



Original article

Time and sex dependency of hemodynamic, renal, and survivability effects of endotoxemia in rats

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ABSTRACT

Widely different exposure times to endotoxic insults have been employed in reported studies. The current experimental study systematically evaluated the time-course and sex influences of endotoxic insult on survivability and cardiovascular and renal functions. Rats received i.p. lipopolysaccharide (LPS, 5 mg/kg) once or twice (over 2 successive days). Systolic blood pressure (SBP), biomarkers of renal function and inflammation, and vasodilator responsiveness of isolated perfused kidneys to acetylcholine (ACh) or N-ethylcarboxamidoadenosine (NECA) were evaluated 6 hr after first LPS injection or 1, 2, or 6 days later. A single 6-hr LPS challenge caused (i) sex-unrelated elevations in serum urea and creatinine and reductions in NECA, but not ACh, vasodilations, (ii) more increases in renal NF- κ B/iNOS expressions in male than in female rats, and (iii) hypotension and tachycardia only in male rats. These parameters, except for hemodynamic changes, were restored to near-control levels 1 day after single LPS dosing. The 2-days dosing with LPS had no effects on renal function biomarkers, but caused hypotension, tachycardia, and increases in renal NF- κ B/iNOS expression and NECA and ACh vasodilations in both rat sexes. None of these parameters were different from control values when measured 6 days after the endotoxic insult. Alternatively, the rat mortality was observed during first 2 days of the study and was notably higher in male than in female rats. Our data suggest that the frequency and time elapsed after LPS exposure as well as rat sex are important determinants of the magnitude and direction of detrimental effects of endotoxemia.

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1. Introduction

Endotoxemia is a complicated disorder with different stages and governed by inflammatory and anti-inflammatory cellular and humoral responses (Monneret et al., 2008). Despite recent advancement of our understanding of endotoxemia, some aspects of its pathophysiological effects are not yet clear, and consequently the mortality and morbidity of endotoxemia and endotoxic shock are still high. Lipopolysaccharide (LPS) exposure is a well-known

experimental method for induction of systemic inflammatory response and endotoxemia (van Lier et al., 2019). LPS activates Toll like receptor-4 on macrophages and monocytes and thus triggering host inflammatory reaction cascade leading to production of multiple pro-inflammatory cytokines including nuclear factor-kappa b (NF- κ B), Tumor necrosis factor-alpha (TNF- α) and interleukin 1-beta (IL-1 β) (Vidya et al., 2018). These systemic inflammatory events culminate into endotoxemia associated multiple organ damage including cardiovascular and renal dysfunction (Lv & Wang, 2016; Morrell et al., 2014).

Sex related disparities in host responses to inflammatory conditions have been identified (Casimir et al., 2018). Previous studies have shown that the subject sex significantly impacts the endotoxic inflammatory, cardiovascular and renal consequences in both human (Bosch et al., 2018) and animal (Losonczy et al., 2000). Likewise, published experimental and clinical studies indicated that the detrimental effects of endotoxemia vary considerably by time. Clinical studies revealed time-dependent hemodynamic, inflammatory, and cumulative endotoxic symptom score, with the disease severity being more pronounced during first 1–3 hr

Abbreviations: SBP, Systolic blood pressure; heart rate, HR; LPS, lipopolysaccharide; NECA, N-ethylcarboxamidoadenosine; ACh, acetylcholine.

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following endotoxin challenge (Fullerton et al., 2016). Similarly, studies in rats demonstrated that LPS caused rapid increases in serum IL-1 β and TNF- α over the first 6 hr of endotoxemia, after which cytokine levels started to decrease thereafter (Fu et al., 2014). Contrarily, Fodor et al. 2015 demonstrated that lung, liver and kidney injuries in rats observed 6 hr after LPS challenge are maintained or even worsened over the following 24 hr depending on the LPS dose employed.

Although the duration of endotoxic insult and animal sex have been proposed as major determinants of cardiovascular and renal responses during endotoxemia (Fodor et al., 2015; Fu et al., 2014; Fullerton et al., 2016; Wedn et al., 2019a; Wedn et al., 2019b), there have been no studies that systemically evaluated the hemodynamic and renal consequences over several days of the endotoxic insult as well as the sex specificity of these interactions. The current study employed biochemical and molecular techniques to investigate the time and sex based differences in survivability, hemodynamic and renal outcomes in a rat model of endotoxemia.

2. Materials and methods

2.1. Animals

Adult male and female Wistar rats (10–13 weeks old, 180–230 g) were housed in a temperature-controlled room with standard 12-hr light/dark cycle at the animal facility, Faculty of Pharmacy, Alexandria University, Egypt. All rats were fed with standard rat chow and tap water ad libitum. Animal experiments were conducted in compliance with institutional guidelines on the care and use of laboratory animals, and were approved by the Animal Care and Use Committee of the Faculty of Pharmacy, Alexandria University (ACUC project # 28/2014).

2.2. Drugs

LPS (E coli 0111: B4), 5'-N-ethylcarboxamidoadenosine (NECA, adenosine analogue), acetylcholine chloride (ACh), phenylephrine hydrochloride (Sigma-Aldrich, MO, USA), thiopental (Biochemie, Vienna, Austria), and heparin (5000 IU/ml; Nile pharmaceutical Co, Egypt) were purchased from commercial vendors. LPS, thiopental and heparin were dissolved in saline (Al-Mottahedoon Pharma Co, Egypt). ACh and NECA were prepared freshly in distilled water (Aqua Chemicals, Egypt) and dimethyl sulfoxide (Loba Chemie Pvt Ltd, India), respectively. All chemicals used to compose Krebs' solution were obtained from Sigma Chemical Co., St Louis, MO, USA.

