SEX DIFFERENCES IN OXIDATIVE STRESS AND INFLAMMATION RESPONSES DURING AND AFTER SIMULATED HEMORRHAGE

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ABSTRACT

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Hemorrhage (i.e., massive blood loss) induces an oxidative stress and inflammatory response that can persist even following hemostasis and resuscitation. Premenopausal females exhibit a survival advantage following hemorrhage compared to young males. In this study, we hypothesized that young males would elicit a greater oxidative stress and inflammatory response compared to young females, both during and after a simulated hemorrhage via lower body negative pressure (LBNP). Young, healthy human subjects (10F; 10M) participated in a stepwise-LBNP protocol to presyncope. Venous blood samples were collected at baseline, presyncope, and 1-h into recovery (i.e., following "resuscitation"). The oxidative stress response was assessed via circulating F_2-isoprostanes (F_2-IsoP) using gas chromatography-negative ion chemical ionization-mass spectrometry. The inflammatory response was assessed via circulating tissue necrosis factor-α (TNF-α), C-Reactive Protein (CRP), thymus and activation-regulated chemokine (TARC), and interleukin (IL)-5, IL-6, IL-7, and IL-10, using a MSD® Multiplex assay. LBNP tolerance time was similar between male and female subjects (Males, 1592±124 s vs. Females, 1437 ± 113 s; $P = 0.37$). There was no effect of time or sex on the absolute or relative change in F_2-IsoP during or after LBNP ($P \geq 0.12$). However, male subjects exhibited a greater pro-and anti-inflammatory response during and after LBNP compared to female subjects (Notable markers at 1-h recovery compared to baseline, IL-6: Males, 101.4 ± 138.9% vs. Females, 12.3 ± 34.0%, $P = 0.06$; IL-10: Males, 71.1 ± 133.3% vs. Females, -2.2 ± 11.8%; $P = 0.06$). These data suggest that there may be a potential sex difference in the inflammatory response to simulated hemorrhage.

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I dedicate this practicum report to my wonderful family in honor of their unconditional support of my pursuit to earn this degree. Without their tough yet loving advice, selflessness in providing countless amounts of free meals and coffee, and excitement with each academic milestone, I would not be following this life-long dream. You are my greatest motivation.

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CHAPTER I: BACKGROUND & LITERATURE REVIEW

Despite advances in therapeutic interventions, major blood loss (i.e., hemorrhage) remains one of the leading causes of potentially-survivable death, accounting for 90% of the deaths that occur in the combat casualty care setting, and up to one-third of the deaths that occur in the civilian trauma setting globally (19, 26, 27, 70). Severe loss of blood volume and reduced oxygen carrying capacity can lead to tissue ischemia and hypoxia, which is particularly concerning if these effects impact the vital organs. This hypoxic state and subsequent reperfusion (due to fluid resuscitation, for example) can lead to reactive oxygen species (ROS) production and an upregulation of the systemic inflammatory response (5, 12, 14, 30, 57, 66, 68, 75, 79, 80, 88). This acute increase in ROS can exhaust innate antioxidant mechanisms, resulting in cell damage via lipid peroxidation (52, 60), reduced mitochondrial function (61), microcirculation impairment (41, 71) and ultimately, multiple organ dysfunction (MODS) (10, 18, 35, 43, 50).

Prior clinical studies demonstrate that acute systemic oxidative stress and inflammatory responses after hemorrhage and resuscitation can persist from 2 to 24 hours after the initial traumatic injury (43, 52). Interestingly, this response is associated with an increased risk of MODS after traumatic injury and treatment (43, 52). For example, Jastrow *et al.* demonstrate that the inflammatory markers, tissue necrosis factor α (TNF-α), interleukin (IL)-6, and IL-10, are associated with MODS (43). It has been shown that TNF-α can activate nuclear factor kappa B (NF-κB), induce ROS formation in mitochondria within minutes (30, 36), and consequently, create a positive feedback loop in the oxidative stress and inflammation responses that can persist for some time following the initial stimulus (6, 8). As such, it is necessary to address the increases in

both oxidative stress and inflammation when developing treatment strategies following hemorrhage.

Very few studies have investigated oxidative stress and inflammatory responses immediately following hemorrhage, and prior to initiation of resuscitation. In an animal model of trauma and hemorrhage, Tsai *et al.* bled rats to a mean arterial pressure of 30-40 mmHg, maintained this pressure for 30-min, and then performed fluid resuscitation with lactated Ringer's or shed blood (74). A sham group underwent surgical preparation but were not hemorrhaged nor resuscitated. Liver, lung, heart, and spleen tissues were collected following hemorrhage or following hemorrhage plus resuscitation, and oxidative stress (via the ratio of oxidized/reduced glutathione) and inflammation (via measurement of NF-κB DNA binding activity) were assessed. Following hemorrhage only, oxidative stress was not elevated compared to the sham group in the lung and spleen, but was elevated following resuscitation with lactated Ringer's and/or shed blood. Inflammation increased with hemorrhage compared with the sham condition in all tissues, and this response persisted with Lactated Ringer's and/or shed blood in the lung, spleen, and heart. These data suggest that tissue reperfusion may induce an augmented oxidative stress and inflammatory response following hemorrhage and resuscitation.

These data demonstrate the paradoxical effect of fluid resuscitation in increasing oxidative stress and inflammation, and sustained tissue ischemia followed by tissue reperfusion can cause mitochondrial uncoupling and cell death (47). Additionally, restoring blood flow to ischemic tissues can result in systemic circulation of locally-produced ROS, inducing an amplified oxidative stress response throughout the body (3). Numerous animal studies of hemorrhage have been used to examine how therapies with antioxidant properties, such as pyruvate and cytochrome c, can impact oxidative stress and inflammatory responses following fluid resuscitation (29, 60). These

therapies suppressed ROS formation and attenuated the systemic inflammatory response to hemorrhage and resuscitation (29, 60). Furthermore, Flaherty *et al*. demonstrated that pyruvate-fortified Ringer's solution stabilized mean arterial pressure more so than lactated Ringer's during the recovery period from hemorrhage and fluid resuscitation, which may have been associated with beneficial cardiac and vascular effects of the anti- oxidant and anti-inflammatory properties of the pyruvate solution (29). Again, these data demonstrate the interdependent relationship between oxidative stress, inflammation, and survival from hemorrhagic injury.

In the experimental setting, hemorrhage can be non-invasively simulated in healthy, conscious humans via the lower body negative pressure (LBNP) technique. By applying negative pressure to the pelvis and lower limbs, LBNP translocates blood from the upper body into the lower body, causing reduced venous return, cardiac output, arterial pressure, and cerebral blood velocity (22, 38, 44, 63). This central hypovolemia elicits compensatory hemodynamic responses similar to actual blood loss, including increased heart rate and peripheral resistance (22, 38, 44, 63). LBNP is a well-validated model of the early stages of hemorrhage, as demonstrated in two recent studies. In a baboon model, Hinojosa-Laborde *et al*. progressively removed 25% of total blood volume in 6.25% aliquots. Four weeks later, the same animals were subjected to a progressive LBNP protocol, where chamber pressures were reduced to elicit the same central venous pressure responses to the hemorrhage protocol. Systolic arterial pressure, heart rate, stroke volume, cardiac output, and vascular resistance responses were similar in both interventions (38). From this study, the authors estimated that a LBNP stimulus of approximately -70 mmHg is equivalent to a blood loss of 17.8 ml/kg (i.e. 1.25 l in a 70 kg subject) (38). Similarly, Johnson *et al*. examined cardiovascular responses to graded blood loss (up to 1,000 ml) and progressively decreasing LBNP (to -45 mmHg) in human subjects during a single experimental session. The

trajectory of central venous pressure, heart rate, mean arterial pressure, and stroke volume were similar between LBNP and actual hemorrhage (44).

Our laboratory routinely utilizes a presyncopal-limited LBNP protocol where we progressively decrease the chamber pressure in a stepwise fashion until the subject reaches presyncope, as defined by a systolic arterial pressure of <80 mmHg, acute bradycardia, or onset of presyncopal symptoms such as lightheadedness, visual disturbances, sweating, or nausea. With progressively decreasing LBNP, and subsequent central hypovolemia and hypotension, peripheral resistance will increase to protect blood flow to the vital organs (21, 67). The resulting peripheral tissue ischemia and reduced oxygen delivery may be associated with a systemic oxidative stress response, as recently demonstrated by our laboratory, where circulating F_2-Isoprostanes (a systemic marker of oxidative stress) increased by ~28% at the termination of presyncopal-limited LBNP, coincident with a ~9% reduction in peripheral tissue oxygenation (as indexed by forearm oxygen saturation) (59). Additionally, LBNP has been shown to elicit an immune response through an increase in white blood cells (77).

The recovery from LBNP can also be utilized as a model of fluid resuscitation following hemorrhage. Once pressure within the LBNP chamber is released to atmospheric pressure, venous return is restored, quickly reperfusing ischemic tissues and potentially eliciting an oxidative stress and inflammatory response. In our previous study (59), neither inflammation nor oxidative stress markers were measured during recovery from the simulated hemorrhage stimulus of LBNP. Accordingly, in the current study we explore if these responses occur during fluid resuscitation after a simulated hemorrhage, as previously demonstrated with fluid resuscitation after actual blood loss (43, 66, 79).

There is a large body of evidence suggesting that young females have lower tolerance to the central hypovolemia induced via LBNP compared with young males (20, 37, 55, 81). There are a number of potential underlying mechanisms that may account for this difference in tolerance. For example, Convertino observed that males elicited a greater sympathetic response (indexed by higher circulating plasma norepinephrine) to presyncopal LBNP than females (20), which might account for their higher stroke volume, cardiac output, and mean arterial pressure at presyncope compared to females (20). Interestingly, however, females exhibited an augmented heart rate response to LBNP compared to males, despite lower circulating norepinephrine (20). After accounting for body surface area in male and female subjects, Fu *et al.* also observed lower stroke volume and increased heart rate in females compared to males, despite similar concentrations of circulating norepinephrine (33). Both studies also reported similar increases in systemic vascular resistance between male and female subjects, leading Fu *et al.* to conclude that the lower tolerance to LBNP observed in females was likely due to reduced cardiac filling rather than reduced systemic vascular resistance (33). Despite extensive investigations elucidating the sex differences in cardiovascular responses during LBNP, it remains unknown if sex hormones influence the oxidative stress and inflammatory responses to this stress. Preliminary analysis of our previously published data (59) suggest that males exhibited a greater oxidative stress response at the termination of LBNP compared to female subjects (Males: 37.0 ± 15.4%; Females: 5.0 ± 10.1%; P = 0.11); however, this analysis was inconclusive due to the small sample size (N = 15) and skewed sex distribution (Males = 11; Females = 4).

