Fig. 2C), the same timepoint at which the hypotension reached its nadir (Fig. 2A).

Spectral analysis of HRV showed that cardiac autonomic function was not affected by LPS in sham-operated rats. Changes in the R-R oscillations in the LFnu range (0.25–0.75 Hz, Fig. 3A) or in the HFnu range (0.75–3 Hz; Fig. 3B) in sham-operated rats treated with LPS or saline were not statistically different from saline values. The LF/HF ratio, spectral measure of cardiac sympathovagal balance, also remained unaffected by LPS in sham-operated rats (Fig. 3C). In OVX rats, LPS produced significant increases and decreases in normalized units of LF and HF spectra of HRV, respectively. As a consequence, the LF/HF ratio was significantly increased in these rats (Fig. 3C), denoting a shift in the cardiac autonomic control towards sympathetic dominance.

### 3.2. Gonadal hormone supplementation variably modulates cardiovascular manifestations of endotoxemia in OVX rats

The effects of replacing OVX rats with estrogenic or progestin compounds on cardiovascular actions of LPS in OVX rats are shown in Figs. 4 and 5. The hypotensive effect induced by LPS in OVX rats was completely abolished after prior administration of E<sub>2</sub> (10  $\mu$ g/kg, i.v.) (Fig. 4A). In this case, changes in MAP observed in E<sub>2</sub>/LPS-treated rats were similar to those caused by saline administration. LPS hypotension was also significantly attenuated when OVX rats pretreated with PPT (selective ER $\alpha$  agonist, 10  $\mu$ g/kg, i.v.) or DPN (selective ER $\beta$  agonist, 10  $\mu$ g/kg, i.v.). Nevertheless, in this latter setting the hypotensive action of LPS was not completely eliminated as a residual fall in MAP was

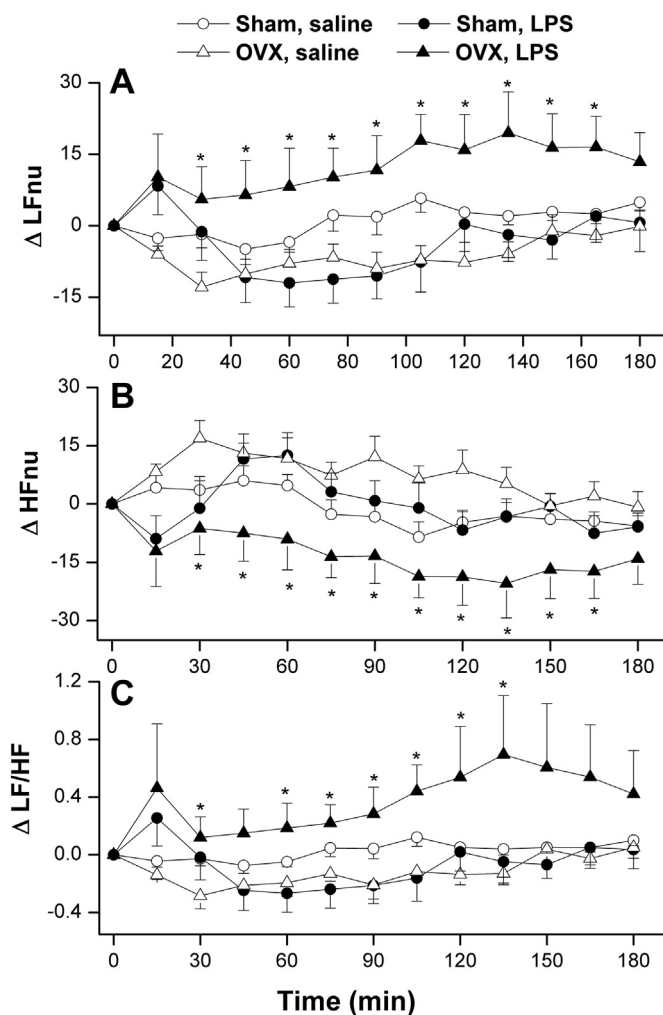


Fig. 3. Effects of LPS (10 mg/kg, i.v.) on changes in spectral indices of heart rate variability (HRV) in conscious sham and OVX rats. Values are means  $\pm$  S.E.M. of 6–8 observations. \*P < .05 vs. sham/saline values.

still evident in presence of PPT or DPN (Fig. 4A). In contrast to estrogenic compounds, prior exposure to MPA (10 mg/kg, i.v.) had no effect on LPS-induced hypotension (Fig. 4A).

Fig. 4B shows that the tachycardic profile in endotoxic rats was inconsistently affected by hormonal preparations. PPT, DPN and MPA were more effective than E<sub>2</sub> in reversing the rises in HR caused by LPS (Fig. 4B). Moreover, the pretreatment with the selective ER-agonists (PPT or DPN) significantly attenuated the increase in myocardial contractility ( $dP/dt_{max}$ ) induced by LPS in OVX rats while other interventions (E<sub>2</sub> or MPA) failed to do so (Fig. 4C).  $\tau$  remained unchanged in all endotoxic OVX rats with or without hormone supplementation (Fig. 4D).