2.3. Protocol and experimental groups

We investigate the sex-related effects of single-dose or two-dose LPS on survivability, hemodynamic and renal outcomes. In single-dose LPS model (Fig. 1A), 6 groups of male and female rats (n = 6–8 each) were utilized and divided into 2 control groups (1 male and 1 female) and 4 LPS groups (2 males and 2 females). Rats in LPS groups received a single i.p. dose of 5 mg/kg LPS (El-Mas et al., 2006; McKenna et al., 2018; Wedn et al., 2019a) and measurements were taken after 6 hr (2 groups, 1 male and 1 female) or 24 hr (2 groups, 1 male and 1 female). The experiments in control rats were undertaken 6 hr after injection of saline.

Another 6 groups of rats were employed to examine the effects of two-day LPS model in which rats received two i.p. doses of LPS (5 mg/kg/day) on 2 consecutive days (Fig. 1B). In the first 2 groups (1 male and 1 female), measurements were conducted 48 hr after first LPS dose (24 hr after second LPS dose). The same parameters were studied 6 days after first LPS challenge in another two groups

(1 male and 1 female). The remaining two groups (1 male and 1 female) served as controls (two saline doses, 24 hr apart) and subjected to experimental measurements 48 hr. One additional group of male rats (n = 5) was administered a single dose of LPS (5 mg/kg) and measurements were followed for 6 days. Fig. 1 illustrates the time schedules used for investigation of hemodynamic and renal effects of single and two-dose LPS.

At the specified time points, the mortality rate was determined, systolic blood pressure (SBP) was measured using tail-cuff plethysmography. Rats were then anesthetized with thiopental (50 mg/kg i.p.) and retro-orbital blood samples were collected for serum urea and creatinine analyses. Left kidneys were isolated and perfused to evaluate renovascular responsiveness to ACh (0.01–7.29 nmol) and NECA (1.6–100 nmol). Right kidneys were harvested for immunohistochemical determination of NF- κ B and inducible nitric oxide synthase (iNOS) expression. Euthanasia was induced by an overdose of sodium thiopental (100 mg/kg i.p.). During the time elapsed between LPS administration and measurement time-points, rats were housed under controlled laboratory conditions and maintained on a 12-hr light–dark cycle with free access to rat chow and tap water. Rats that died before their scheduled measurement time-points were excluded from the experiments and used only to compute the mortality rate.

2.4. Tail-cuff plethysmography

Non-invasive SBP measurement in conscious rats was carried out using tail cuff method as reported previously (Hammoud et al., 2017). Rats were allowed to acclimate to the tail cuff procedure for at least two sessions prior to actual BP recording. A tail pressure transducer (Pan Lab, Spain) was placed on the base of the tail, and connected with computerized data acquisition system coupled with LabChart-7 pro software (Power Lab 4/35, model ML866/P, AD Instruments, Bella Vista, Australia). Heart rate (HR) was computed as a cyclic measurement of BP waveforms and showed on a second channel of LabChart recording system. The reported SBP and HR values are the average of three or four consecutive readings per rat taken at 15 min intervals.

2.5. The rat isolated perfused kidney

The isolated perfused kidney technique was employed to evaluate renovascular reactivity to vasodilators, ACh and NECA, as outlined in our previous publications (El-Mas et al., 2004; Gohar et al., 2014). Following induction of anesthesia (thiopental, 50 mg/kg i.p.) and collection of blood samples, abdominal surgery was performed, left renal artery was cannulated and left kidney was rapidly isolated from its surrounding tissues. After its relocation to a temperature controlled glass chamber, the kidney was perfused with carbogenated Krebs' solution at a constant flow rate of 5 ml/min by means of a peristaltic pump (Model P3-Pharmacia Fine Chemicals). The changes in renal perfusion pressure were monitored using a pressure transducer attached to computerized data acquisition system (AD Instruments, Bella Vista, Australia). The renal perfusion pressure was allowed to stabilize for 30 min at the commencement of the experiment prior to its elevation by continuous infusion of the α_1 -adrenoceptor agonist phenylephrine (10 μ M). Cumulative dose response curves to bolus injections of ACh (0.01–7.29 nmol) and NECA (1.6–100 nmol) were then established in which each dose of acetylcholine or NECA was injected when the preceding dose has achieved its maximal vasodilatory response.

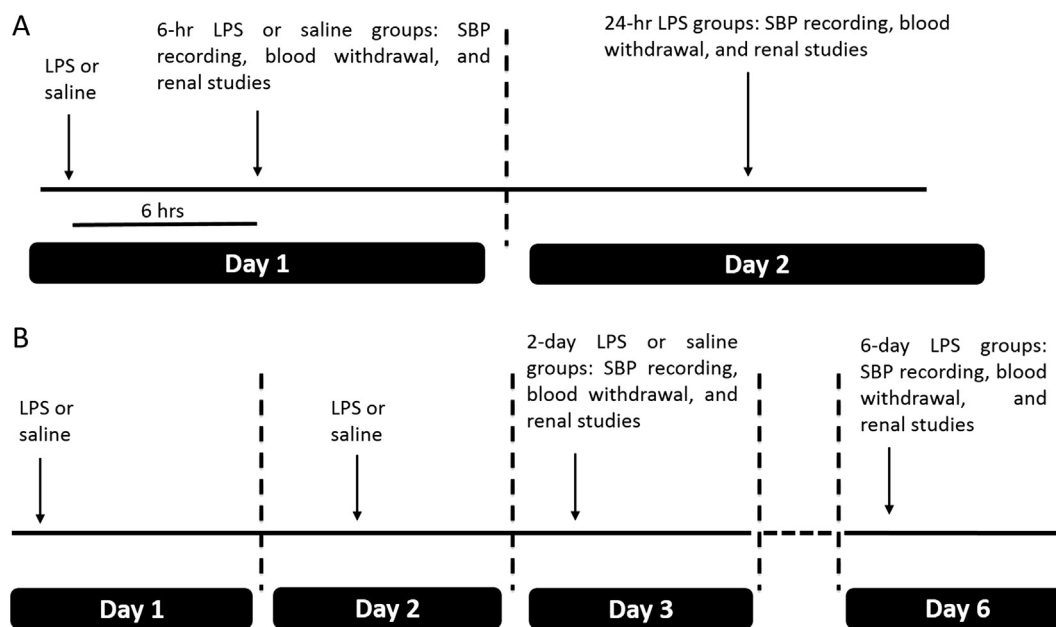


Fig. 1. Time schedules for the investigation of the effects of single (5 mg/kg, panel A) or double (5 mg/kg/day for 2 days, panel B) LPS dosing on SBP, survivability, renal function and inflammation in male and female rats. Abbreviations: LPS, lipopolysaccharide; SBP, systolic blood pressure.