Following severe blunt trauma and hemorrhage in the clinical setting, premenopausal women demonstrate attenuated systemic inflammation and decreased mortality compared to males of the same age with similar traumatic injuries (mechanism, severity, and pattern) (see **Figure 1**)

(9, 32, 34, 84). Additionally, young males were more likely to experience more severe episodes of MODS and longer recovery times than premenopausal females (51). This survival advantage is likely due, at least in part, to higher systemic 17β-estradiol in females compared to males (9, 86). This mechanism of protection is supported by numerous studies using rodent and porcine hemorrhagic shock models where male animals exhibited suppressed systemic inflammation (72), improved myocardial function (2, 87), and attenuated reductions in mean arterial pressure after treatment with exogenous estradiol (40, 49, 53). These benefits are related to the localization of estrogen receptors (both ER-α and ER-β) on mitochondria in multiple tissues (including myocardial tissue) which may protect against ATP depletion and ROS generation (78, 85). Hsieh *et al.* demonstrated that this estrogen receptor localization is critical for the restoration of cardiac function following hemorrhage and resuscitation, as binding of ER-β upregulates mitochondrial respiratory function and consequently decreases ROS production (39). Additionally, circulating estradiol increases endothelium-independent vasodilation in the coronary arteries, potentially facilitating increased blood flow and oxygen delivery to the heart in female pigs during hemorrhage (82). As such, it is important to investigate the mechanisms that underpin the survival advantage in premenopausal females following acute hemorrhage, as this knowledge may facilitate development of sex-specific therapies to treat these injuries.

Therefore, this research study has been designed to addresses the following three questions:

1. Does simulated hemorrhage via application of LBNP in healthy human subjects elicit an inflammation response?
2. Do the oxidative stress and inflammation responses anticipated during this simulated hemorrhage persist into recovery (i.e., during “resuscitation” from blood loss)?

3. Is there a sex difference in the oxidative stress and inflammatory responses during and after simulated hemorrhage (via application and release of LBNP)?

Our **central hypothesis** is that young males (compared with young premenopausal females) will exhibit a greater oxidative stress and inflammatory response throughout simulated hemorrhage and resuscitation, via application of LBNP and during recovery from this stress.

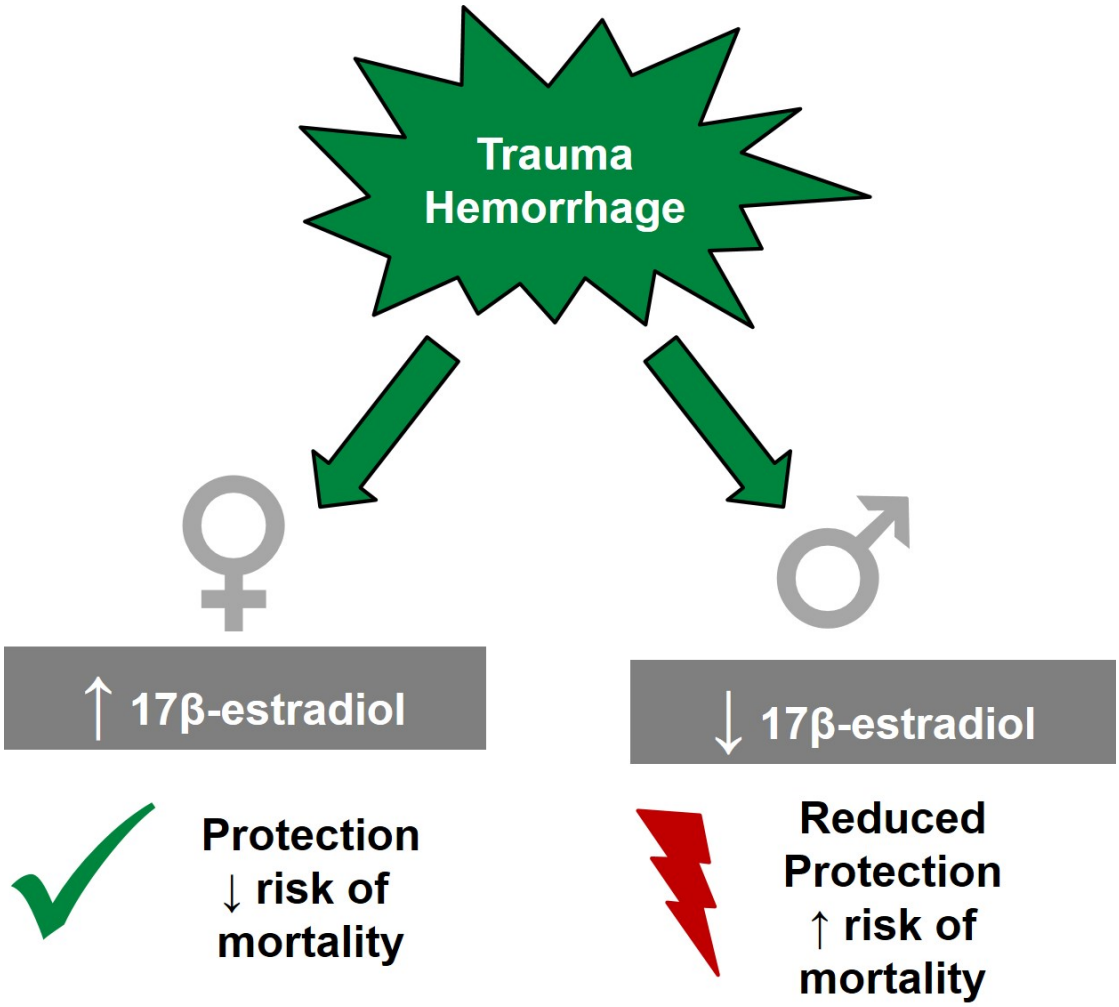

Figure 1. Increased 17β-estradiol in females may decrease mortality risk after traumatic hemorrhage.

CHAPTER II: RESEARCH PROJECT

Specific Aims

Specific Aim 1*:* Examine sex differences in oxidative stress and inflammation responses during simulated hemorrhage in humans. **Hypothesis:** Male subjects will exhibit a greater oxidative stress and inflammatory response during progressive LBNP to presyncope compared with female subjects.

Specific Aim 2*:* Examine sex differences in oxidative stress and inflammation responses during recovery from simulated hemorrhage in humans. **Hypothesis:** Male subjects will exhibit a greater oxidative stress and inflammatory response during recovery from simulated hemorrhage compared with female subjects.

Significance

This proposal is **significant** because it will provide insight into whether there is a sex difference in oxidative stress and inflammation responses during and after acute tissue hypoperfusion induced via LBNP. An effect of acute hemorrhage is cell ischemia and hypoxia due to a decreased circulating blood volume and oxygen delivery. This hypoxic state and subsequent reperfusion (due to fluid resuscitation, for example) can induce an oxidative stress and inflammatory response which may lead to further damage of these tissues, and multiple organ dysfunction (32, 42, 50, 58, 66). Premenopausal female patients demonstrate attenuated systemic inflammation (32), enhanced pressor responses to pharmacological interventions (49), and decreased mortality (9) compared to their male counterparts after traumatic hemorrhage, most likely due to the elevated presence of systemic 17β-estradiol (9).

Despite exploration of this survival advantage in females, and development of interventions following acute hemorrhage (e.g. fluid resuscitation and pharmacological therapies (15)), a critical barrier remains in the assumption that male and female patients will have equivalent physiological and pathological responses to the initial blood loss injury, and subsequent treatments. In this proposal, we will assess both oxidative stress and inflammatory responses during both simulated hemorrhage (via LBNP) and throughout a 1-h recovery period, and also examine if there is a sex difference in these responses. The findings from this study may provide critical insight into the development of resuscitation treatments for both male and female patients.

Innovation

This study is **innovative** for three key reasons: 1) We utilized the novel experimental model of LBNP for assessing oxidative stress and inflammatory responses as a result of simulated hemorrhage and subsequent tissue reperfusion (upon release of the negative pressure). LBNP is a well-validated model to simulate the hemodynamic responses evoked during acute hemorrhage (38, 44), and as our laboratory has previously demonstrated, LBNP can serve as a model to induce oxidative stress responses (59). Furthermore, the use of LBNP is innovative due to the controlled nature of the central hypovolemia, and the reversibility of symptoms induced by this stimulus. Actual blood loss experiments in healthy humans imposes ethical and logistical challenges, while studying patients with traumatic hemorrhage is difficult due to the critical nature of rapid clinical interventions, with scientific investigation being a lesser priority. As such, LBNP provides an ethical and timely alternative to blood loss and tissue reperfusion experiments in human subjects; 2) Oxidative stress and inflammatory markers were assessed at multiple time points of both the simulated hemorrhage protocol and during recovery. This is a novel approach as no previous

studies have measured this comprehensive panel of markers during or after simulated hemorrhage induced by LBNP; 3) We recognize the potential impact that sex differences in oxidative stress and inflammatory responses may have on development of interventions for treatment of hemorrhagic injuries. Current resuscitation therapies can be used to suppress oxidative stress and inflammatory responses to acute hemorrhage in human patients; however, studies of these interventions often fail to address the influence of sex hormones on patient outcomes (12, 45, 56, 65, 73). The investigation of a potential sex difference in oxidative stress and inflammatory responses may further refine the implementation of these existing interventions.

Materials & Methods

Subjects. Twenty-nine healthy, young subjects were recruited to participate in this study conducted at the University of North Texas Health Science Center (UNTHSC) in Fort Worth, TX. The experimental protocol was reviewed and approved by the North Texas Regional Institutional Review Board (IRB #2018-120). Subjects participated in one familiarization session, and one experimental session. During the familiarization session, subjects were briefed on the study aims, the experimental protocol and equipment, and associated risks, and were given multiple opportunities to ask questions. Informed, written consent and a HIPAA authorization form were completed by each subject. Female subjects completed a menstrual cycle questionnaire, and a urine pregnancy test (OSOM® hCG Card Pregnancy Test, Sekisui Diagnostics, Burlington, MA, USA) to ensure they were not pregnant. All subjects completed a medical history questionnaire, a sleep quality questionnaire (Pittsburg Sleep Quality Index, (13)), and a questionnaire for assessment of obstructive sleep apnea risk (STOP-BANG, (16)). Height, weight, neck circumference, sex, and age were recorded. Seated and standing 12-lead electrocardiogram (ECG; Cardiovit AT-10 Plus,

Schiller, Baar, Switzerland) and blood pressure assessments were performed. Following these screening assessments, subjects were sealed supine in the LBNP chamber (VUV Analytics Inc. Austin, TX, USA) and exposed to a -15 mmHg LBNP stimulus for 5-min in order to familiarize them to the sensation of the experimental protocol.

At least 24 hours following the familiarization session, subjects returned to the laboratory between 7:00 AM and 9:00 AM for the experimental session. Female subjects were tested during the early follicular phase (i.e., low estrogen and progesterone phase; days 1-4) of their menstrual cycle (self-reported), or for subjects who were taking oral contraception, within the first four days of the sugar-pill phase. Subjects were asked to refrain from exercise, alcohol, caffeine, prescription and non-prescription medications, herbal medications, and dietary supplements for at least 24 hours prior to experimentation. For at least 2 hours prior to experimentation, subjects refrained from food consumption, but were asked to drink un-caffeinated fluids *ad libitum*. Upon arrival for the experimental session, subject height, weight, and age were measured and recorded. Female subjects completed another urine pregnancy test to ensure they were still not pregnant.