Spectral analysis of HRV showed that cardiac autonomic dysfunction caused by LPS in OVX rats was indiscriminately rectified in rats pretreated with all hormonal preparations. As shown in Fig. 5, the pretreatment with E<sub>2</sub>, PPT, DPN, or MPA abolished the LPS-evoked increases and decreases in the normalized units of LF and HF spectra of HRV, respectively. This resulted in the disappearance of the increases in LF/HF ratios and cardiac sympathetic dominance caused by LPS (Fig. 5).

### 3.3. Activation of ER $\alpha$ preferentially abolishes BRS<sub>SNP</sub> dysfunction caused by LPS in OVX rats

The effects of OVX and/or LPS on baroreflex curves generated by

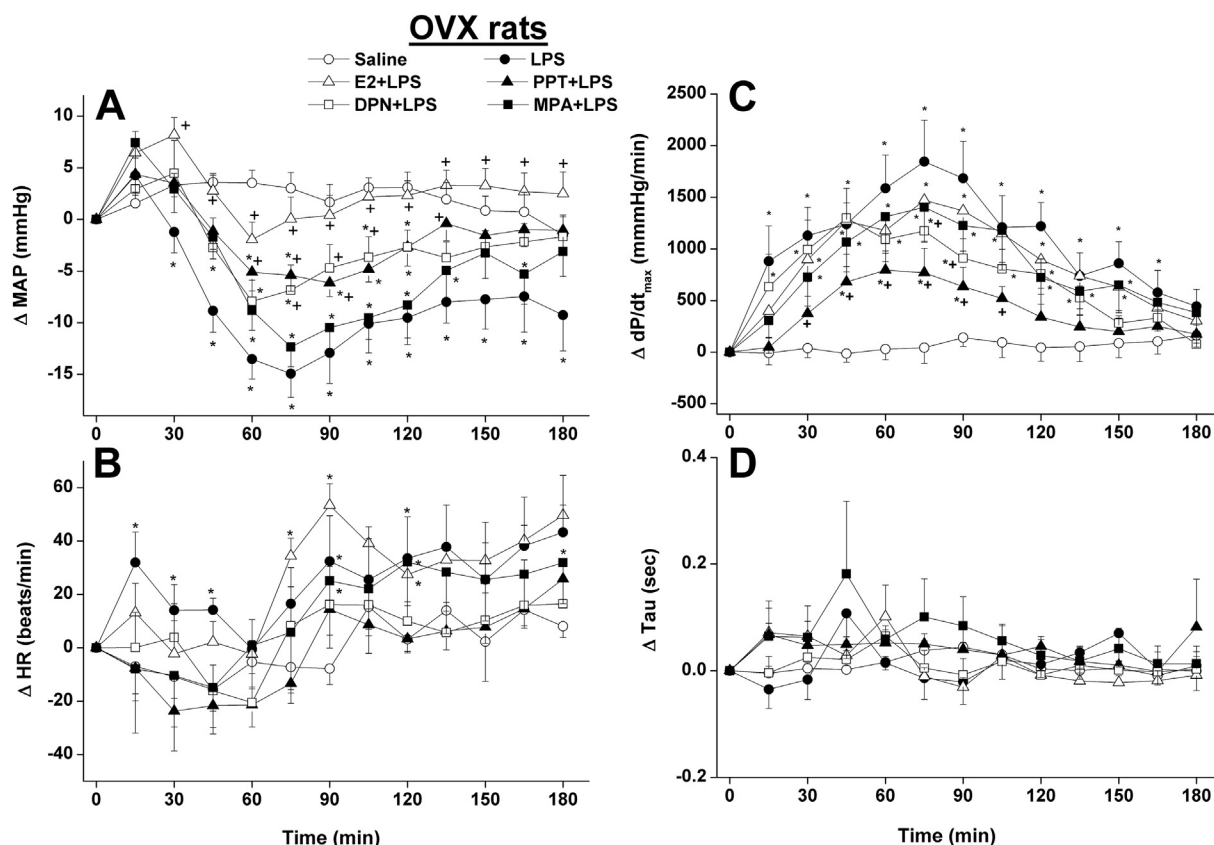


Fig. 4. Effects of i.v. pretreatment with estradiol (E2, 10  $\mu$ g/kg), PPT (estrogen receptor- $\alpha$  agonist, 10  $\mu$ g/kg), DPN (estrogen receptor- $\beta$  agonist, 10  $\mu$ g/kg), medroxyprogesterone acetate (MPA, 10 mg/kg) or saline on LPS (10 mg/kg i.v.)-evoked changes in mean arterial pressure (MAP, panel A), heart rate (HR, panel B), maximum rate of rise in left ventricular pressure (dP/dt<sub>max</sub>, panel C) and left ventricular relaxation time constant (tau, panel D) in conscious OVX rats. Values are means  $\pm$  S.E.M. of 6–8 observations. \*P < .05 vs. saline values, +P < .05 vs. saline/LPS values.