2.6. Measurement of serum urea and creatinine

Next to induction of anesthesia, approximately 2 ml of blood samples were withdrawn by the aid of a glass capillary tube which was utilized to puncture the rat *retro*-orbital venous plexus. Following their collection, blood samples were permitted to coagulate for 15 min at room temperature, centrifuged for 10 min at 1200 g and then the resultant supernatant serum layer was translocated into Eppendorf tubes and stored at -80°C for later biochemical analyses. Serum urea and creatinine were measured colorimetrically (Helmy et al., 2015a; Helmy et al., 2015c).

2.7. Immunohistochemistry

The glomerular and tubular protein expressions of NF- κ B and iNOS were examined using immunohistochemistry technique delineated in our previously published studies (Helmy et al., 2015a; Helmy et al., 2015b). Shortly, paraffinized kidney sections (4 μm in thickness) were placed on positively charged slides (Thermo Scientific[®], Berlin, Germany), and then in xylene for deparaffinization purpose. Afterwards, gradual rehydration was carried out by immersing slides in ethanol (100, 95, and 70%) and phosphate buffered saline (PBS). Antigen retrieval was accomplished by placing slides in citrate buffer (pH 6, Thermo Scientific) which was incubated in a microwave at power 100 and 30 for 1 and 9 min, respectively. Following epitope recovery process, sections were incubated 10 min with hydrogen peroxide (3%) to quench endogenous peroxidases, overnight with polyclonal antibody for NF- κ B p65, or iNOS (1:200 dilutions, Bioss Inc, USA), and 30 min with HRP-secondary antibody (EnVision[™] FLEX, Dako Agilent, CA, USA). The chromogen 3,3'-diaminobenzidine and hematoxylin were employed for protein visualization and counterstaining, respectively. Slides were then dehydrated with ethanol (95 and 100%) and xylene. Images of glomeruli and tubules were captured by computer-assisted microscopy (Optika[®] Microscopes, Italy) in which the protein expression signals were quantified using Fiji ImageJ software (Schindelin et al., 2012) (version 1.51w, National Institutes of Health, Bethesda, Maryland, USA).

Each image was analyzed for area fraction which represents the percentage of image area with positive staining.

2.8. Statistical analysis

All experimental values are expressed as means \pm SD. The vasodilatory responses of acetylcholine and NECA were expressed as the percentage from precontraction induced by phenylephrine. Alternatively, the cumulative vasodilatory effects of acetylcholine and NECA were indicated by calculating the area under the curve (AUC) for individual experiments using trapezoidal integration and zero line as the baseline (Gohar et al., 2014; Hammoud et al., 2017). Statistical significance was tested by conducting the repeated measures analysis of variance (ANOVA) test followed by Tukey's post hoc with the level of significance set at $P < 0.05$. Statistical analysis of the mortality data was performed with the Chi-square test. The Pearson correlation coefficient was used to measure the linear correlation between the hypotensive response and associated increases in renal NF- κ B expression in the LPS (6 hr)-treated male rats. All of the statistical tests and calculations in the current study were performed using Graph pad prism software, version. 6.01.

3. Results

In the rat isolated perfused kidney experiments, no statistically significant differences were observed in the average basal renal perfusion pressure (~ 100 mmHg) in various experimental groups under a constant flow rate of 5 ml/min.

3.1. Time and sex-related hemodynamic effects of single and two-day LPS models

Fig. 2 illustrates the impact of single (5 mg/kg) or 2-day (5 mg/kg/day) LPS injection or an equal volume of its vehicle saline on tail cuff measurement of SBP and HR in male and female rats at different time intervals. Significant decreases and increases in SBP and HR, respectively, were observed in male rats 6 hrs following LPS administration. Such hypotensive and tachycardic effects caused

