Original Research

Hemorrhage simulated by lower body negative pressure provokes an oxidative stress response in healthy young adults

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Impact statement

We characterize the systemic oxidative stress response in young, healthy human subjects with exposure to simulated hemorrhage via application of lower body negative pressure (LBNP). Prior work has demonstrated that LBNP and actual blood loss evoke similar hemodynamic and immune responses (i.e. white blood cell count), but it is unknown whether LBNP elicits oxidative stress resembling that produced by blood loss. We show that LBNP induces a 29% increase in F_2-isoprostanes, a systemic marker of oxidative stress. The findings of this investigation may have important implications for the study of hemorrhage using LBNP, including future assessments of targeted interventions that may reduce oxidative stress, such as novel fluid resuscitation approaches.

Abstract

Hemorrhage is a leading cause of potentially preventable death in both civilian and military trauma settings. Lower body negative pressure (LBNP) is a validated, non-invasive, and reproducible approach to simulate hemorrhage by inducing central hypovolemia in healthy conscious humans. The oxidative stress response to simulated hemorrhage via LBNP has not been quantified. We hypothesized that systemic markers of oxidative stress would increase with application of maximal, pre-syncopal limited LBNP. Fifteen healthy human subjects (11 M/4 F; age 27 ± 1 y) were recruited for a single LBNP experiment to presyncope (chamber pressure was progressively reduced every 5-min in a stepwise manner). Heart rate was assessed via ECG, arterial pressure and stroke volume (SV) were measured continuously via finger photoplethysmography, muscle oxygen saturation (SmO_2) was measured via near-infrared spectroscopy, and venous blood samples were collected at baseline and presyncope. Plasma samples were analyzed for F_2-isoprostanes (F_2-IsoP), a global marker of oxidative stress. The magnitude of central hypovolemia, indexed by the maximal decrease (%Δ) in SV, ranged from 27 to 74% ($53.5 \pm 3.9\%$; $P < 0.001$), and mean arterial pressure (MAP) decreased by $12.6 \pm 2.6\%$ ($P < 0.001$ vs. pre-LBNP baseline). F_2-IsoP increased by $28.5 \pm 11.6\%$ ($P = 0.05$) from baseline (24 ± 2 pg/mL) to presyncope (29 ± 3 pg/mL). The increase in F_2-IsoP was not associated with %Δ SV ($r = 0.21$, $P = 0.46$), %Δ MAP ($r = 0.05$, $P = 0.86$), %Δ SmO_2 ($r = 0.05$, $P = 0.90$), or the maximum level of LBNP attained ($r = 0.35$, $P = 0.20$). Simulated hemorrhage induced by LBNP to presyncope elicited an increase in oxidative stress, but this response was not associated with the magnitude of central hypovolemia, hypotension, or the decrease in peripheral muscle tissue oxygen saturation. These findings have important implications for the study of hemorrhage using LBNP, and future investigations of interventions targeting oxidative stress.

Keywords: F2-isoprostanes, blood loss, lower body negative pressure, human subjects, oxidative, cardiovascular

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Introduction

Hemorrhage is a leading cause of potentially preventable death in both civilian[1–3] and military trauma settings.[4–7] Hemorrhage can also result from non-traumatic injury including childbirth, surgery, ruptured ulcers, and hemorrhagic viruses.[8] In an analysis of combat casualty records from the Armed Forces Medical Examiner Service Mortality Surveillance Division from 2001 to 2011, of the 4016 battlefield fatalities occurring in the pre-medical treatment facility environment, 24.3% ($n = 976$) of deaths were potentially survivable, of which 90.9% ($n = 888$) were primarily attributed to hemorrhage.[6] Even in a relatively well-controlled clinical

setting, hemorrhage was still the second leading cause of death behind head injury in a Level 1 civilian trauma center, accounting for 36% of deaths.[1] Hemorrhage still accounted for 25% of deaths even after a six-year transition to a new bleeding control bundle of care treatment approach.[1]

Physiological and pathophysiological responses to hemorrhage are complex and vary across multiple time scales. Loss of blood volume decreases venous return, which subsequently decreases stroke volume (SV), cardiac output, and arterial pressure. Afferent inputs from the peripheral and central baro- and chemoreceptors to the medullary cardiovascular control center initiate compensatory efferent sympathetic responses to increase heart rate and contractility, constrict systemic arteries and veins, and activate the renin-angiotensin system, in an effort to restore central blood volume and arterial pressure. If the blood loss is not treated and the severity of hemorrhage progresses, these compensatory mechanisms eventually fail, and metabolic demand of the tissues can no longer be met by the reduced blood and oxygen supply. This state of hemorrhagic shock may impose ischemia,[9–11] culminating in multiple organ dysfunction and death.[10,12,13]