Instrumentation. Subjects lay supine in the LBNP chamber (VUV Analytics Inc. Austin, TX, USA) so that their iliac crest aligned with the opening of the chamber. Subjects were sealed into the chamber with heavy-duty plastic and a neoprene band around the waist. Instrumentation included the following: 1) 3-lead ECG (shielded leads, cable and amplifier, AD Instruments, Bella Vista, NSW, Australia) for continuous ECG recording; 2) a finger cuff (Finometer^{TM}, Finapres Medical Systems, Amsterdam, The Netherlands) was placed on the left middle finger for non-invasive, continuous arterial blood pressure recordings via finger photoplethysmography, and calculation of stroke volume via the pulse contour method (22); 3) near infrared spectroscopy (NIRS; OxiplexTS Model 99200, Version 3.1, ISS, Champaign, IL, USA) for continuous forearm muscle (flexor carpi

ulnaris) tissue oxygenation monitoring; and 4) a flexible antecubital venous catheter (BD Insyte Autoguard Shielded IV Catheter, Franklin Lakes, NJ, USA) for serial blood sampling. An aortic diameter measurement was made using duplex Doppler ultrasound (3SC-RS Ultrasound Probe and Vivid T8 Ultrasound, GE Healthcare, Milwaukee, WI) to improve the accuracy of Finometer-derived stroke volume estimates.

Protocol. Following instrumentation, subjects completed a 5-min baseline period. Within the last 2-min of baseline, a 10 ml blood sample was taken. The pressure in the LBNP chamber was then progressively decreased every 5-min to -15, -30, -45, -60, -70, -80, -90, and -100 mmHg (**Figure 2**), or until the onset of presyncope, defined by a systolic arterial pressure of <80 mmHg, acute bradycardia, or voluntary subject termination due to presyncopal symptoms (e.g. lightheadedness, visual disturbances, sweating, nausea). It is important to note that presyncopal symptoms usually resolved within 30-60 s after LBNP termination. 10 ml blood samples were taken within the last 2-min of each LBNP stage, and immediately following the onset of presyncope.

Following the release of LBNP, subjects remained in the LBNP chamber for a 60-min recovery period. A blood sample was taken at 5, 15, 30 and 60-min of this recovery period. At the conclusion of the 60-min blood sample retrieval, all instrumentation was removed, and the subject was assisted out of the LBNP chamber. With confirmation that all presyncopal symptoms had subsided, subjects were allowed to leave the laboratory.

Blood Sample Collection & Analysis. Whole blood samples were placed into tubes containing K2 EDTA, an anti-coagulating agent, then centrifuged at 4°C for 15-min at 2000 RPM (858.6 g RCF) for extraction of plasma (Sorvall, Legend RT Centrifuge: Round Buckets, Kendro Laboratory Products, Germany). All plasma samples were extracted into Eppendorf tubes, snap-frozen in

liquid nitrogen, and stored in a freezer at -80°C for subsequent analysis. Blood samples collected at baseline, presyncope, and 1-h into recovery were analyzed.

We selected F_2-isoprostanes (F_2-IsoP) as a marker of oxidative stress in this study, as they are a systemic measure of oxidative stress, generated by ROS-mediated peroxidation of arachidonic acid (3). F_2-IsoP are elevated in porcine models of ischemia-reperfusion injury (5), and have been used as oxidative stress biomarkers in multiple clinical settings, including sepsis (4) and cardiac arrest (5). Additionally, our laboratory recently demonstrated that circulating F_2-IsoP increased by ~28% at the termination of presyncopal-limited LBNP (59). F_2-IsoP were measured by the Eicosanoid Core Laboratory at Vanderbilt University using a gas chromatography-negative ion chemical ionization-mass spectrometry assay method outlined by Milne et al. (54).

To assess the effect of LBNP on the inflammatory response, a select panel of circulating pro- and anti-inflammatory cytokines were measured, to include: TNF-α, IL-5, IL-6, IL-7, IL-10, CRP, and TARC. Jastrow *et al*. previously demonstrated that TNF-α, IL-5, IL-6, IL-7, and IL-10 are detectable in humans after trauma-related hemorrhage (43), and it is well-established that CRP and TARC are associated with increases in TNF-α production (31, 64). Inflammatory marker analysis was completed by the O'Bryant Laboratory at the University of North Texas Health Science Center using a MSD® Multiplex Assay (Meso Scale Diagnostics, Rockville, MD, USA).

Data Analysis. Continuous waveform recordings of ECG, arterial pressure, and muscle oxygen saturation were collected at 1000 Hz (PowerLab and LabChart, AD Instruments, Bella Vista, NSW, Australia) and analyzed offline via specialized data analysis software (WinCPRS, Absolute Aliens, Turku, Finland). Presyncopal time was defined as the moment the LBNP chamber pressure was released and returned to atmospheric pressure. The final 3-min of baseline and 60-min

recovery were averaged and used for analysis, while presyncopal measurements were averaged over the final 1-min of data collection prior to presyncope.

Using WinCPRS, R-waves were detected from the ECG signal and used to measure heart rate (HR) and divide all other continuous waveforms into cardiac cycles. Mean arterial pressure (MAP) was calculated as the area under the arterial pressure waveform. Body surface area (BSA) was calculated using the Du Bois formula (25). Stroke volume was recorded directly from the Finometer into LabChart and divided by BSA, to provide the stroke volume index (SVi). Cardiac output index (COi) was subsequently calculated as SVi multiplied by HR, and systemic vascular resistance index (SVRi) was calculated as MAP divided by COi.

Statistical Analysis. JMP (Version 13.2, SAS Institute Inc, Cary, NC, USA) was used for all statistical analyses. Comparisons between the male and female subject demographics and baseline hemodynamic variables were performed using unpaired t-tests. For all other data, two-factor (time and sex) linear mixed model analysis with repeated measures were performed, followed by Holm-corrected post-hoc tests. All data are presented as mean ± standard error (SE), and exact P-values are reported for all comparisons.

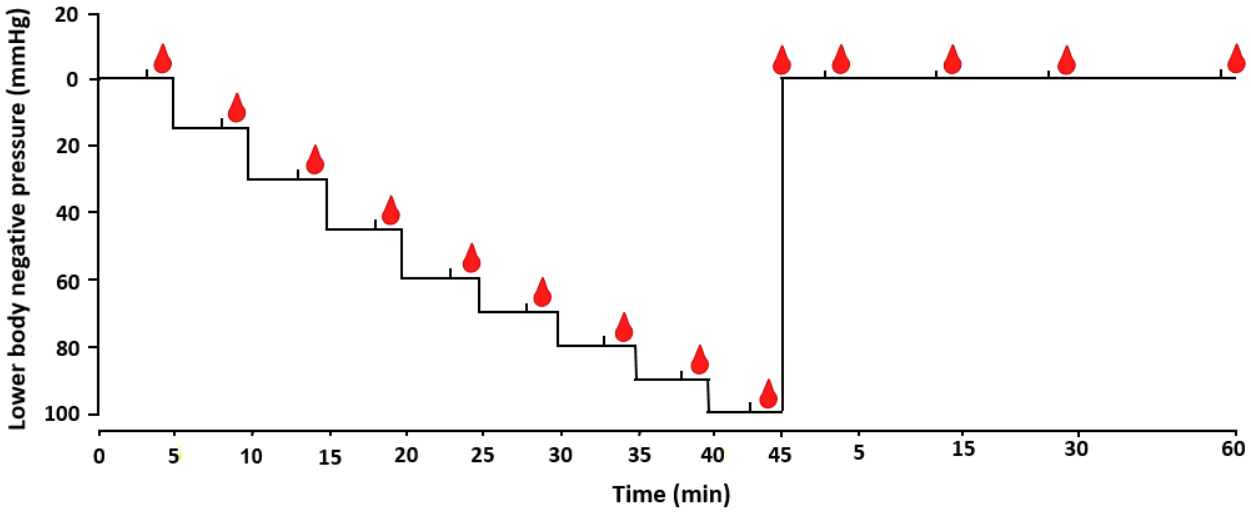

Figure 2: Experimental Protocol. Stepwise LBNP protocol up to -100 mmHg chamber pressure, and a 60-min recovery period. Blood droplets symbolize when blood samples were taken. Blood samples were drawn within the last 2-min of each LBNP stage or recovery stage; however, the presyncopal blood sample was drawn immediately at the start of recovery.

Results

Subject Demographics

Twenty-nine subjects were recruited to participate in this study. A total of 9 subjects were excluded as they did not meet the inclusion criteria (N=4), were lost to follow-up (N=4), or there were technical difficulties in obtaining blood samples (N=1). As such, twenty subjects (10 males, 10 females) completed the full protocol and were included in the final analyses. Males were taller than their female counterparts (P = 0.03), but there were no differences in age, weight, body mass index (BMI), or BSA between male and female subjects. Additionally, baseline measurements of HR, SAP, DAP, MAP, SVi, and SmO_2 did not differ between the sexes (**Table 1**).

Cardiovascular Responses to LBNP

Subjects reached a mean maximal LBNP of -63.0 ± 3.1 mmHg (range: -30.2 to -81.1 mmHg) for a mean tolerance time of 1514 ± 84 s (range: 753-2119 s). Males and females tolerated LBNP for a similar amount of time (Males, 1592 ± 124 s; Females, 1437 ± 113 s; P = 0.37). All subjects reached presyncope, defined either by a SAP < 80 mmHg (N = 13), or voluntary subject termination due to presyncopal symptoms (N = 7). At the time of presyncope, SVi (**Figure 3A:** Males, -50.2 ± 6.3% vs. Females, -49.4 ± 3.2%; P = 1.00), COi (**Figure 3D:** Males, -11.0 ± 8.8%; Females, -17.4 ± 2.2%; P = 1.00), and MAP (**Figure 3C:** Males, -20.6 ± 2.4% vs. Females, -21.8 ± 1.5%; P = 0.96) decreased by a similar magnitude in both sexes, which consequently elicited similar compensatory increases in HR (**Figure 3B:** Males, 91.8 ± 16.5%; Females, 68.1 ± 9.9%; P = 0.16). At presyncope, SmO_2 (**Figure 3F:** Males, -8.0 ± 3.6%; Females, -5.4 ± 0.9%; P = 1.00) decreased by a similar magnitude in both sexes. For both sexes, SVi, COi, MAP, HR, and SmO_2

returned to baseline values by the 1-h recovery time point ($P \geq 0.52$). There was no change in SVRi for both sexes during and after LBNP (all P values = 1.00).

Oxidative Stress and Inflammation Responses to LBNP

Oxidative Stress. When combining the male and female subjects (N=20), as shown in **Figures 4A and 4B**, there was no effect of time on the absolute ($P = 0.85$) or %Δ F_2-IsoP concentration ($P = 0.85$). Baseline plasma F_2-IsoP concentrations between male and female subjects were not different (Males, 40.7 ± 4.8 pg/ml; Females, 31.9 ± 3.5 pg/ml; P=1.00). As demonstrated in **Figure 4D**, there was no effect of time ($P = 0.82$) or sex ($P = 0.12$) on the %Δ F_2-IsoP concentration during or 1-h after LBNP.