i.v. administration of a series of doses of PE or SNP (1–16  $\mu$ g/kg each) in conscious rats are shown in Fig. 6. Compared with SO rats, OVX caused upward shifts in the baroreflex curves of PE (Fig. 6A) and significantly reduced the slopes of these curves, which represent the baroreflex sensitivity (BRS<sub>PE</sub>, Fig. 6C). By contrast, the baroreflex curves of SNP (Fig. 6B) or their slopes (BRS<sub>SNP</sub>, Fig. 6C) were not influenced by OVX. Alternatively, the way the baroreflex curves was affected by the endotoxic insult depended on the hormonal state of rats (OVX vs. SO) and nature of the baroreflex response (bradycardia vs. tachycardia). Whereas LPS had no effects on PE or SNP curves or BRS in SO rats, it caused downward shifts in SNP, but not PE, curves and significantly reduced BRS<sub>SNP</sub> in OVX rats (Fig. 6).

Estrogenic or progestin replacement of OVX rats differently affected the LPS-baroreflex interaction. The downward shift in the SNP curves (Fig. 7C) and BRS<sub>SNP</sub> suppression (Fig. 7D) caused by LPS in OVX rats disappeared in rats supplemented with PPT in contrast to no effect for other hormonal supplements (E2, DPN, or MPA). To the contrary, with the exception of PPT, all hormonal supplements caused downward shift in the PE baroreflex curves (Fig. 7A) and significantly increased BRS<sub>PE</sub> (Fig. 7B).

### 3.4. Molecular effects of hormonal supplementation on cardiac and aortic inflammation in endotoxic rats

Immunohistochemical staining was employed to determine the effects of LPS on protein expression of inflammatory (NF- $\kappa$ B p65 and iNOS) and anti-inflammatory (HSP70) cytokines in hearts and aortas of SO and OVX rats (Figs. 8 and 9, Panel A). Upon treatment with LPS, NF- $\kappa$ B p65 increases both in cardiomyocytes (heart) smooth muscle cells (aortic arch). The data also showed that OVX significantly increased the

ventricular expression of iNOS (Fig. 8B) compared to SO rats. More progressive and significant elevations in iNOS expression resulted when OVX rats were challenged with LPS. This contrasted with the effects of LPS in SO rats in which the same LPS regimen failed to elicit any changes in ventricular expression of iNOS.

Pretreatment of OVX rats with E<sub>2</sub> or selective ER agonists (PPT or DPN) significantly blunted the LPS-evoked increases in ventricular expression of NF- $\kappa$ B p65 and iNOS. Among the three estrogenic compounds, PPT was the most effective in alleviating the inflammatory response to endotoxemia. PPT caused more significant reductions in myocardial expression of NF- $\kappa$ B p65 and iNOS than E<sub>2</sub> or DPN and brought the ventricular expressions of these proteins to levels that were not significantly different from those found in ventricular tissues of SO rats. Alternatively, MPA had no effect on the protein expression of NF- $\kappa$ B p65 (Fig. 8).

Paradoxically, LPS increased the ventricular and aortic expression of HSP70 in SO by 2.5 folds compared with saline-treated values, while it had no effects on HSP70 expression in ventricular and aortic tissues of OVX rats. Representative images from immunostained ventricular tissues are shown in Figs. 8 and 9. Moreover, the ventricular expression of HSP70 was significantly and comparably increased by all estrogenic compounds in endotoxic OVX rats.

Unlike estrogenic products, the ventricular and aortic expression of none of the inflammatory (NF- $\kappa$ B p65 or iNOS) or anti-inflammatory (HSP70) mediators in endotoxic OVX rats was influenced by the prior treatment with MPA (Figs. 8 and 9).