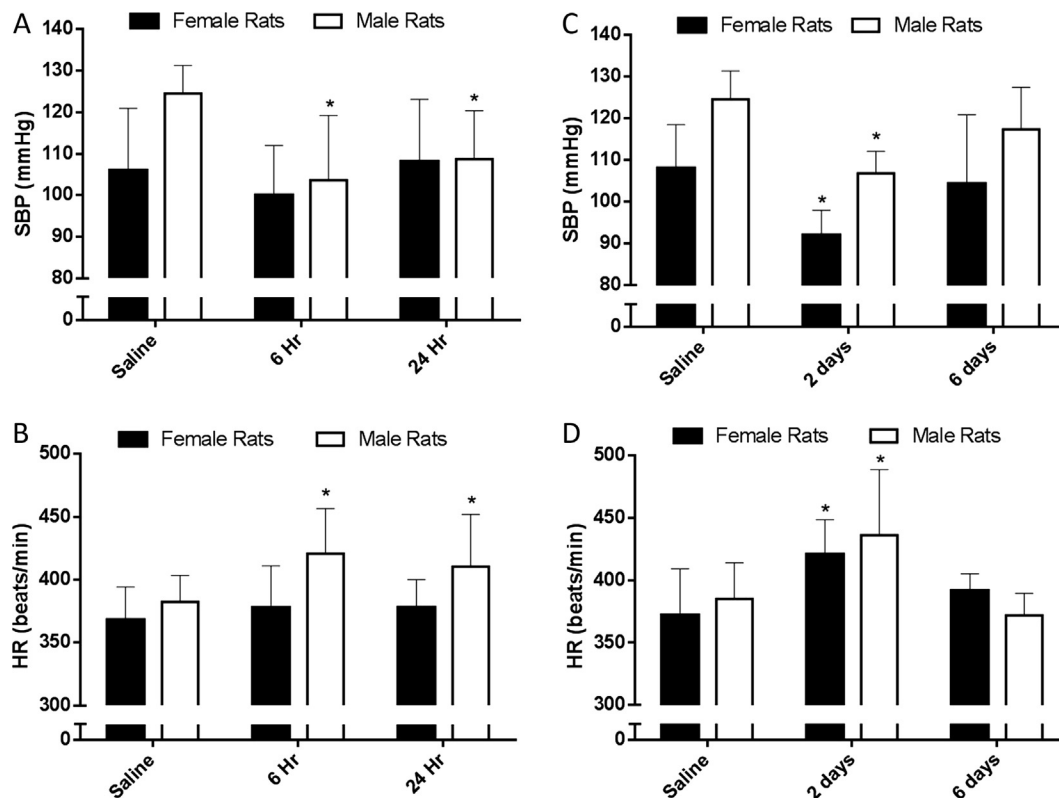


Fig. 2. Effect of single (5 mg/kg, 6 and 24 hr, panels A and B) or double (5 mg/kg/day for 2 days, 2 and 6 days, panels C and D) LPS on tail-cuff measurements of SBP and HR in male and female rats. Values are means \pm SD. of 6–8 observations. The one-way ANOVA followed by the Tukey's post hoc was employed to measure statistical significance. * $P < 0.05$ vs. saline values in the same rat sex. Abbreviations: HR, heart rate; LPS, lipopolysaccharide; SBP, systolic blood pressure.

by single LPS dosing were still manifested when measured 24 hrs later. Unlike male rats, the same LPS regimen elicited no hemodynamic alterations at 6 or 24 hrs in female rats (Fig. 2A and B). By contrast, the 2-day regimen of LPS (5 mg/kg/day) caused significant falls in SBP and rises in HR in rats of either sex. Complete hemodynamic recovery in both rat sexes, nonetheless, was observed 6 days following the 2-day LPS regimen (Fig. 2C and D).

Alternatively, six-day SBP measurements in male rats that received a single 5 mg/kg dose of LPS showed significantly lower SBP on day 2 (112 ± 3 mmHg), but not day 6 (121 ± 3 mmHg), compared with control values (120 ± 3 mmHg).

3.2. Time and sex-related renal effects of single LPS injection

Fig. 3 demonstrates the effect of single LPS dosing (5 mg/kg) on renal vasodilations and biochemical and molecular markers of kidney function and inflammation. The biochemical studies carried out at 6 hr after single LPS challenge revealed significant and sex unrelated elevations in serum urea and creatinine (Fig. 3A and B). Despite the failure of 6-hr LPS exposure to affect renal vasodilatory responses to cumulative bolus injections of ACh (0.01–7.29 nmol) and AUCs of ACh dose-vasodilatory response curves (Fig. 3C and D), the cumulative vasodilatory responses as well as AUCs of dose response curves of NECA were significantly attenuated (Fig. 3E and F). Alternatively, the immunohistochemical analysis showed substantial increases in glomerular and tubular protein expressions of NF κ B (Fig. 3G, supplementary Fig. 1) and iNOS (Fig. 3H, supplementary Fig. 2) in renal tissues harvested from male and female rats sacrificed 6 hr after LPS challenge. However, compared with respective female expression values, the LPS

evoked elevations of renal expression of inflammatory markers (NF κ B and iNOS) were significantly higher by almost twofold in male tissues. The linear correlation analysis performed by the Pearson correlation coefficient revealed a significant statistical correlation between the hypotensive response and the elevations in renal NF- κ B expression in male LPS 6-hr group (correlation coefficient of 0.83, p -value of 0.028). Remarkably, all of the abovementioned LPS acute renal manifestations receded in rats of both sexes when investigated 24 hrs after LPS exposure (Fig. 3A–H).

Notably, under the same experimental settings, serum creatinine (0.3 ± 0.01 , 0.27 ± 0.2 , 0.3 ± 0.01 mg/dl, respectively) or urea (25 ± 1 , 27 ± 2 , 29 ± 2 mg/dl, respectively) were not statistically different on days 2 and 6 of single LPS dosing compared with control values.

3.3. Time and sex-related renal effects of two-dose LPS administration

The effects of two-day LPS administration (5 mg/kg/day) on renal injury, inflammation and vasodilator reactivity are shown in Fig. 4. Although the 2-day LPS regimen failed to affect serum urea and creatinine (Fig. 4A and B), it resulted in significant enhancement of renal responsiveness to ACh and NECA (Fig. 4C–F). The LPS related facilitation of renal vasodilator capacity was accompanied by increases in renal expression of NF κ B and iNOS. Unlike the acute effects of single LPS challenge on renal protein expression which were limited to male population, the enhancing effect of 2-day LPS administration was equally demonstrated in rats of the two sexes (Fig. 4G and H, supplementary Figs. 1 and 2). Furthermore, none of the above renal parameters were different