Inflammation. Pro-inflammatory responses are represented by TNF-α, IL-6, IL-7, CRP, and TARC, while anti-inflammatory responses are represented by IL-5 and IL-10. Presyncopal LBNP elicited both pro- and anti-inflammatory responses.

Regarding the pro-inflammatory markers for the group of 20 subjects, TARC increased from baseline for both the absolute (P=0.03; **Figure 5A**), and relative responses ($P = 0.02$, **Figure 5B**). There was no difference between the %Δ of IL-6, IL-7, or CRP at presyncope from baseline ($P \geq 0.11$). At the 1-h recovery time point, there was an increase IL-6 (%Δ from baseline; P=0.04, **Figure 6B**), while %Δ CRP ($P = 0.01$, **Figure 7B**) and TARC ($P = 0.02$, **Figure 5B**) decreased. There was no change in the absolute or relative concentration of TNF-α during or after LBNP (time effect, $P \geq 0.14$, **Figures 8A&B**). Regarding the anti-inflammatory markers, there was no change in the absolute or relative concentrations of IL-5 or IL-10 during or after LBNP (time effect, $P \geq 0.12$, **Figures 9A&B and 10A&B**).

There were no differences between males and females at baseline for any of the inflammatory markers ($P \geq 0.12$). Regarding the pro-inflammatory responses at presyncope compared with baseline, males did not exhibit a change in the %Δ IL-6 (**Figure 6D**) and CRP (**Figure 7D**), but there was an increase in the %Δ TARC ($P = 0.01$, **Figure 5D**) and IL-7 ($P = 0.04$, **Figure 11D**), which were also higher compared with female subjects ($P \leq 0.07$). During recovery, males exhibited a decrease in the %Δ TARC ($P = 0.04$, **Figure 5D**), CRP ($P = 0.09$, **Figure 7D**), and IL-7 ($P = 0.05$, **Figure 11D**) compared with presyncope. Interestingly, males exhibited an increase in the %Δ IL-6 during recovery compared to baseline ($P = 0.02$), which was higher than the response of female subjects ($P = 0.06$, **Figure 6D**). There was no effect of sex ($P = 0.48$) on the %Δ TNF-α during or after LBNP (**Figure 8D**).

The anti-inflammatory response was also greater in males than in females. In male subjects, the %Δ IL-5 increased from baseline to presyncope ($P = 0.05$) but then decreased from presyncope to recovery ($P = 0.02$, **Figure 9D**), while the %Δ IL-10 increased from baseline to recovery only ($P = 0.08$, **Figure 10D**); this response was also higher compared with female subjects (Males, 71.1 ± 42.2% vs. Females, -2.2 ± 3.9%; $P = 0.06$, **Figure 10D**).

Table 1. Subject demographics and baseline hemodynamic measurements.

	N=20	Males (N=10)	Females (N=10)	P-value
Age (y)	26.0 ± 0.9	26.8 ± 1.3	25.1 ± 1.3	0.36
Height (cm)	166.0 ± 1.7	169.7 ± 1.8	162.3 ± 2.5	0.03
Weight (kg)	68.9 ± 2.2	71.2 ± 3.1	66.6 ± 3.1	0.31
BSA (m^2)	1.76 ± 0.03	1.82 ± 0.04	1.71 ± 0.05	0.12
BMI (kg/m^2)	25.0 ± 0.6	24.7±1.0	25.2 ± 0.8	0.69
HR (bpm)	61.6 ± 1.4	61.6 ± 2.3	61.7 ± 1.7	0.49
SAP (mmHg)	130.7 ± 2.5	133.6 ± 4.0	127.8 ± 2.8	0.13
DAP (mmHg)	72.9 ± 1.4	73.9 ± 2.2	71.9 ± 1.9	0.25
MAP (mmHg)	96.2 ± 1.9	97.4 ± 2.8	95.1 ± 2.7	0.29
SVi (ml)	45.2 ± 1.5	44.5 ± 2.1	46.0 ± 2.3	0.32
SmO_2 (%)	73.3 ± 1.5	75.6 ± 2.6	71.5 ± 1.7	0.10

All data are presented as mean ± SE. Two-tailed unpaired t-tests were applied to compare male and female subjects. Body surface area (BSA); body mass index (BMI); heart rate (HR); systolic arterial pressure (SAP); diastolic arterial pressure (DAP); mean arterial pressure (MAP); stroke volume index (SVi), muscle oxygen saturation (SmO_2).

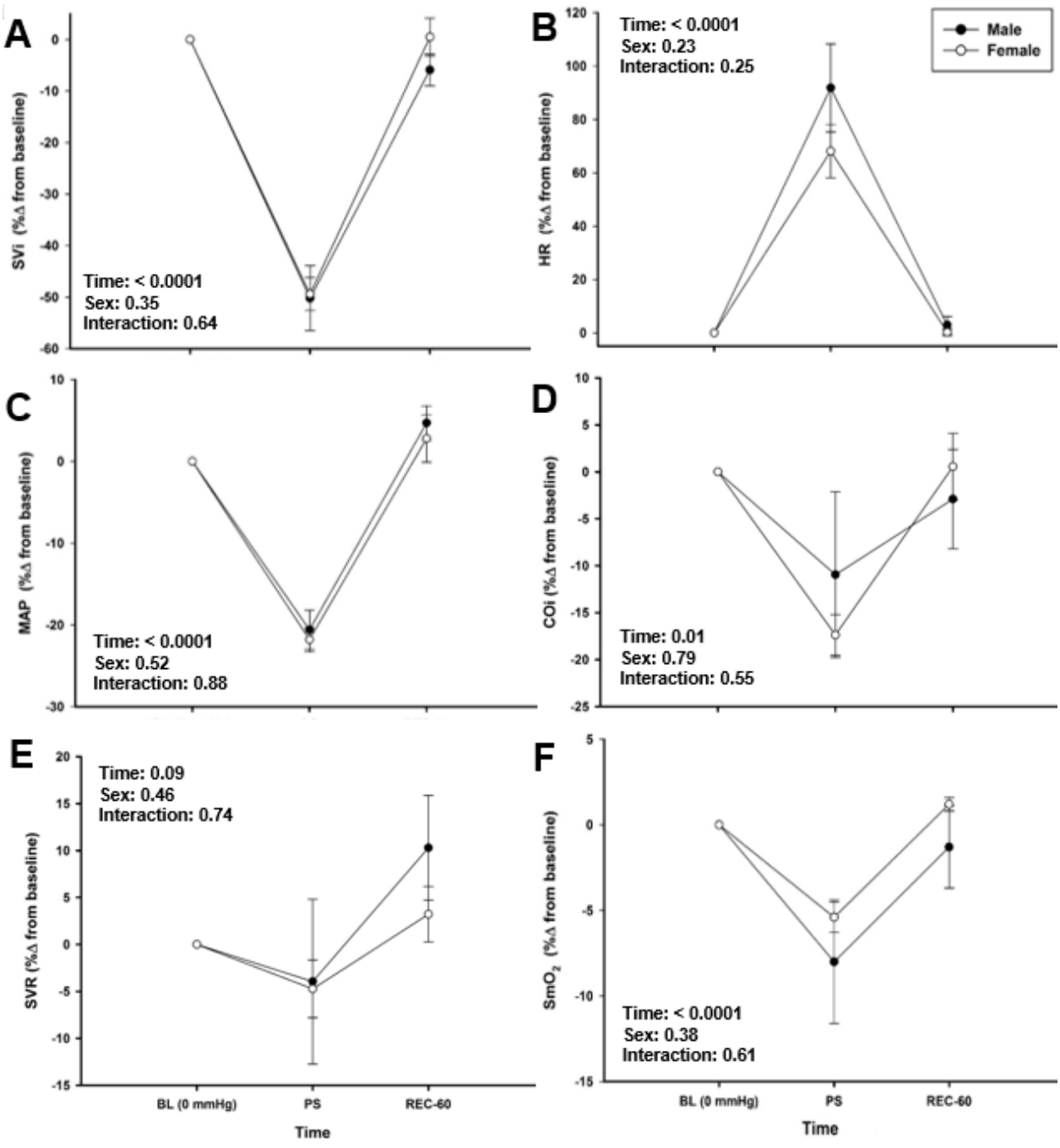

Figure 3. Sex differences (10 males, 10 females) in hemodynamic responses at presyncope (PS) and 60-min into recovery (REC-60); % change from baseline (BL) for stroke volume index (SVi; Panel A), heart rate (HR; Panel B), mean arterial pressure (MAP; Panel C), cardiac output index (COi; Panel D), systemic vascular resistance (SVR; Panel E), and muscle tissue oxygen saturation

(SmO_2; Panel F). Two-factor (time and sex) linear mixed model analyses with repeated measures were performed to compare hemodynamic variables over time and between males and females.