## 4. Discussion

The current experimental study reports on the roles of female

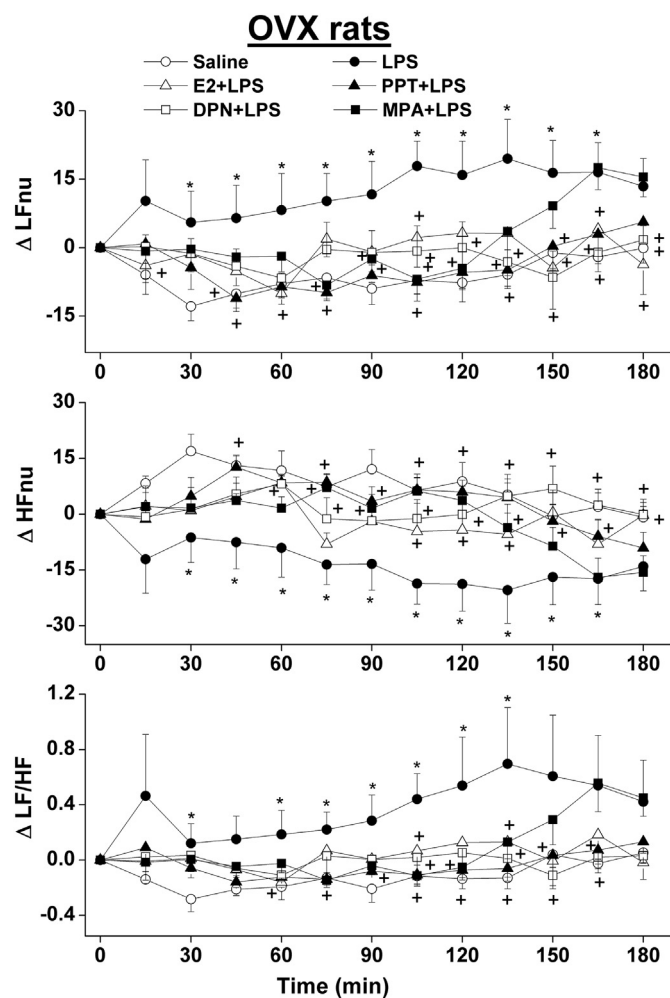


Fig. 5. Effects of i.v. pretreatment with estradiol (E2, 10  $\mu$ g/kg), PPT (estrogen receptor- $\alpha$  agonist, 10  $\mu$ g/kg), DPN (estrogen receptor- $\beta$  agonist, 10  $\mu$ g/kg), medroxyprogesterone acetate (MPA, 10 mg/kg), or saline on LPS (10 mg/kg)-evoked changes in spectral indices of heart rate variability (HRV) in conscious OVX rats. Values are means  $\pm$  S.E.M. of 6–8 observations. \*P < .05 vs. saline/saline values, +P < .05 vs. saline/LPS values.

gonadal hormones and their receptors in inflammatory and cardiovascular complications of endotoxemia. The data showed that the classic features of endotoxemia such as robust tissue inflammation, hypotension, and cardiac autonomic and baroreflex dysfunctions appeared in female rats subjected to bilateral OVX but not sham operation, suggesting key roles for gonadal hormones in guarding against endotoxic insult. Hormone replacement studies showed that this privileged hormonal status in females is primarily attributed to E<sub>2</sub> rather than progesterone. More specifically, receptor agonistic and molecular studies revealed more prominent cardioprotective and antiinflammatory role for ER $\alpha$  than ER $\beta$  because selective activation of ER $\alpha$  caused more effective counteraction of the LPS-induced (i) suppression of baroreflex activity, (ii) elevation of systolic contractility (dP/dt<sub>max</sub>), and (iii) up-regulation of myocardial expressions of NF- $\kappa$ B and iNOS.

The current observation that LPS reduced BP in OVX, but not sham, rats is consistent with reported studies (Shen et al., 2017) and suggests a protective role for female gonadal hormones against endotoxemia. Along with the falls in BP, HRV and arterial baroreceptor studies revealed clear signs of cardiac autonomic dysfunction in LPS-treated OVX rats. Spectral HRV analysis, which reflects the global cardiac sympathovagal balance (Stein et al., 1994; El-Mas and Abdel-Rahman, 2007; El-Mas and Abdel-Rahman, 2013), demonstrated reductions in the total power of HRV along with increases in LF/HF ratios. These

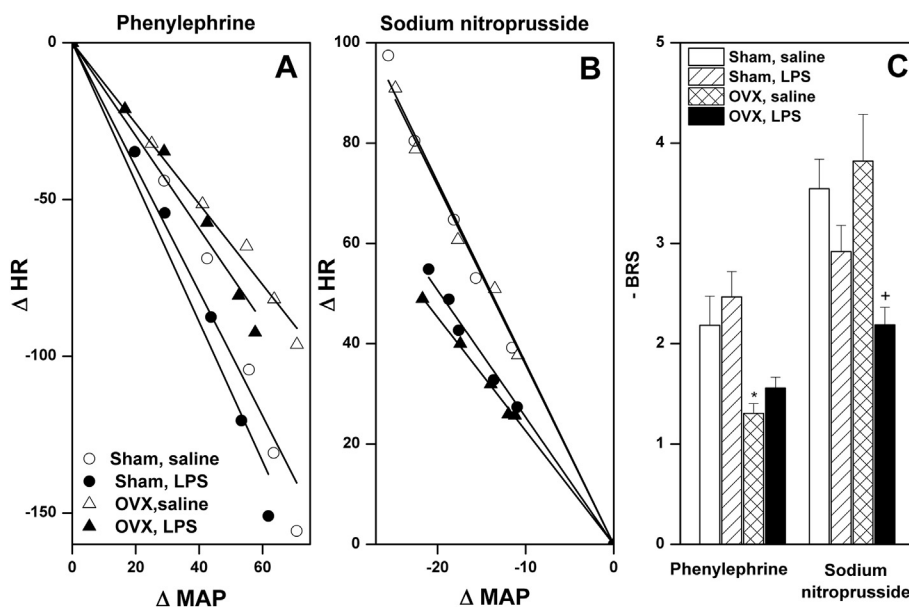
effects point to an increased cardiac sympathetic drive in OVX rats in response to the endotoxic insult. The latter might well explain the timely increases in HR and LV contractility (dP/dt<sub>max</sub>) seen in the same rat model. Clinically, impaired HRV is believed to positively correlate with cardiovascular morbidity and mortality in endotoxemia as well as in other pathological conditions such as diabetes and heart failure (Malave et al., 2003; Gonzalez-Clemente et al., 2007).