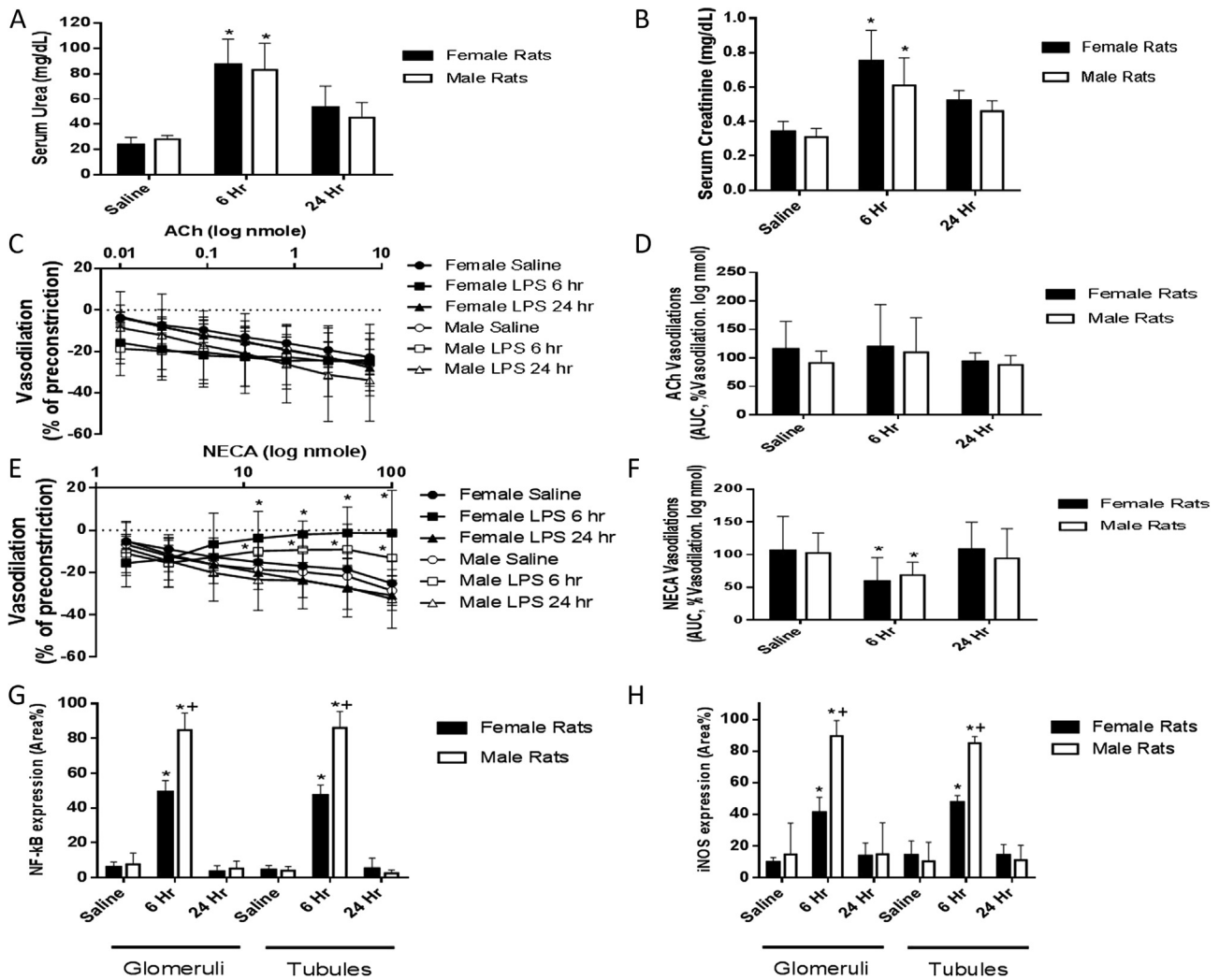


Fig. 3. Effects of single-dose LPS (5 mg/kg) or saline on (A) serum urea, (B) serum creatinine, (C) cumulative vasodilatory effects of ACh in phenylephrine-precontracted isolated perfused kidneys, (D) AUCs of the ACh dose-vasodilatory response curves, (E) cumulative vasodilatory effects of NECA in phenylephrine-precontracted isolated perfused kidneys, (F) AUCs of the NECA vasodilatory dose response curves, (G) immunohistochemical NF- κ B protein expressions in renal cortical glomeruli and medullary tubules and (H) immunohistochemical iNOS protein expressions in renal cortical glomeruli and medullary tubules. These parameters are measured 6 and 24 hr post LPS administration in male and female rats. Values are means \pm SD. of 6–8 observations. The one-way ANOVA (panels A, B, D, F, G and H) or repeated measures ANOVA (panels C and E) followed by the Tukey's post hoc was employed to measure statistical significance. * $P < 0.05$ vs. saline values in the same rat sex. + $P < 0.05$ vs. female LPS 6-hr. Abbreviations: ACh, acetylcholine; AUC, area under the curve; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NECA, N-ethylcarboxamidoadenosine; NF- κ B, nuclear factor-kappa b.

from control values when measured 6 days after the 2-day LPS exposure in both male and female rats (Fig. 4A–H).

3.4. Time and sex-related survivability effects of single and two-day LPS models

The mortality rate among endotoxic rats at different time points following single or 2-day LPS challenge is depicted in Fig. 5. The LPS administration raised the mortality rate significantly when compared with the saline group, which displayed 0% mortality. Interestingly, the rat mortality was considerably increased following second LPS dose compared with mortality after single LPS injection. Of note, all of the mortality events after single LPS injection took place within first 6 hr as the mortality rate did not differ between 6 and 24 hr. The same observation can be made on second LPS injection of 2-day regimen as mortality rate at 2 and 6 days were equal. The male sex appears to be more susceptible to endotoxemia related lethal events as the mortality rate in males was almost double that of female population at all timepoints (Fig. 5).