Loss of blood volume and the persistent oxygen supply-demand mismatch with hemorrhage can elicit an oxidative stress response.[14] Basal physiological production of reactive oxygen species (ROS) is readily managed by endogenous antioxidant enzymes and cofactors.[15] However, severe blood loss, the resultant ischemia of under-perfused tissues, and tissue reperfusion effected by resuscitation, provoke massive ROS overproduction.[16] This ROS tsunami can exhaust the endogenous antioxidant mechanisms, leaving the reperfused tissues vulnerable to mitochondrial permeability transition, lipid peroxidation, protein carbonylation and oxidation, and DNA damage.[14,15] Consequently, excessive ROS contribute to cell death and organ failure following hemorrhagic injury.

Circulating F_2-Isoprostanes (F_2-IsoP) generated by ROS-mediated peroxidation of polyunsaturated fatty acids,[17,18] provide a measure of systemic oxidative stress.[19] These byproducts of arachidonic acid metabolism[19,20] are preferentially generated under low oxygen conditions,[20,21] and their circulating concentrations increase in a variety of clinical settings,[19] including stroke,[22,23] subarachnoid hemorrhage,[24] traumatic brain injury,[24] sepsis,[25] and cardiac arrest.[26] Accordingly, F_2-IsoPs are the primary markers of oxidative stress assessed in the present study.

The urgency of stabilizing hemorrhage victims limits studies of direct pathophysiological effects of blood loss and resuscitation in the emergency setting.[27] However, animal and human models of hemorrhage, whether by actual blood loss or simulated hemorrhage [such as via application of lower body negative pressure (LBNP)], afford rigorous experimental study of hypovolemia. LBNP is a well validated, non-invasive, controlled, and reproducible method to study the effects of progressive central hypovolemia in healthy conscious humans.[28–31] While LBNP can be used to isolate the hemodynamic effects of central hypovolemia, it is unknown whether LBNP elicits oxidative stress resembling that produced by actual blood loss.

Isolated central hypovolemia may elicit oxidative stress by provoking peripheral vasoconstriction, and thus, tissue ischemia.[32,33] To our knowledge, no previous studies have evaluated the oxidative stress response to LBNP, and only one study has assessed this response to actual exsanguination in humans. Corcoran *et al*.[34] measured plasma F_2-IsoPs and isofurans in humans after controlled moderate blood withdrawal up to 20% of estimated total blood volume. These investigators reported that the moderate central hypovolemia, which did not induce hypotension, was insufficient to elicit oxidative stress.[34] Accordingly, the aim of the current study was to assess whether simulated hemorrhage, induced by progressive application of LBNP to presyncope (to include hypotension), elicits systemic oxidative stress in healthy conscious human subjects. We hypothesized that plasma F_2-IsoP, a systemic marker of oxidative stress, would increase with application of LBNP in the absence of exsanguination. The findings of this investigation may have important implications for the study of hemorrhage using LBNP, including future assessments of targeted interventions that may reduce oxidative stress.

Materials & methods

Subjects

The protocol was reviewed and approved by the UNTHSC Institutional Review Board. Twenty-five subjects were recruited from the University of North Texas Health Science Center (UNTHSC), and the surrounding community. Inclusion criteria were: 18–45 years of age, both males and females, non-tobacco/nicotine users, resting systolic arterial pressure (SAP) <140 mmHg and diastolic arterial pressure (DAP) <90 mmHg, normal 12-lead ECG (seated and standing), normal clinical results from a medical exam reviewed by a physician, and a body mass index (BMI) $\leq 30\,kg/m^2$ unless athletic/muscular build. Exclusion criteria were: use of antihypertensive medications, beta-adrenoreceptor antagonists, bronchodilators, use of medications or dietary supplements that alter autonomic function, history of hyperthyroidism, known cerebrovascular abnormalities, atherosclerosis, autonomic dysfunction, respiratory illness, orthostatic hypotension, abnormal blood clotting, and blood donation within the last 60 days. Females taking hormonal contraceptives were tested during the first four days of the sugar pill/no pill phase. All female subjects not taking hormonal contraceptives were tested during days 1–4 of the early follicular/low-hormone phase of their menstrual cycle. All female subjects, regardless of contraception status, completed a urine pregnancy test immediately before experimentation. Written informed consent was obtained from all participants prior to the study. Subjects attended a familiarization session during which they were shown all of the equipment, guided through the informed consent document, completed medical history documentation, and had resting blood pressure and ECG recordings taken.