The vasoactive method was adopted to assess the reflex control of cardiac autonomic control. Electrophysiologic and pharmacologic studies indicated that while reflex bradycardic responses to BP increments are predominantly mediated via facilitated central vagal discharges, reflex tachycardia induced by BP decrements marks central sympathetic outflow (Smyth et al., 1969; El-Mas and Abdel-Rahman, 1998; El-Mas et al., 2002). Based on this, the current findings of maintained reflex bradycardia (BRS<sub>PE</sub>) and depressed reflex tachycardia (BRS<sub>SNP</sub>) in endotoxic OVX rats infer selective attenuation of baroreflex-mediated cardiac sympathetic activity in this model system. Such effect is obviously specific to the gonadal hormone depleted state because no similar situation was observed in rats with intact hormonal dominion (Fig. 6). Surprisingly, the presumed suppression of reflex cardiac sympathetic activity might be at odds with our spectral HRV data that highlighted facilitated cardiac sympathetic activity in OVX rats. Of note, the cardiac sympathovagal balance measured by spectral analysis reflects the net of all central and peripheral functions involved in cardiac autonomic control. It is likely, therefore, that the depressed reflex sympathetic activity in endotoxic OVX rats might have been overruled by the simultaneous baroreflex-unrelated exaggeration in cardiac sympathetic control.

Although the counterbalancing effects of estrogenic compounds against endotoxic inflammation and hypotension have been recognized (Speyer et al., 2005; Shen et al., 2017), little information is available regarding the role of E<sub>2</sub> in cardiac autonomic and left ventricular dysfunctions provoked by the endotoxic shock. Likewise, progesterone supplementation has been shown in one study to confer protections against the endotoxic hypothermic response in OVX rats (Saia et al., 2008), but whether progesterone favorably affects cardiovascular aberrations triggered by endotoxemia in the same rat model remains unclear. The current study depicts distinct and inconsistent effects for female gonadal hormones on the intensified endotoxic manifestations in OVX rats. Whereas the heightened spectral index of cardiac sympathetic drive observed in endotoxic rats was similarly blunted after E<sub>2</sub> or MPA supplementation, the concomitant decreases in BP was inhibited by E<sub>2</sub> only. By contrast, the LPS-evoked increases in LV contractility (dP/dt<sub>max</sub>) or attenuated reflex tachycardia was affected by neither hormonal therapy.

The reason(s) for the heterogeneous effects of E<sub>2</sub> on endotoxic responses in the current study is not clear. E<sub>2</sub> is believed to produce its effects via multiple sites, among which ER $\alpha$  and ER $\beta$  are the most important. These receptor subtypes differ in their anatomic distribution (Grzegorzewski et al., 2010), encoding genes (Pastore et al., 2012), and molecular pathways (O'lonne et al., 2007). Such discrepancies permit variable and sometimes opposite roles for the two receptor sites in arbitrating the biological effects of E<sub>2</sub> (Jobe et al., 2010; Sobrino et al., 2010; Pastore et al., 2012; Xiong et al., 2015). Interestingly, agonistic studies of the current investigation revealed more advantageous effects for ER $\alpha$  in combating endotoxic manifestation in OVX rats. Whereas selective activation of ER $\alpha$  and ER $\beta$  by PPT and DPN, respectively, produced similar counteraction of LPS hypotension and enhanced cardiac sympathetic activity, the LPS-mediated increase in LV contractility and decrease in reflex tachycardia were abrogated by PPT only.

Immunohistochemical protein expression studies were employed to test the hypothesis that inflammatory pathways of NF $\kappa$ B and iNOS account for distinct roles of gonadal hormones and their receptors in endotoxemia. Reported studies indicate that the upregulation of NF $\kappa$ B during endotoxemia is followed by the activation of a wide array of cytokines, chemokines, and iNOS (Goren et al., 2004; Natoli, 2006).