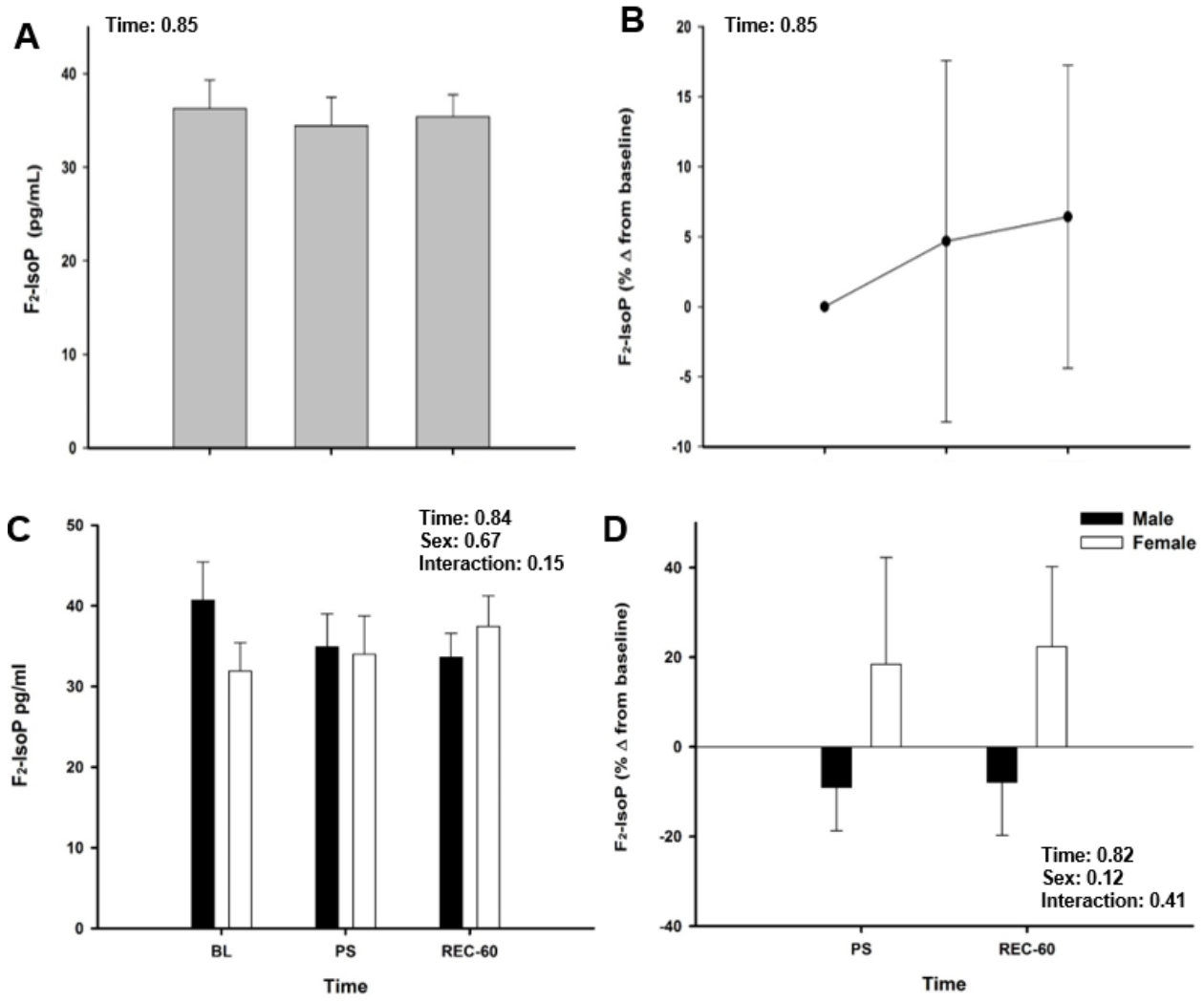

Figure 4. F_2-Isoprostanes (F_2-IsoP) at baseline (BL), presyncope (PS), and 60-min into recovery (REC-60). *Panel A:* Absolute F_2-IsoP concentrations during and after LBNP for N=20. *Panel B:* Percent change from baseline F_2-IsoP concentrations for N=20. *Panel C:* Sex differences in absolute F_2-IsoP concentrations during and after LBNP. *Panel D:* Sex differences in percent change from baseline F_2-IsoP concentrations. Two-factor (time and sex) linear mixed model analysis with repeated measures were performed to compare F_2IsoP concentrations at different times and between males (N=10) and females (N=10).

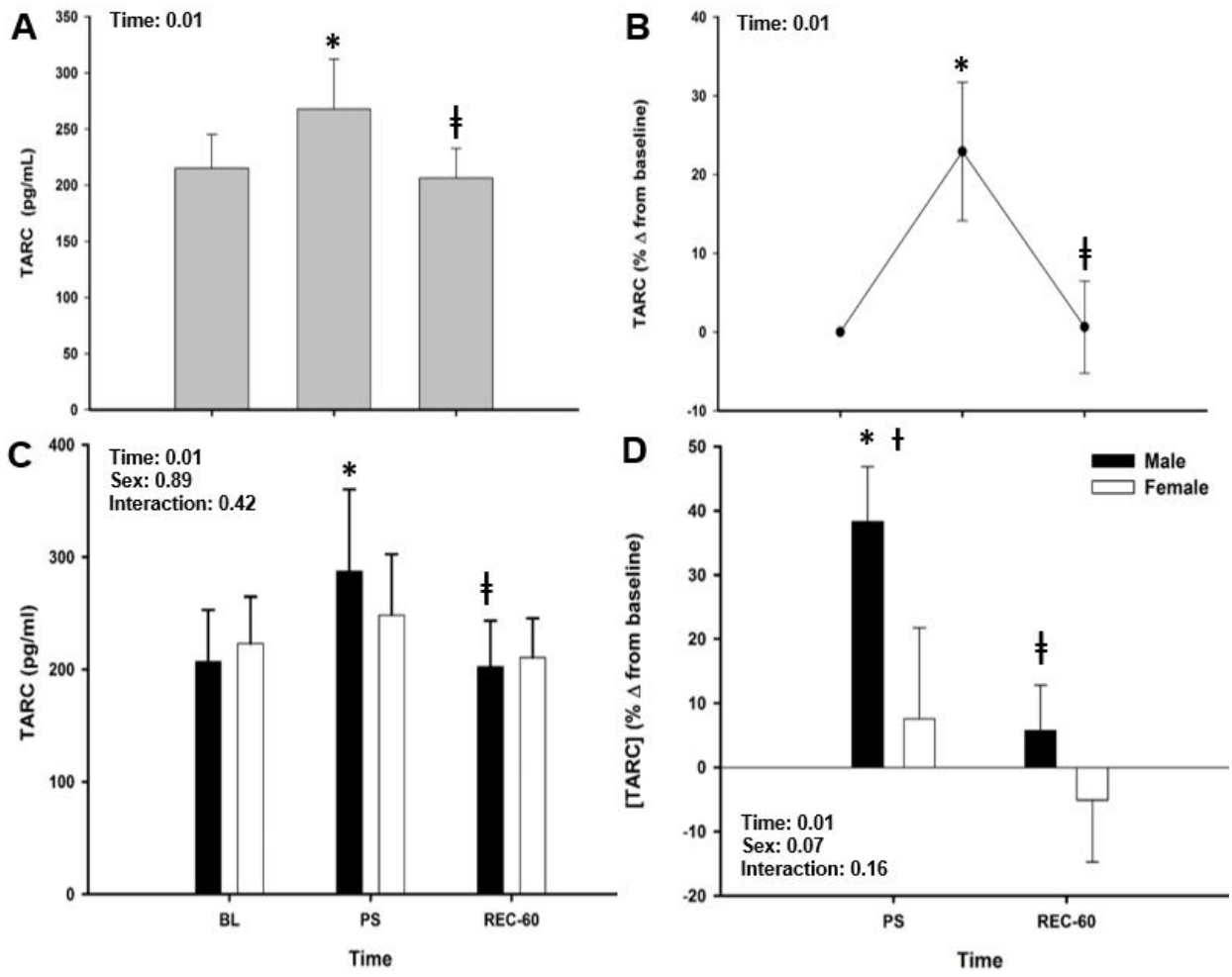

Figure 5. Thymus and activation-regulated chemokine (TARC) at baseline (BL), presyncope (PS), and 60-min into recovery (REC-60). *Panel A:* Absolute TARC concentrations during and after LBNP for N = 20. *Panel B:* Percent change from baseline TARC concentrations for N =20. *Panel C:* Sex differences in absolute TARC concentrations during and after LBNP. *Panel D:* Sex differences in percent change from baseline TARC concentrations. Two-factor (time and sex) linear mixed model analysis with repeated measures were performed to compare TARC concentrations at different times and between males (N=10) and females (N=10). *, vs. baseline, $P \leq 0.08$; ǂ, vs. presyncope, $P \leq 0.06$; †, denotes sex difference, $P = 0.07$.

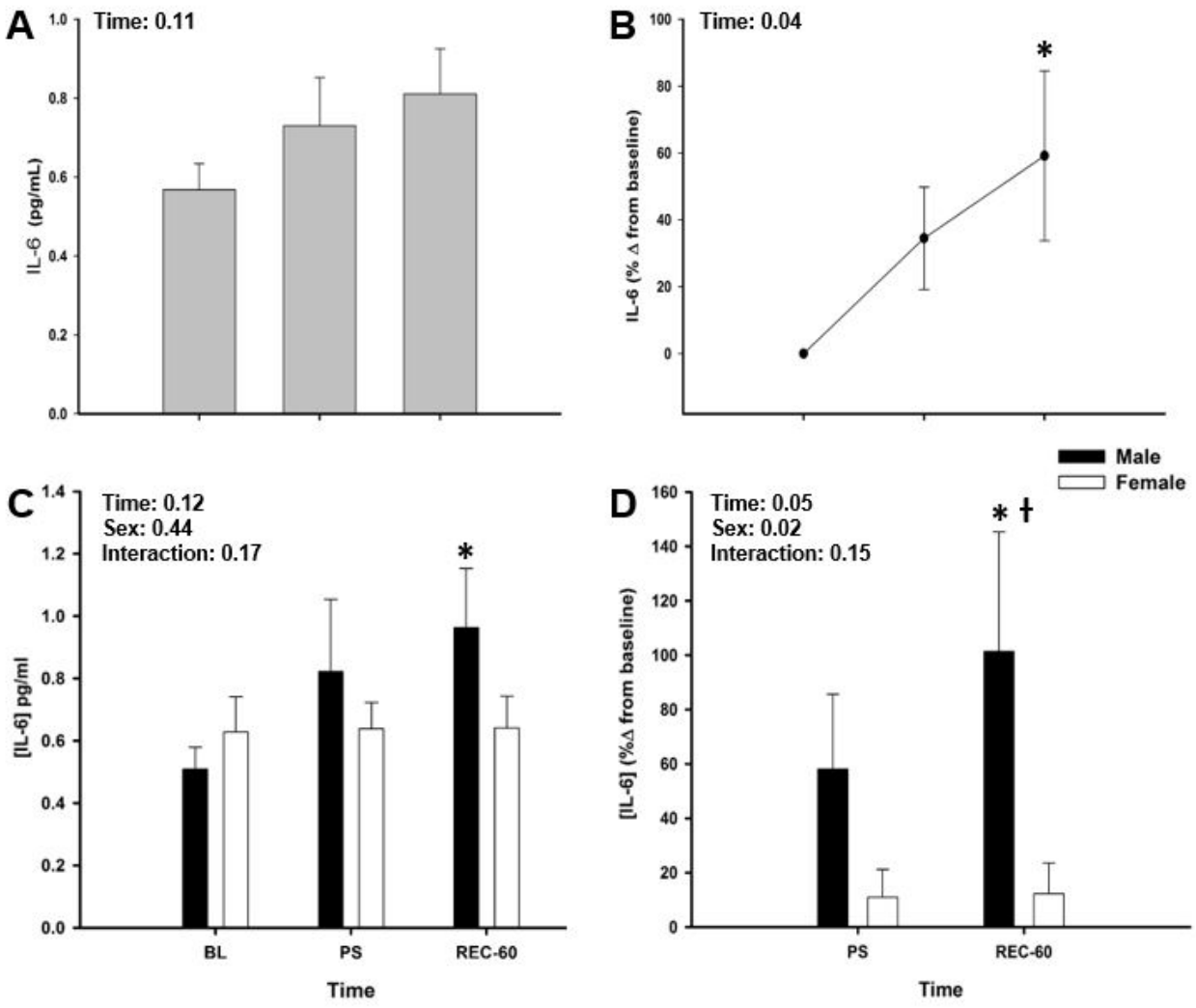

Figure 6. Interleukin 6 (IL-6) at baseline (BL), presyncope (PS), and 60-min into recovery (REC-60). *Panel A:* Absolute IL-6 concentration during and after LBNP for N = 20. *Panel B:* Percent change from baseline IL-6 concentration for N = 20. *Panel C:* Sex differences in absolute IL-6 concentration during and after LBNP. *Panel D:* Sex differences in percent change from baseline IL-6 concentration. Two-factor (time and sex) linear mixed model analysis with repeated measures were performed to compare IL-6 concentration at different times and between males (N=10) and females (N=10). *, vs. baseline, $P \leq 0.07$; †, denotes sex difference, $P = 0.06$.