Instrumentation

Subjects were placed supine with their iliac crest aligned with the opening of the LBNP chamber (VUV Analytics Inc. Austin, TX, USA). Subjects were instrumented for continuous measurement of: (1) heart rate (HR) via three lead ECG (shielded leads, cable and amplifier, AD Instruments, Bella Vista, NSW, Australia), (2) non-invasive arterial pressure and SV via finger photoplethysmography (Finometer™, Finapres Medical Systems, Amsterdam, The Netherlands), and (3) muscle oxygen saturation (SmO_2) of the flexor carpi muscles of the forearm via near-infrared spectroscopy (CareGuide™, Reflectance Medical, Westborough, MA, USA). Venous blood samples were collected from a peripheral venous catheter (BD Insyte Autoguard Shielded IV Catheter, Franklin Lakes, NJ, USA) placed in an antecubital vein. Approximately 1–2 mL of heparinized saline was flushed through the catheter between sample collections.

Study design

All subjects recruited for this LBNP experiment had participated in a number of previous LBNP experiments as part of a larger study.[35–37] At least one month intervened following the last LBNP test for all subjects. Upon arrival in the laboratory, subjects were positioned supine in the LBNP chamber and instrumented (as described in the Instrumentation section). After a 5-min baseline period, the pressure in the LBNP chamber was decreased by 15 mmHg increments every 5-min until −60 mmHg was reached. From that point onwards, the pressure in the LBNP chamber was decreased by 10 mmHg every 5-min to a maximum pressure of −100 mmHg.[38,39] The LBNP protocol progressed until the onset of presyncope, defined by at least one of the following criteria: (1) instantaneous SAP below 80 mmHg, (2) completion of 5-min at the maximum LBNP level of −100 mmHg, or (3) voluntary subject termination due to subjective symptoms such as dizziness, nausea, blurred vision, or general discomfort. Blood samples were collected at baseline (10 mL) and at the end of each 5-min LBNP step including maximum LBNP (5 mL per sample). The presyncopal blood sample was defined as the last sample collected prior to the onset of presyncope.

Sample and data analysis

Waveform analysis. Continuous waveform data (ECG, arterial pressure, SV) were collected at 1000 Hz (LabChart, AD Instruments, Bella Vista, NSW, Australia) and analyzed offline via specialized software (WinCPRS software, Absolute Aliens, Turku, Finland). HR was determined from R-waves detected from the ECG signal. SAP and DAP were detected from the continuous arterial pressure recordings, and mean arterial pressure (MAP) was calculated as the area under the arterial pressure waveform via the WinCPRS software. SV was recorded directly to LabChart via the Finometer. SmO_2 data were extracted from the NIRS device into an Excel spreadsheet, and analyzed offline. Baseline waveform data were analyzed using the last 4-min of the baseline period just prior to the start of the first LBNP step. At presyncope, waveform data were analyzed using the 1-min prior to pre-syncope.

Hematocrit & oxidative stress. Hematocrit was measured in duplicate from the baseline blood sample only (Clay Adams MHCT II, Parsippany, NJ, USA). The remaining blood samples were transferred into EDTA-coated tubes (BD Vacutainer, Franklin Lakes, NJ, USA) containing 1.23 mg glutathione/1 mL whole blood. Whole blood samples were centrifuged at 4°C for 15-min at 1500 r/min. The plasma was extracted and placed in Eppendorf tubes in ~1 mL aliquots, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Baseline and presyncopal plasma samples were analyzed for the oxidative stress marker F_2-IsoP via gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) according to a well-established analytical approach[20] at the Eicosanoid Core Laboratory at Vanderbilt University.

Statistical analysis. Two-tailed paired *t*-tests were performed to assess hemodynamic responses between baseline and presyncope (Table 1). A one-tailed paired *t*-test was used to compare oxidative stress responses from baseline to presyncope, as per our *a priori* hypothesis that markers of oxidative stress would increase with application of maximal LBNP. Two-tailed unpaired *t*-tests were performed between responder and non-responder sub-groups (described in the Results section). Linear regression was used to assess the relationships between changes in oxidative stress and hemodynamic responses, with Pearson correlation coefficients and *P*-values reported for each comparison. Subject demographics are presented as mean ± SD, and all other data are presented as mean ± SEM.