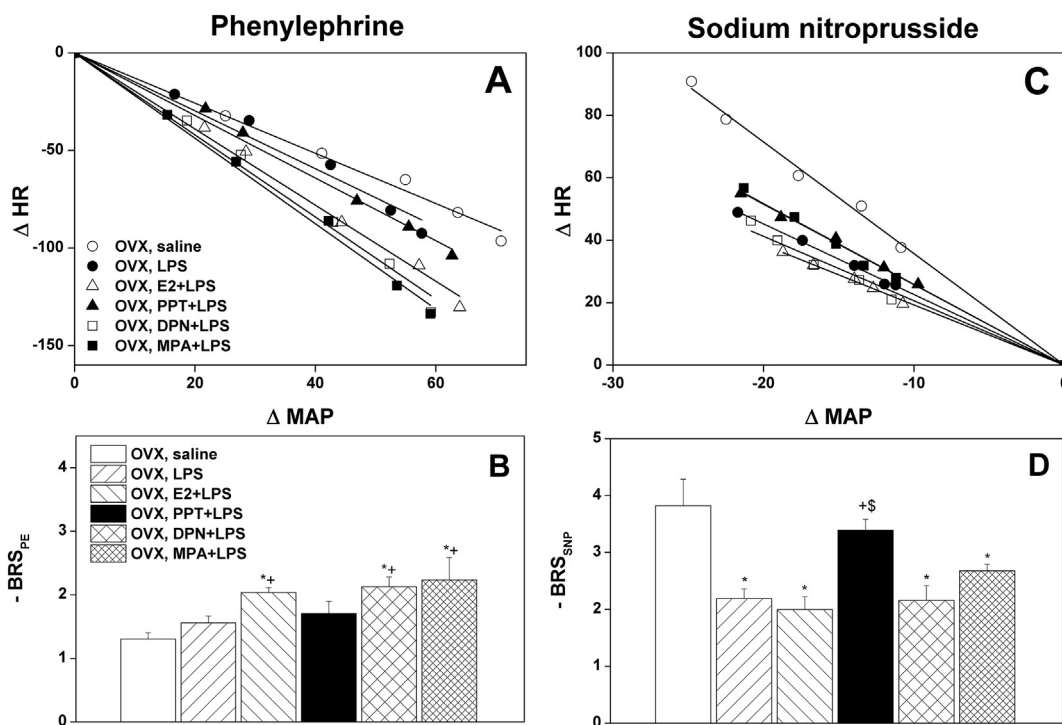


**Fig. 6.** Effects of LPS (10 mg/kg, i.v.) on baroreflex curves generated by phenylephrine (panel A) and sodium nitroprusside (panel B) and their slopes (baroreflex sensitivity, BRS, panel C) in conscious sham and OVX rats. \*P < .05 versus sham/saline values, +P < .05 versus OVX/saline values.

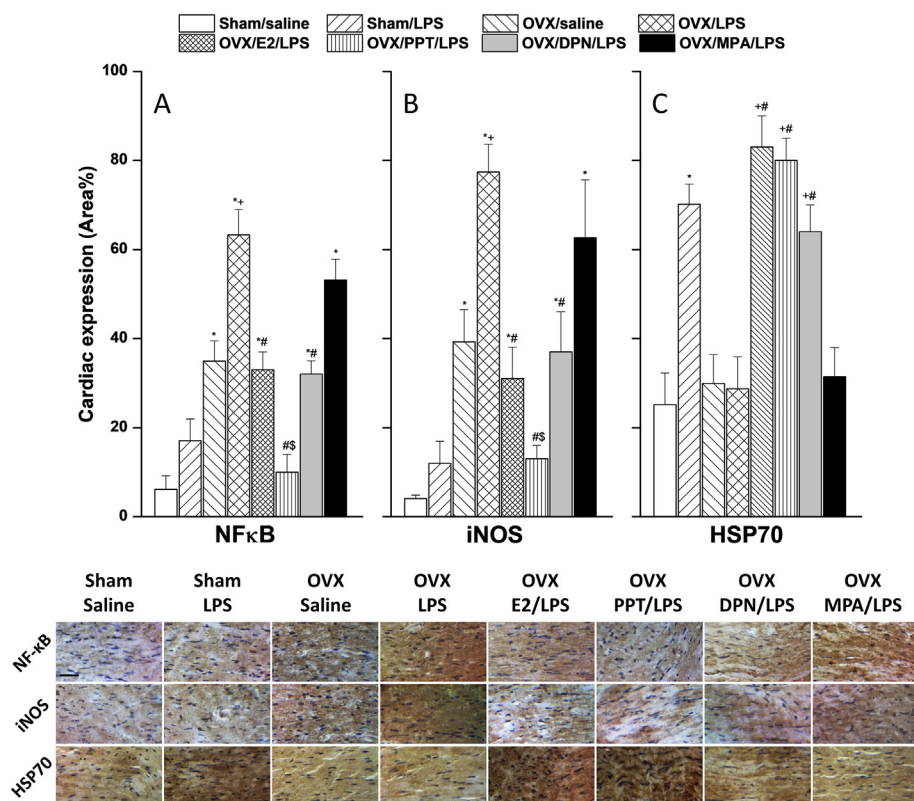
These inflammatory products strongly correlate with poor prognosis, myocardial dysfunction, and mortality known to accompany endotoxic shock (O'Neill, 2011; Kakihana et al., 2016). Two important molecular observations emerged from current studies. Firstly, in contrast to no effect in sham rats, LPS treatment of OVX rats produced exaggerated inflammatory response as indicated by the tremendous elevations in ventricular and aortic expressions of NFκB and iNOS. These inflammatory events may very likely explain the endotoxic response set off by LPS in rats with depleted gonadal hormones. Secondly and

equally important, among all replacement therapies employed in the present study, the ERα agonist PPT was the most effective in eliminating the inflammatory response and restoring protein expressions of NFκB and iNOS to near-control levels particularly in ventricular tissues.