4. Discussion

In previous studies on hemodynamic and renal consequences of endotoxemia, measures have been made at single time points or repeatedly within few hours of the endotoxic insult (Farmer et al., 2003; Gholamnezhad & Fatehi Hassanabad, 2018; Martin et al., 1993; Pastor, 1999; Peters & Lewis, 1996; Piepot et al., 2000; Piepot et al., 2003; Vaschetto et al., 2010; Waller et al., 1994; Yamaguchi et al., 2006). Amazingly, no attempts have been made to systematically assess hemodynamic and renal effects of endotoxemia in the same animal model for longer durations (days) and how they could be influenced by the animal sex. These issues were investigated in the present study, which showed that 6-hr LPS exposure caused hypotension, tachycardia, impaired renal adenosinergic vasodilation along with rises in rat mortality and levels of renal injury and inflammation markers. These manifestations mostly disappeared one day after LPS dosing. Despite the similarity in hemodynamic and lethal profile of 6-hr and 2-day regimens, the renal profile was intriguingly different since the 2-day endotoxin

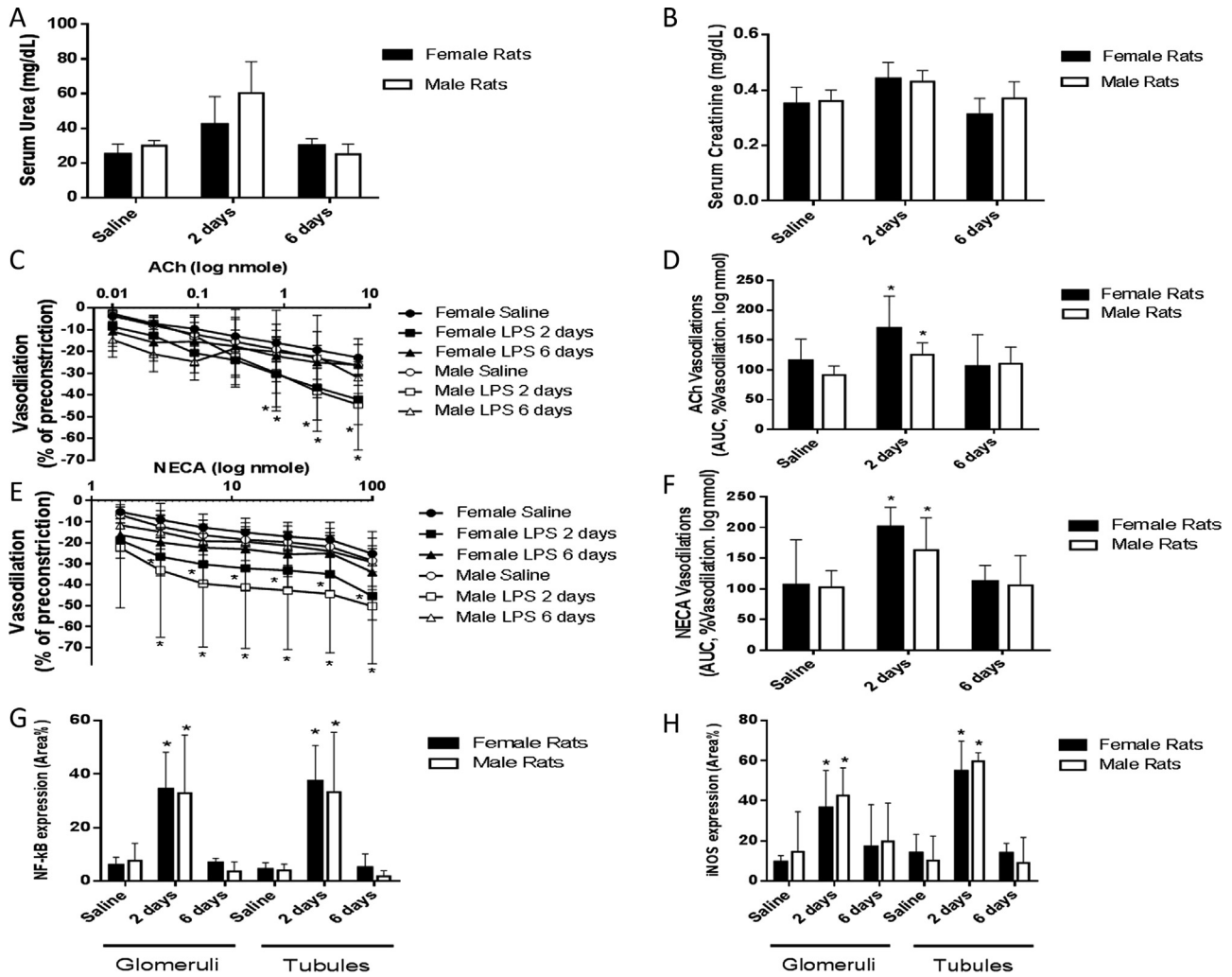


Fig. 4. Effects of two-dose LPS (5 mg/kg/day for 2 days) or saline on (A) serum urea, (B) serum creatinine, (C) cumulative vasodilatory effects of ACh in phenylephrine-precontracted isolated perfused kidneys, (D) AUCs of the ACh vasodilatory response curves, (E) cumulative vasodilatory effects of NECA in phenylephrine-precontracted isolated perfused kidneys, (F) AUCs of the NECA vasodilatory dose response curves, (G) immunohistochemical NF-κB protein expressions in renal cortical glomeruli and medullary tubules and (H) immunohistochemical iNOS protein expressions in renal cortical glomeruli and medullary tubules. These parameters are measured 2 and 6 days post LPS administration in male and female rats. Values are means ± SD, of 6–8 observations. The one-way ANOVA (panels A, B, D, F, G and H) or repeated measures ANOVA (panels C and E) followed by the Tukey's post hoc was employed to measure statistical significance. *P < 0.05 vs. saline values in the same rat sex. Abbreviations: ACh, acetylcholine; AUC, area under the curve; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NECA, N-ethylcarboxamidoadenosine; NF-κB, nuclear factor-kappa b.