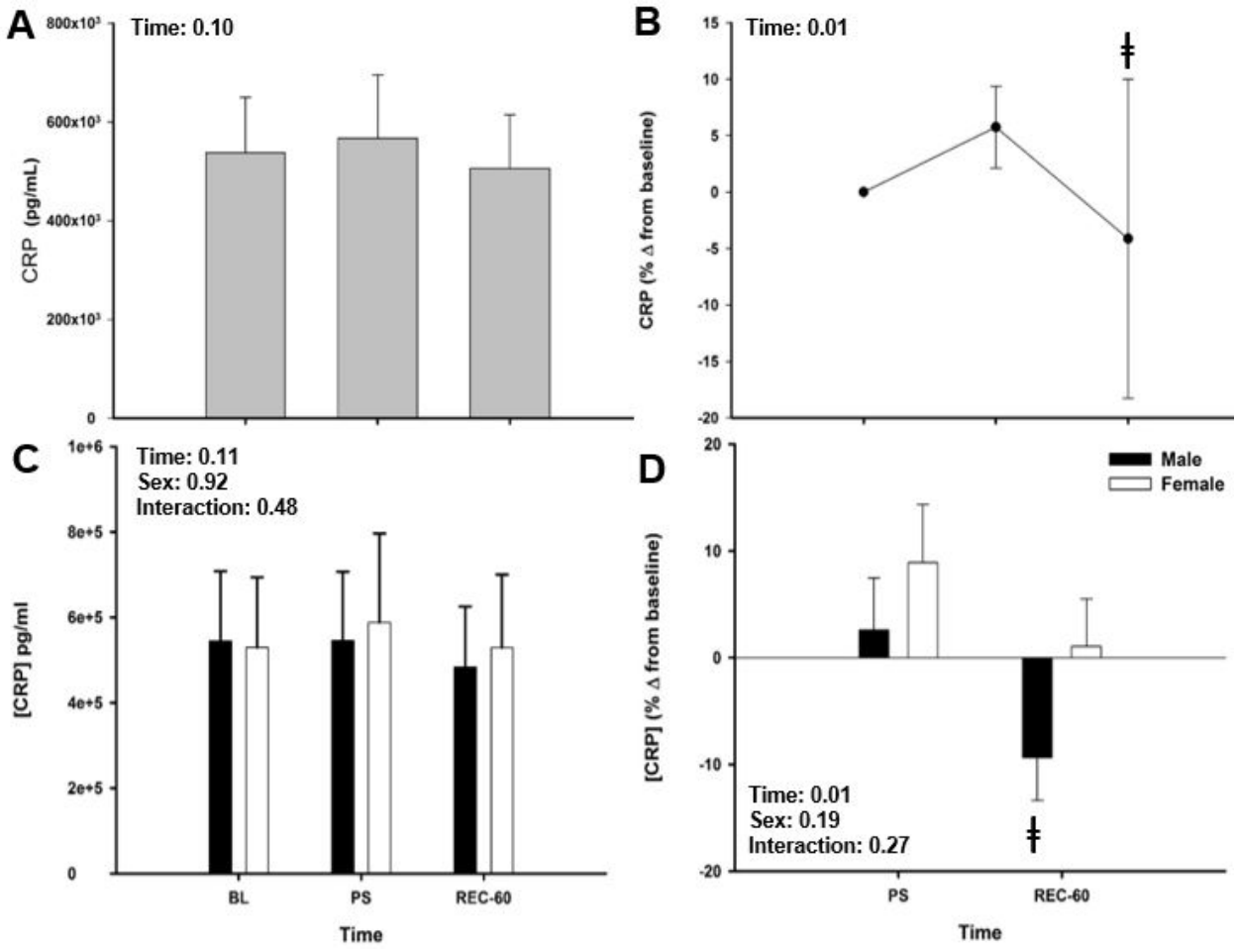

Figure 7. C-Reactive Protein (CRP) at baseline (BL), presyncope (PS), and 60-min into recovery (REC-60). *Panel A:* Absolute CRP concentration during and after LBNP for N = 20. *Panel B:* Percent change from baseline CRP concentration for N = 20. *Panel C:* Sex differences in absolute CRP concentration during and after LBNP. *Panel D:* Sex differences in percent change from baseline CRP concentration. Two-factor (time and sex) linear mixed model analysis with repeated measures were performed to compare CRP concentration at different times and between males (N=10) and females (N=10). ǂ, vs. presyncope, $P \leq 0.09$.

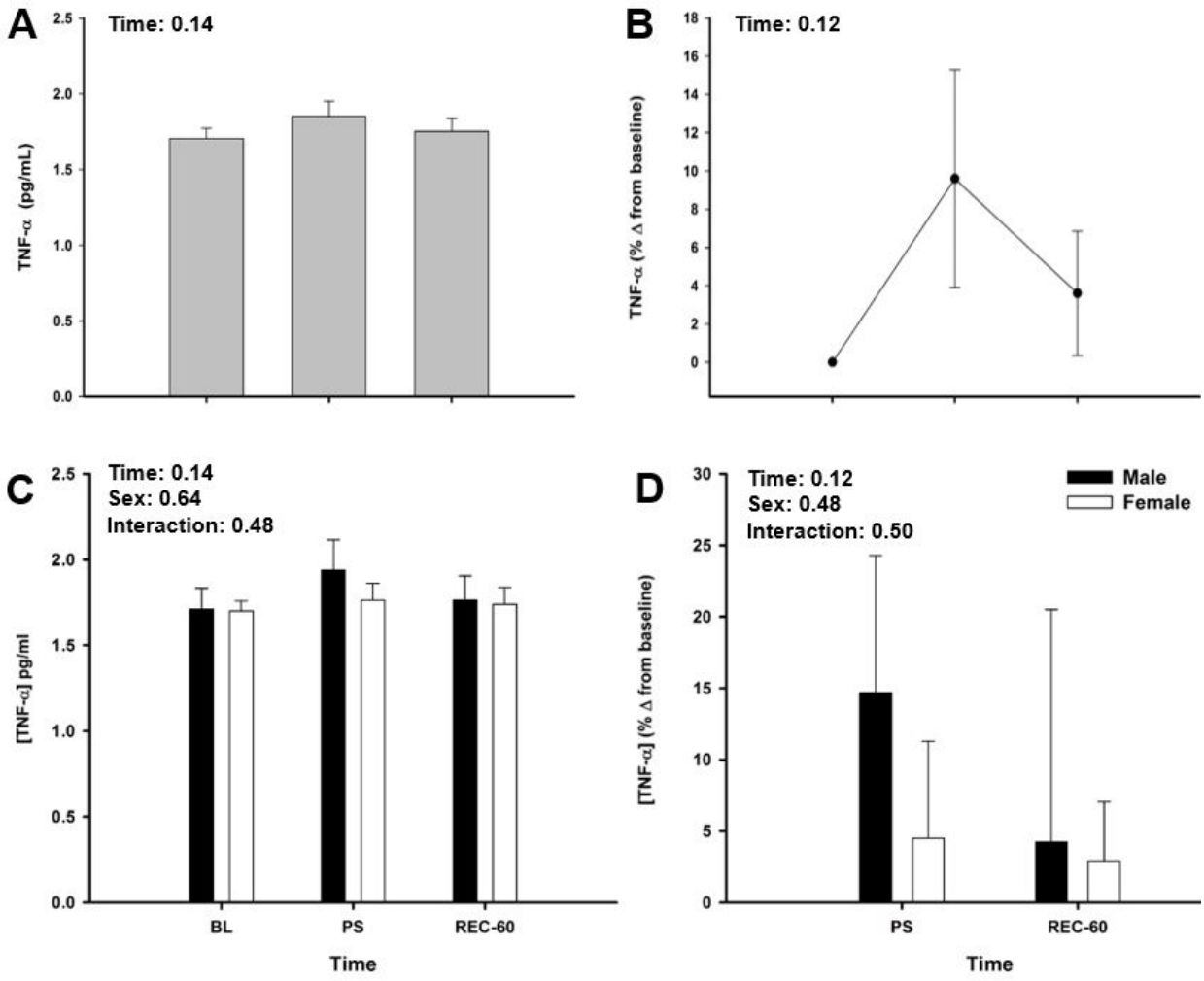

Figure 8. Tissue necrosis factor-α (TNF-α) at baseline (BL), presyncope (PS), and 60-min into recovery (REC-60). *Panel A:* Absolute TNF-α concentration during and after LBNP for N = 20. *Panel B:* Percent change from baseline TNF-α concentration for N = 20. *Panel C:* Sex differences in absolute TNF-α concentration during and after LBNP. *Panel D:* Sex differences in percent change from baseline TNF-α concentration. Two-factor (time and sex) linear mixed model analysis with repeated measures were performed to compare TNF-α concentration at different times and between males (N=10) and females (N=10).