HSPs are a family of chaperone proteins produced in response to stress conditions (Moseley, 2000; Lanneau et al., 2008). HSP70 produces its cytoprotective effect via the inhibition of the production of NF-κB and iNOS and reducing IL-1β toxicity (Kiang et al., 2004; Cao et al., 2012). Here, we report that the LPS effect on tissue abundance of



**Fig. 7.** Effects of i.v. administration of estradiol (E2, 10 μg/kg), PPT (estrogen receptor-α agonist, 10 μg/kg), DPN (estrogen receptor-β agonist, 10 μg/kg) or medroxyprogesterone acetate (MPA, 10 mg/kg) on LPS (10 mg/kg, i.v.)-evoked changes in baroreflex curves generated by phenylephrine (panel A) and sodium nitroprusside (panel C) and their slopes (baroreflex sensitivity, BRS, panels B and D) in conscious OVX rats. \*P < .05 versus OVX/saline values, +P < .05 versus OVX/LPS values, \$P < .05 versus OVX/E2/LPS or OVX/DPN/LPS values.



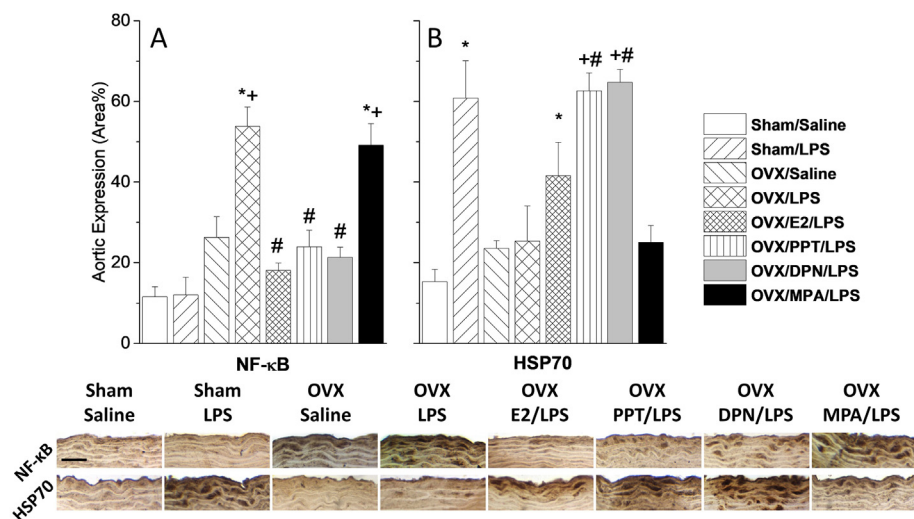
**Fig. 8.** Effects of LPS (10 mg/kg, i.v.) on the protein expression of NF-κB p65 (Panel A), iNOS (Panel B) and HSP70 (Panel C) in cardiomyocytes in transverse sections of the heart apex of sham and OVX rats. The effects of i.v. pretreatment with estradiol (E2, 10 μg/kg), PPT (estrogen receptor-α agonist, 10 μg/kg), DPN (estrogen receptor-β agonist, 10 μg/kg) or medroxyprogesterone acetate (MPA, 10 mg/Kg) on LPS-induced changes in cardiac expression of the same proteins in OVX rats are also shown. Representative images for immunostained sections are shown. Scale bar is 100 μm. \*P < .05 versus sham/saline values, +P < .05 versus OVX/saline values, #P < .05 versus OVX/LPS values, #P < .05 versus OVX/E2/LPS or OVX/DPN/LPS values.

HSP70 depended on the gonadal hormonal state of the rat. Remarkably, LPS produced a 3-fold increase in HSP70 expression in ventricular and aortic tissues of sham, but not OVX, rats. Such elevations in tissue HSP70 presumably appeared to have served as an adaptive mechanism that switched off the inflammatory response and cardiovascular damage induced by endotoxemia in sham rats. Similar increases in ventricular and aortic HSP70 expressions were noted when LPS was administered to OVX replaced with estrogenic drugs. In this latter case, the increased tissue HSP70 does not seem to contribute to the more predominant ERα-mediated protection against endotoxemia because comparable increases in tissue HSP70 were caused by all estrogenic compounds (E<sub>2</sub>, PPT, and DPN).

It is worth noting that the current study measured the p65 subunit of the NF-κB transcription factor family, which is a component of the canonical NF-κB inflammatory pathway (Napetschnig and Wu, 2013).

In unstimulated cells, NF-κB p65 and p50 subunits exist in the cytoplasm due to inhibitory effects of κB (IκB), which binds to the p50/p65 heterodimer preventing their nuclear translocation (Manavalan et al., 2010). The exposure to inflammatory stimuli as LPS induces NF-κB signaling through activation of IκB kinase that phosphorylates IκB and facilitates its disassociation from p50/p65 heterodimer. The free dimer locates to the nucleus and bind to specific gene promoters to modulate the expression of inflammatory proteins including iNOS (Jeon et al., 1999; Napetschnig and Wu, 2013). Alternatively, the cytoprotective activity of HSP70 is mediated via the inhibition of NF-κB, iNOS, and IL-1β signaling (Kiang et al., 2004; Cao et al., 2012). The data concerning the balance between these pro-inflammatory (NF-κB and iNOS) and anti-inflammatory (HSP70) mediators in stimulated versus unstimulated cells is scarce and warrants further investigations.