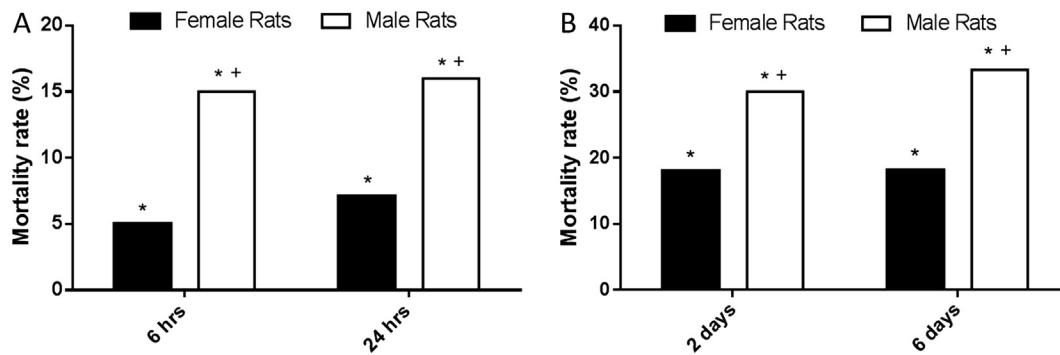


Fig. 5. Effect of single (5 mg/kg, panel A) or two-dose (5 mg/kg/day for 2 days, panel B) LPS on the mortality rate percentage in male and female rats. The mortality rate among saline-treated male or female rats was 0%. The variation in mortality rate was analyzed using chi-square test. *P < 0.05 vs. saline value (zero mortality) in the same rat sex. + P < 0.05 vs. female value at same time interval. Abbreviations: LPS, lipopolysaccharide.

exposure (i) failed to alter biomarker of renal function, (ii) facilitated cholinergic and adenosinergic renal relaxation, and (iii) induced a lesser increase in renal expression of inflammatory

mediators. By 6 days post-LPS administration in the one- or two-dose LPS models, the measured hemodynamic and renal parameters had returned to normal levels. The mortality rate, hemody-

dynamic aberrations at 6 and 24-hr intervals and renal inflammatory derangements at 6 hr were more evident in the male population.

After 6 hr of LPS exposure, rats exhibited notable signs of renal dysfunction such as elevated serum levels of urea and creatinine and reduced renovascular reactivity to NECA. These endotoxic consequences were sex independent as they were equally demonstrated in male and female rats. To the contrary, only male rats showed greater vulnerability to hypotension, tachycardia and death associated with 6-hr endotoxemia. Such sexual dimorphism in the hemodynamic and lethal complications was similarly demonstrated in other animal models of endotoxemia (El-Lakany et al., 2018; Zellweger et al., 1997). Molecularly, immunohistochemical measurements revealed more powerful inflammatory responses in endotoxic male rats as suggested by the significantly greater increases in tubular and glomerular protein expression of NF- κ B and iNOS compared with female rats. These observations, which are in line with previous reports (Angele et al., 2014; El-Lakany et al., 2018), might indicate that the increased susceptibility of male rodents to acute endotoxic mortality and hypotension positively correlate to, and possibly induced by, the heightened inflammatory response to endotoxemia. Additionally, it has been proposed that the female protection from trauma induced decline in vascular responsiveness is closely related to estrogen upregulation of Rho kinase and protein kinase C pathway (Li et al., 2014). Recently, data from our work on renovascular contractility during endotoxemia implicate the heme oxygenase-1 (HO-1) as a possible contributing factor to the female resistance against renovascular actions of LPS (Wedn et al., 2019c).

Our finding of preserved renal responsiveness to ACh appears to be at odds with published studies (Gholamnezhad & Fatehi Hassanabad, 2018; Pastor, 1999; Peters & Lewis, 1996; Piepot et al., 2000; Piepot et al., 2003; Vaschetto et al., 2010; Waller et al., 1994), which reported impaired cholinergic vasorelaxation in endotoxic renal vasculature. Such controversy might presumably be attributed to differences in the time and duration of endotoxic challenge. Remarkably, most of these studies measured renal responses to ACh within 3 hr of endotoxemia while our observations started after 6 hr. Exceptions are present in few studies which showed decreased cholinergic responses after 5–6 hr; however these studies employed more aggressive endotoxic insult including long infusion periods (Pastor, 1999) or large doses of LPS (Gholamnezhad & Fatehi Hassanabad, 2018). Thus, it seems that endotoxin suppression of renal ACh responsiveness is manifested only during early hours of LPS exposure after which normal renal cholinergic responsiveness tends to be restored. This assumption receives support from the observation that ACh reactivity is diminished after 1–2 hr of LPS injection and regained most of its relaxing action after 3–6 hr (Peters & Lewis, 1996; Waller et al., 1994). Moreover, others have shown complete normalization of endotoxic renal cholinergic relaxation within 6–24 hr (Martin et al., 1993; Waller et al., 1994). Unlike ACh, the adenosinergic relaxation was shown to be diminished very early (at 1.5 hr) and later (after 6 hr) following non-aggressive LPS regimens (Jolly et al., 2008). This is consistent with our data that renovascular reactivity to NECA was suppressed 6 hr after LPS challenge. Therefore, it is likely that LPS related impairment of renal cholinergic responses tend to have faster recovery adenosinergic responses. Given the differences in underlying machinery of the two vasodilators, with the vasorelaxant action of NECA being dependent on both endothelial (Rekik et al., 2002; Teng et al., 2005) and non-endothelial (Prentice & Hourani, 1996) factors, our data imply a more powerful downregulatory effect for LPS on renal adenosinergic signaling.