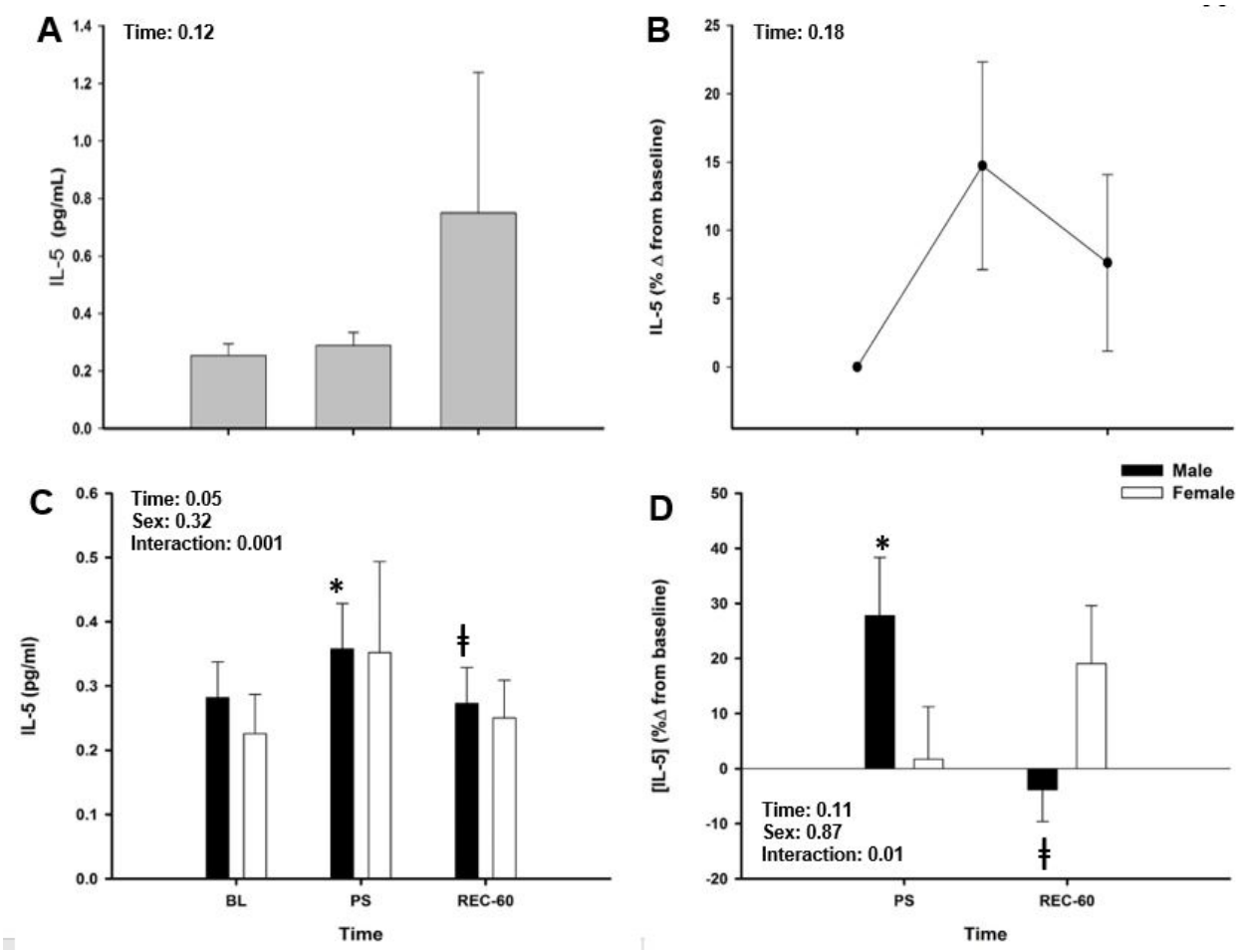

Figure 9. Interleukin 5 (IL-5) at baseline (BL), presyncope (PS), and 60-min into recovery (REC-60). *Panel A:* Absolute IL-5 concentration during and after LBNP for N = 20. *Panel B:* Percent change from baseline IL-5 concentration for N = 20. *Panel C:* Sex differences in absolute IL-5 concentration during and after LBNP. *Panel D:* Sex differences in percent change from baseline IL-5 concentration. Two-factor (time and sex) linear mixed model analysis with repeated measures were performed to compare IL-5 concentration at different times and between males (N=10) and females (N=10). *, vs. baseline, $P \leq 0.05$; ǂ, vs. presyncope, $P \leq 0.02$.

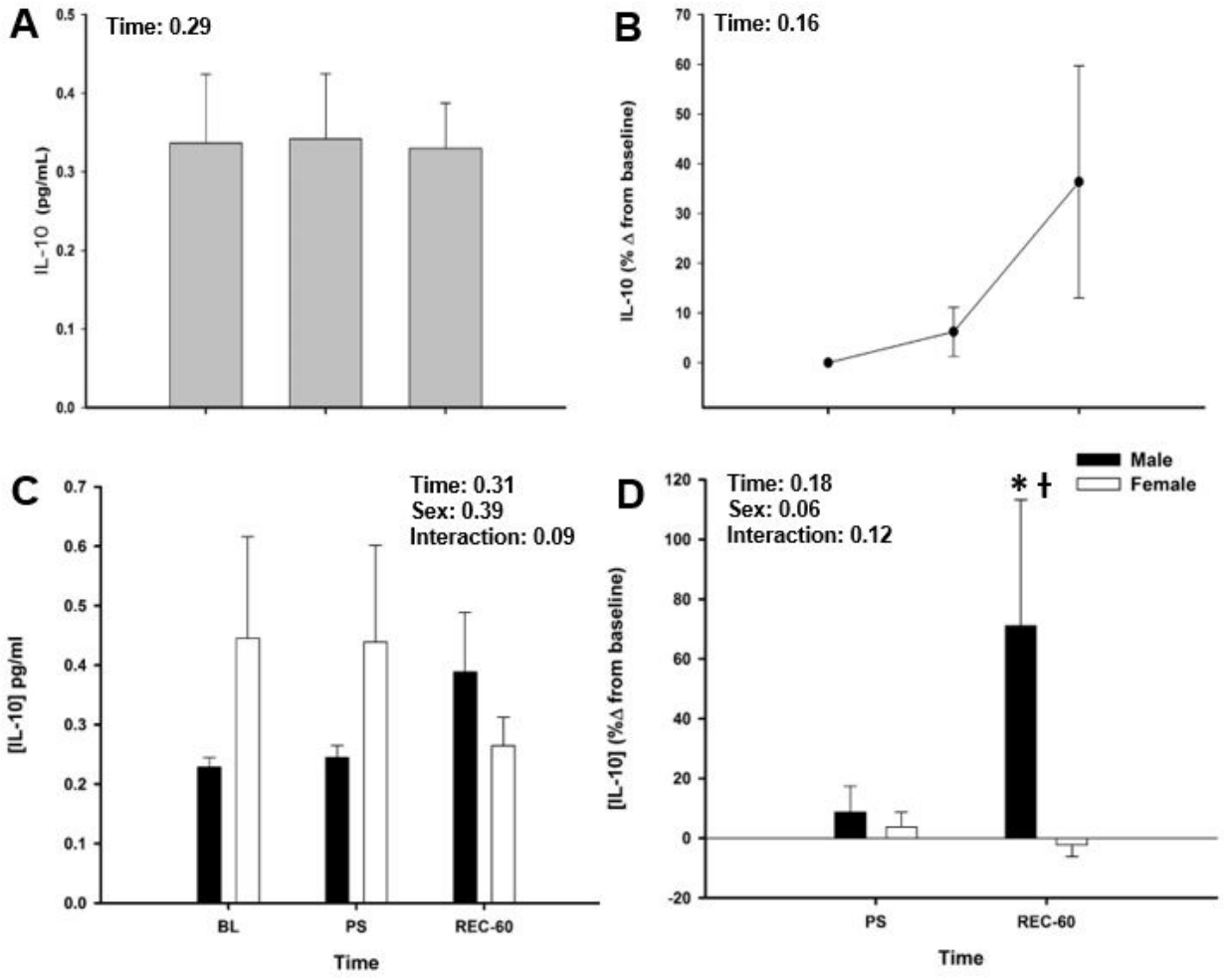

Figure 10. Interleukin 10 (IL-10) at baseline (BL), presyncope (PS), and 60-min into recovery (REC-60). *Panel A:* Absolute IL-10 concentration during and after LBNP for N = 20. *Panel B:* Percent change from baseline IL-10 concentration for N = 20. *Panel C:* Sex differences in absolute IL-10 concentration during and after LBNP. *Panel D:* Sex differences in percent change from baseline IL-10 concentration. Two-factor (time and sex) linear mixed model analysis with repeated measures were performed to compare IL-10 concentration at different times and between males (N=10) and females (N=10). *, vs. baseline, P = 0.08; †, denotes sex difference, P = 0.06.

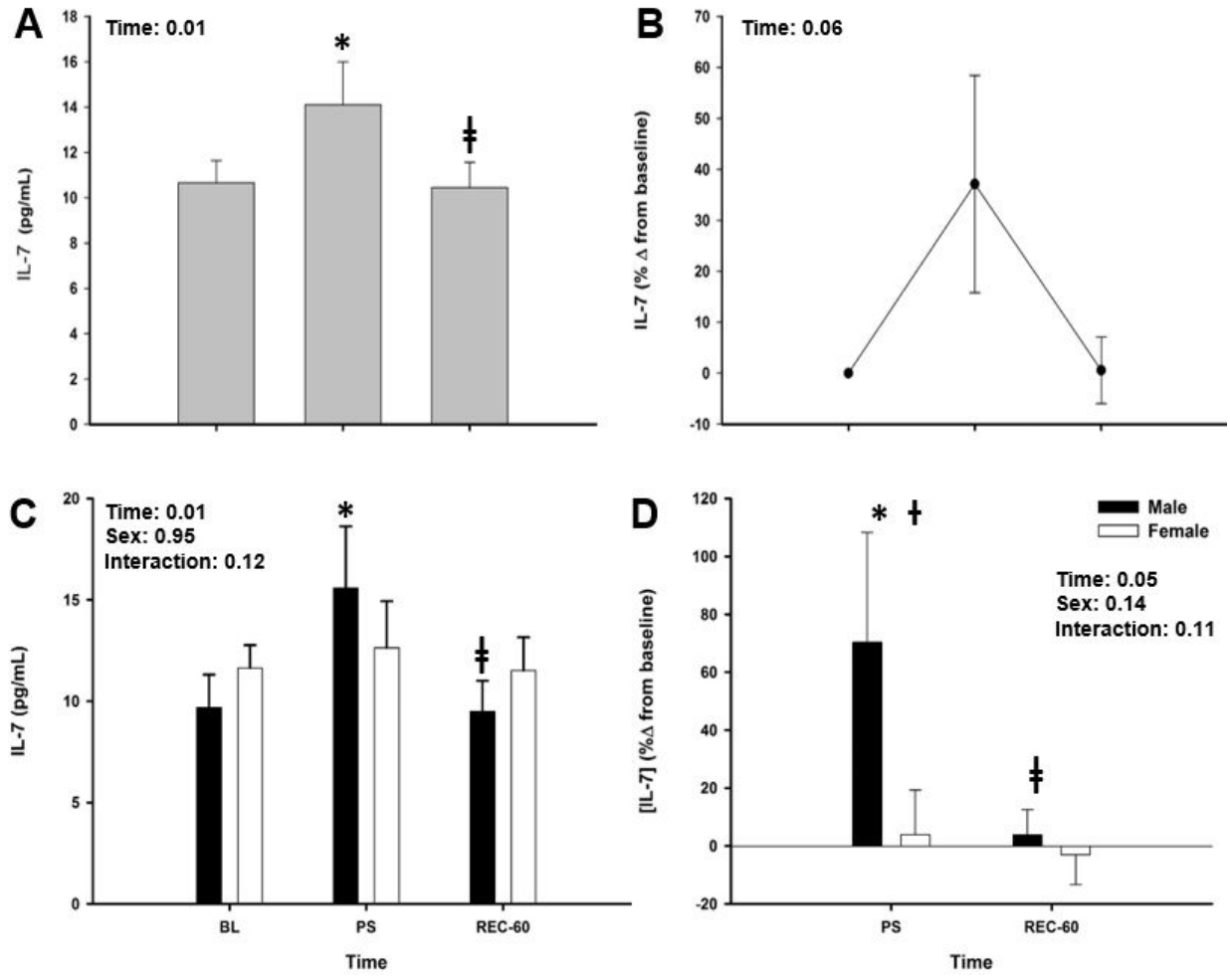

Figure 11. Interleukin 7 (IL-7) at baseline (BL), presyncope (PS), and 60-min into recovery (REC-60). *Panel A:* Absolute IL-7 concentration during and after LBNP for N = 20. *Panel B:* Percent change from baseline IL-7 concentration for N = 20. *Panel C:* Sex differences in absolute IL-7 concentration during and after LBNP. *Panel D:* Sex differences in percent change from baseline IL-7 concentration. Two-factor (time and sex) linear mixed model analysis with repeated measures were performed to compare IL-7 concentration at different times and between males (N=10) and females (N=10). *, vs. baseline, $P \leq 0.04$; ǂ, vs. presyncope, $P \leq 0.05$; †, denotes sex difference, $P = 0.06$.