Although pharmacologic and molecular data of the present study



**Fig. 9.** Effects of LPS (10 mg/kg, i.v.) on the protein expression of NF-κB (Panel A) and HSP70 (Panel B) in transverse sections in the walls of the aortic arch of sham and OVX rats. The sections show the tunica intima and tunica media, which consist of bundles of white connective tissue and circular smooth muscles arranged in layers. The protein expression is mainly localized in the smooth muscle cells. The effects of i.v. estradiol (E2, 10 μg/kg), PPT (estrogen receptor-α agonist, 10 μg/kg), DPN (estrogen receptor-β agonist, 10 μg/kg) or medroxyprogesterone acetate (MPA, 10 mg/Kg) on LPS-induced changes in aortic expression of the same proteins in OVX rats are also shown. Representative images for immunostained sections are shown. Scale bar is 100 μm. \*P < .05 versus sham/saline values, +P < .05 versus OVX/saline values, #P < .05 versus OVX/LPS values.

denote a key role for ER $\alpha$  in guarding against endotoxic manifestations, the cardioprotective and antiinflammatory actions conferred by ER $\beta$  as well have been established. In fact, a downregulatory action for the two receptor types against inflammation has been documented through possibly diverse cellular mechanisms. ER $\beta$  protects endothelial cells from inflammation-induced endothelial apoptosis and dysfunctions (Fortini et al., 2017). On the other hand, pharmacologic and genetic deletion studies demonstrated that ER $\alpha$  is more involved in suppressing TNF $\alpha$  release from macrophages (Campbell et al., 2014). Additionally, the modulation by ER $\alpha$  and ER $\beta$  of the production of inflammatory mediator from macrophages is both age and sex dependent (Stanojević et al., 2018). Along this line, receptor antagonist studies in adult female rats with intact hormonal state highlighted a greater impact for progesterone and ER $\beta$  than ER $\alpha$  in offsetting inflammatory and cardiovascular derangements caused by the LPS challenge (El-Lakany et al., 2018). Together, these findings suggest that the relative antiinflammatory propensities of ER $\alpha$  and ER $\beta$  are influenced by factors such as sex, hormonal state, and cell type involved.

Remarkably, the 4-h post-LPS monitoring period was employed in the current study because earlier experimental studies showed that LPS caused rapid increases in serum cytokines during the first 6 h of endotoxemia and declined thereafter (Fu et al., 2014). (Fu et al., 2014a) In a recent report from our laboratory (Wedn et al., 2019), we found that endotoxic manifestations of hypotension and inflammation were evident at 6 h and returned to normal at 24 h. Likewise, clinical reports revealed time-dependent hemodynamic, inflammatory, and cumulative endotoxic symptom score, with the disease severity being more pronounced during first 1–3 h following endotoxin challenge (Fullerton et al., 2016). Considering such relatively short 4-h monitoring period of our study, it could be argued that nongenomic rather than genomic mechanisms have contributed to the developed cardiovascular anomalies. Losel et al. (2002) reported that nongenomic actions of steroidal receptors appeared within few seconds to minutes from the exposure to the steroidal hormone, whereas genomic actions required hours to manifest. That said, the role of genomic mechanisms cannot be overlooked especially with the consideration that the expression of inflammatory proteins during endotoxemia is rather a rapid process. The time course for iNOS and NF- $\kappa$ B protein expression showed an increase at 3 h, a further increase at 6 h, and a return to control level at 24 to 48 h after LPS in cardiac and renal tissues (Wang et al., 2000; Wedn et al., 2019). We also demonstrated that elevations in protein expression of cardiac and aortic iNOS are reported within 90 min of endotoxemia (El-Mas et al., 2006; El-Mas et al., 2008). These reports are consistent with the present study in which significant increases in cytokine protein expression were noted 4 h after LPS exposure. However, further studies are needed to determine whether these changes in protein levels arose due to transcriptional or post-translational events.

## 5. Conclusions

The present study produced novel information regarding the roles of gonadal hormones in the privileged state of female rats against inflammatory and cardiovascular irregularities caused by endotoxemia. Molecular and integrative research in hormone depleted and repleted rats implicate estrogen, but not progesterone, in the protection against inflammatory, hypotensive, LV, and cardiac autonomic effects of endotoxemia. Cellular pathways of ER $\alpha$  play more integral roles than those of ER $\beta$  in alleviating responses to endotoxemia. Clinically, selective agonists at ER $\alpha$  might be exploited therapeutically to improve endotoxic manifestations in female with compromised hormonal function.

## Declaration of Competing Interest

None.

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