Aside from hypotension, all of the abovementioned molecular and functional renal manifestations caused by 6-hr endotoxemia in male rats appear to be transient as they dissipated after 24 hr. Consistent with this view, full recovery of renal disturbances in

time periods ranging from 16 to 24 hr after endotoxin administration was shown by previous authors (Sade et al., 1999; Waller et al., 1994). However, reports of persistent renal perturbations after 24 hr from LPS injection exist in the literature (Tan et al., 2019; Xin et al., 2016). Notably, these studies often employ more aggressive LPS dosing regimens. Since most of the adverse cardiovascular effects of LPS receded after 24 hr, we then investigated the renal and hemodynamic consequences of supplementation with a second LPS injection 24 hr after first one. While serum urea and creatinine were unaltered, renovascular reactivity to ACh and NECA, contrary to the 6-hr data, was enhanced by 2-day LPS treatment. Although renal expression of inflammatory mediators in male rats was elevated in 2-day measurement, the magnitude of elevation was profoundly less compared with the 6-hr values. In a similar paradigm, Holcombe et al. (Holcombe et al., 2016) showed weakened inflammatory responses to LPS when given as second injection to endotoxic horses. Others have showed that preconditioning animals with endotoxin protected from subsequent renal damage associated with LPS or renal ischemia-reperfusion injury (Dai et al., 2016; He et al., 2018; Kaucsár et al., 2014). The LPS ability to evoke heat shock defensive responses, including upregulation of heat shock protein 70 (HSP70) (Kaucsár et al., 2014; Li et al., 2017), HSP90 (Kaucsár et al., 2014) and HO-1 (Huang et al., 2008; Lee et al., 2014), plays a major role in resistance to inflammatory insults. Therefore, it is tempting to speculate that the first LPS exposure initiated a number of defensive and counter-regulatory mechanisms, which contributed to renal recovery and downregulation of renal injury and inflammation induced by subsequent insults.

The contradictory time-related findings of reduced (6 hr), maintained (1 day), or enhanced (2 days) renal vasodilator capacity in endotoxic rats deserve a comment. As mentioned earlier, the decline in renal vasodilatory responsiveness is believed to take place during early hours of LPS exposure (Peters & Lewis, 1996; Yamaguchi et al., 2006). Alternatively, other studies have indicated a tendency for renal vasoconstriction and increased renal vascular resistance during early stages of endotoxemia (Badr, 1992; Boffa & Arendshorst, 2005; Bougle & Duranteau, 2011; Schrier & Wang, 2004a). The delayed facilitation of renal vasodilatory response might act adaptively to counterbalance the enhanced renal contractility and maintain renovascular homeostasis (Yamaguchi et al., 2006). Others have described progressive and time-dependent increases in renal vascular conductance after LPS infusion (Gardiner et al., 1995, 1996). It could be speculated, therefore, that the abovementioned machinery may be the underlying factor that contributed to normalizing renal vasodilator profile after 24 hr and its further enhancement after second LPS injection in this study.

It is notable, however, that the presumed tolerability to repeated exposures to endotoxic insults might not be visualized with all biological functions. For instance, unlike renal data, we found that the first exposure to LPS did not diminish hemodynamic and lethal outcomes triggered by subsequent LPS dosing. Indeed, the hemodynamic and mortality findings of 48-hr study in male rats mimicked those produced 6 hr after the first exposure to LPS. Additionally, the resistance of female rats to hemodynamic manifestations of endotoxemia observed during the first 24 hr of single LPS exposure, was no longer manifested in rats treated with LPS for two consecutive doses. In the latter instance, the hallmarks endotoxic manifestations of hypotension and tachycardia were clearly featured in female rats. In this regard, it has been reported that sexual dimorphism of endotoxic cardiovascular dysfunction observed with low LPS doses (3 mg/kg) was abolished by more severe endotoxic insult elicited by a 3-fold higher dose of LPS (Chen et al., 2014). Thus, results of the current and previous studies ascertain that the female protection from acute hemodynamic and car-

diac LPS consequences appears to recede following more aggressive or prolonged LPS exposure.

It is imperative also to comment on two issues related to the current study. First, despite its convenience and simplicity, the rodent LPS model of endotoxemia used in this study is not a perfect model of human sepsis or bacterial infection given the fact that LPS is just a single component of the complex pathogen associated molecular patterns generated and released by gram negative organisms (Poli-de-Figueiredo et al., 2008). Having said that, it should also be remembered that the rodent endotoxemia reproduces several features of humans sepsis such as kidney injury, hypotension, impaired left ventricular function and cardiac sympathovagal imbalances, with probably some differences in kinetic and magnitude profiles (Angele et al., 2014; Fink, 2014; Goncalves et al., 2014; Sallam et al., 2018; Schrier & Wang, 2004b). It should also be noted that the clinical relevance of repeated LPS exposure, which gives rise to a condition known as endotoxin tolerance, has been shown recently in patients with sepsis (Pena et al., 2014). Sepsis is not only characterized by initial phase of hyper-inflammation but also by later stages of immune dysfunction or immunosuppression (Hotchkiss et al., 2013). Many authors suggested a role for endotoxin tolerance in the later stages of sepsis since some resemblance in cytokines production features exist between cells isolated from later stage septic patients and in vitro LPS tolerance models (Cavaillon et al., 2005; Otto et al., 2011; Schefold et al., 2008). Furthermore, recent findings support critical role for endotoxin tolerance throughout the clinical course of sepsis and its relation to disease severity (Pena et al., 2014). Second, the molecular entities investigated in the current study (NF- κ B and iNOS) were chosen due to their pivotal contribution to the pathophysiology of endotoxemia. On the one hand, NF- κ B represents a prototypical proinflammatory cytokine whose activation causes downstream upregulation of several other inflammatory genes like those encoding for TNF α , IL-1 β , and IL-6 (Alvarez et al., 2016; Sallam et al., 2016). On the other hand, the iNOS-derived NO is a secondary and probably a final effector of the endotoxic response that is blamed for the endotoxemia-induced fall in BP, vascular hyporeactivity, multiple organ failure and high mortality rate (Cinelli et al., 2019).

Overall, our data demonstrate variable and directionally opposite effects for single or double LPS dosing on hemodynamic, renal, inflammatory and survivability profiles in rats. Along the same context, female rats appear to be less vulnerable to the endotoxic insults compared with the male population.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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