Discussion

In this study, we evaluated whether oxidative stress and inflammatory responses could be induced during (blood loss) and after (resuscitation) presyncopal LBNP in healthy humans, and if there were any sex differences in these responses. The key findings of this study are: 1) Presyncopal LBNP elicited a pro- and anti-inflammatory response, but not an oxidative stress response; 2) Inflammatory responses persisted during the 1-h recovery period, and 3) Males elicited a greater inflammatory response both during and after LBNP compared to females, despite a similar magnitude of central hypovolemia and hypotension, and similar LBNP tolerance.

Contrary to our previous study, we did not detect a change in F_2-IsoP with LBNP or during recovery, and there was no sex difference in this response. We previously reported a ~28% increase in F_2-IsoP with presyncopal LBNP (59). When examining the magnitude of central hypovolemia (via stroke volume) and peripheral ischemia (via SmO_2) between these two studies, stroke volume (-53% vs. -49% in the current study) and SmO_2 (-9.2% vs. -6.5% in the current study) decreased similarly. There was a greater decrease in MAP in the current study (-21% vs. -13%), which, theoretically, should have led to a more robust oxidative stress response. However, in previous work, Corcoran *et al.* showed no change in F_2-IsoP after inducing a 20% blood loss and blood reinfusion in young healthy human males (23). Due to the variability in the F_2-IsoP response, Park *et al.* dichotomized subjects based on a threshold of 10% increase or decrease in F_2-IsoP at presyncope (i.e., responders vs. non-responders), yet there was still no difference in the cardiovascular responses (%Δ stroke volume, SmO_2, or MAP) or LBNP tolerance between groups. Accordingly, it does not appear as though the magnitude of central hypovolemia will elicit predictable increases in F_2-IsoP.

As noted by Park *et al.*, F_2-IsoP is just one marker of oxidative stress, so it is possible that other markers of oxidative stress (e.g. reduced/oxidized glutathione ratio, conjugated dienes, malondialdehyde) may increase with the central hypovolemia, hypotension, and peripheral ischemia induced by LBNP. F_2-IsoPs (specifically, 8-isoprostaglandin $F_{2\alpha}$) can also be produced via prostaglandin endoperoxide synthase enzymes, which are elevated with inflammation (76), and are independent of ROS-mediated lipid peroxidation (62). Moreover, although we reported relative changes from baseline in F_2-IsoP concentrations, baseline antioxidant capacity may modulate the F_2-IsoP response during and after LBNP. Indeed, as seen in **Figure 4B**, there is high variability in the relative F_2-IsoP response (N=20) at presyncope and 1-h into recovery, which may be due to variability in antioxidant capacity between subjects; this potential explanation requires further investigation.

As our previous work was underpowered to explore sex differences (only 4 females and 11 males) (59), we specifically addressed this question in the current study. This aforementioned variability in the F_2-IsoP response persisted when examining sex differences (**Figure 4D**), with F_2-IsoP decreasing by 9.1 ± 9.7% in males and increasing by 18.4 ± 23.8% in females at presyncope compared to baseline. Similarly, there was a decrease in F_2-IsoP of 7.9 ± 11.9% in males, and an increase of 22.3 ± 17.9% in females at the 1-h recovery time point vs. baseline. However, there were no statistically meaningful differences between the sexes for either the F_2-IsoP responses or any cardiovascular responses during or after LBNP.

Male subjects exhibited an overall greater inflammation response both during and after LBNP compared to female subjects. Multiple studies have measured inflammation after hemorrhage and fluid resuscitation in humans, yet due to practical limitations (e.g., rapid treatment), inflammatory markers are often not measured during hemorrhage but prior to fluid

resuscitation (32, 43). Accordingly, the inflammatory markers explored in this study were selected from markers that are elevated following traumatic-hemorrhage plus resuscitation in clinical populations (32, 43, 65, 69). It is notable, however, that confounding factors, such as tissue injury and oxygen supply-demand mismatch (i.e., shock), are likely to augment these markers during injury and fluid resuscitation. For example, Halbgebauer *et al*. observed an augmented increase in circulating heparin sulfate (a vital proteoglycan for extracellular matrix health) and IL-6 concentration in patients being treated for both poly-trauma and hemorrhagic shock compared to patients treated for poly-trauma only, without hemorrhage (35). These data suggest that tissue injury likely augments the inflammatory response to hemorrhage and resuscitation (35). However, our data demonstrate that central hypovolemia alone will also elicit an inflammatory response in male subjects, but not in female subjects.

Frink *et al.* previously showed that following poly-traumatic injuries, females exhibited attenuated increases in IL-6 and IL-10 within the first 24-h after injury and treatment compared to males with similar severity of injuries and treatment (32). Similarly, Lopez *et al.* measured circulating IL-6, IL-10, and TNF-α 12-h after similar traumatic injuries in 1,285 male and 643 female patients (51), and showed that there was not a sex difference in circulating TNF-α (consistent with the current study), but males appeared to have higher circulating IL-6 within the first 30-min following injury, and higher IL-10 at 60-min post-injury; due to large variability in these measurements in the male patients, the authors report that these responses were not statistically meaningful (no exact p-values given) (51).

This sex difference in the inflammatory responses to trauma in human patients is also observed in animal models. For example, in a murine model of traumatic hemorrhage, Kahlke *et al.* hemorrhaged animals to a MAP of 35 mmHg over the course of 90-min and resuscitated with

shed blood and lactated Ringer's solution (46). Twenty-four hours after resuscitation, young male mice had depressed IL-6 and enhanced IL-10 concentrations compared to young female mice (46). In a male Sprague-Dawley rat model of traumatic hemorrhage, Suzuki *et al.* acutely hemorrhaged animals to a MAP of 35-40 mmHg within 10-min, and resuscitated to four times the shed blood volume with lactated Ringer's within 60-min (72). One group of animals received an estradiol injection 30-min into resuscitation. Twenty-four hours after fluid administration, the hemorrhaged group demonstrated a four-fold increase in IL-6, IL-10, and TNF-α compared to the sham group, and this inflammatory response was abolished in the hemorrhage plus estradiol injected rats (72). In line with this previous work, we demonstrated an increase in both IL-6 (pro-inflammatory) and IL-10 (anti-inflammatory) at the 1-h recovery time point in male subjects only. Ultimately, it may be the balance of these opposing responses in male subjects (and patients) that dictates poor outcomes to these injuries and treatments; more work is needed in this area to elucidate this effect. In parallel, we did not observe a pro- or anti-inflammatory response in the female subjects during or after central hypovolemia.

While IL-5 and IL-7 were measured in this study based on observed increases in patients 24-h following hemorrhage and resuscitation (43), it may be inappropriate to measure these markers within the short timeframe of the experimental intervention. Due to their influence on T cell proliferation and maturation (which peaks within two weeks after initial antigen stimulation), IL-5 and IL-7 may not be synthesized de novo within the 1-2 hours of this study (48, 61, 75). However, these cytokines did increase in males at presyncope, which may be due to local release from organs such as the spleen into the circulation. In future studies, assessment of IL-5 and IL-7 at more prolonged time points after the simulated hemorrhage and resuscitation stress (e.g., 24, 48, 72-h) may be beneficial, to be in line with previous clinical studies (43).

Inflammation responses to hemorrhage and resuscitation as observed in both animal studies and human clinical studies were also observed with LBNP in the current study. Indeed, LBNP appears to be a valid model for eliciting these inflammatory responses to both central hypovolemia and subsequent resuscitation, and could be used as a model to explore treatments to attenuate this response.

Methodological Considerations.

There is evidence suggesting that moderate to high-intensity exercise alters oxidative stress, with acute bouts of exercise increasing oxidative stress for 48-h, while long-term regular exercise subdues this acute response (1, 28). As such, subjects self-reported their physical activity patterns, and were asked to refrain from exercise for 24-h prior to each laboratory session. Furthermore, due to evidence that some acute and chronic dietary factors (e.g. consumption of dark soy sauce, olive oil, soy protein, vitamin C or vitamin E) decreases circulating F_2-IsoP in healthy individuals (7, 17, 24, 48, 83), subjects refrained from alcohol, caffeine, and dietary and nutritional supplements for 24-h prior to the experiment. Detailed assessment of dietary patterns may be considered in future studies, as well as examining baseline antioxidant enzyme capacity as this may impact oxidative stress and inflammation responses during and after the LBNP stimulus.

In the current study, we assumed that all female subjects had relatively similar baseline concentrations of estrogen, and higher concentrations of estrogen compared with male subjects. We also assumed that all male subjects had relatively similar baseline concentrations of testosterone, and higher concentrations of testosterone compared to female subjects. To account

for these potential differences in future studies, we will measure baseline sex hormones, as these may modulate the oxidative stress and inflammation responses we observed in this study.

Finally, LBNP is not a comprehensive model of traumatic hemorrhage as this technique does not induce tissue trauma, pain, or the oxygen supply-demand mismatch indicative of shock, with subsequent metabolic acidosis (22). Despite these limitations, use of LBNP does allow for the assessment of hemodynamic, oxidative stress, and inflammatory responses to acute central hypovolemia and tissue hypoperfusion without confounding factors that exist with actual blood loss. Furthermore, acute hemodynamic responses are similar to actual blood loss, as demonstrated in recent studies conducted in humans and non-human primates (22, 38, 44).

Future Directions. The results from the current study provide data describing sex differences in inflammation responses during and after a simulated hemorrhage. Future studies should continue to explore if phase of menstrual cycle or use of contraception influences the likelihood of premenopausal females to exhibit an attenuated systemic inflammatory response during both simulated and actual blood loss injuries. With increasing popularity of long-acting contraceptives by premenopausal women (11), investigating the influence of menstrual cycle and contraceptive use on oxidative stress and inflammation responses during and after a simulated hemorrhage may facilitate the development of more effective fluid resuscitation therapies, which may aid in reducing mortality rates from these injuries in both women and men.

SUMMARY & CONCLUSIONS

We examined sex differences in oxidative stress and inflammation responses during and after simulated hemorrhage in young, healthy humans. There was no difference in the oxidative stress response to a simulated hemorrhage between the sexes; however, males exhibited a greater inflammatory response during and 1-h after presyncopal LBNP, reflecting the responses to hemorrhage and resuscitation. These findings are consistent with previous studies in both animals and humans demonstrating that males typically have a greater inflammatory response to hemorrhage and resuscitation compared with females. Investigating sex differences in oxidative stress and inflammation responses may elucidate why young females have a survival advantage following acute hemorrhagic injuries. With our findings that LBNP can be a model to study this sex difference in inflammation during and after simulated hemorrhage, we can further the development of therapies to treat these injuries and potentially reduce tissue and organ damage following actual blood loss injuries.

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