Results

Fifteen subjects were selected for final analysis (11 M, 4 F; age 27 ± 1 y; height 172 ± 3 cm; weight 78 ± 4 kg). Of the original 25 subjects, 4 were excluded as the venous sampling line became non-patent during the experiment, and 6 subjects requested that no blood sampling be performed. Hematocrits were within the normal clinical ranges for female (39.3 ± 0.3%, range: 39–40%) and male subjects (43.3 ± 0.6%, range: 40–46%).[40] All subjects reached true

Table 1. Hemodynamic responses to pre-syncopal limited lower body negative pressure (LBNP).

	Baseline	Pre-syncope	*P*
HR (bpm)	62 ± 2	113 ± 6	<0.001
SAP (mmHg)	124 ± 2	100 ± 3	<0.001
DAP (mmHg)	71 ± 2	69 ± 3	0.29
MAP (mmHg)	92 ± 2	80 ± 3	<0.001
SV (mL)	103 ± 5	48 ± 4	<0.001
SmO_2 (%)	76.1 ± 1.5	69.2 ± 1.9	<0.001

Note: Values are presented as mean ± SE; all data were collected on $N = 15$, except for SmO_2 where $N = 11$.
Two-tailed paired *t*-tests were applied to compare baseline with presyncope.
HR: heart rate; SAP: systolic arterial pressure; DAP: diastolic arterial pressure; MAP: mean arterial pressure; SV: stroke volume; SmO_2: muscle oxygen saturation.

presyncope, defined as (1) a minimum instantaneous SAP < 80 mmHg, (2) reporting of subjective presyncopal symptoms combined with mean SAP < 100 mmHg for the 1 min prior to presyncope, or (3) minimum SAP ≤ 90 mmHg within the 1 min prior to presyncope. Subjects reached a mean maximum LBNP of -64.7 ± 4.2 mmHg (range: -32.9 to -90.3 mmHg) for a mean tolerance time of 1581 ± 110 s (range: 911–2392 s).

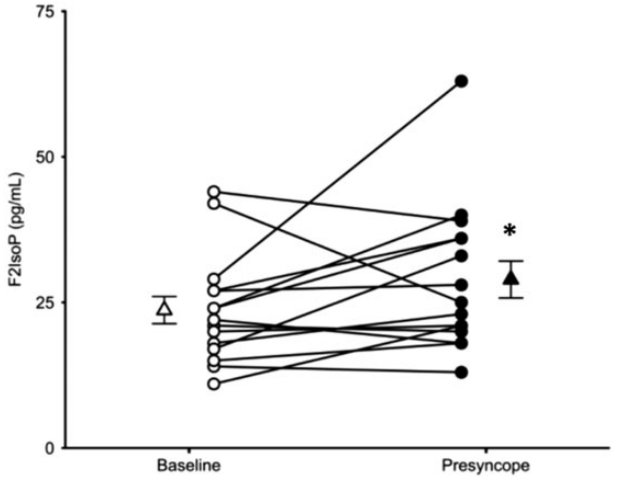

Figure 1. Mean (▲) and individual (○) F_2-Isoprostane (F2-IsoP) concentrations (pg/mL) at baseline (open symbols) and pre-syncope (closed symbols). Mean concentrations increased from baseline to pre-syncope (*, denotes $P = 0.05$). $N = 15$.

At the time of presyncope, SV decreased by $53.5 \pm 3.9\%$ from baseline, and MAP decreased by $12.6 \pm 2.7\%$, which in turn elicited a compensatory increase in HR by $84.1 \pm 10.9\%$ (Table 1). SmO_2 decreased by $9.2 \pm 1.3\%$ from baseline to presyncope. Presyncopal LBNP induced an increase in F_2-IsoPs from 23.7 ± 2.4 pg/mL at baseline to 28.9 ± 3.3 pg/mL at presyncope, a $28.5 \pm 12.0\%$ increase ($P = 0.05$, Figure 1). This increase in F_2-IsoP concentration was not associated with %Δ SV ($r = 0.21$, $P = 0.46$), %Δ MAP ($r = 0.05$, $P = 0.86$), %Δ SmO_2 ($r = 0.05$, $P = 0.90$), or the maximum level of LBNP attained ($r = 0.35$, $P = 0.20$) (Figure 2).

As demonstrated in the data presented in Figure 1, some subjects exhibited an increase in oxidative stress with LBNP, while others exhibited a decrease. We dichotomized subjects based on a threshold of a 10% increase in F_2-IsoP from baseline to presyncope, where Responders ($n = 8$) exhibited a $\geq 10\%$ increase in F_2-IsoP from baseline to presyncope, and Non-Responders ($n = 7$) exhibited a $< 10\%$ increase or a decrease in F_2-IsoP from baseline to pre-syncope. As a result, the average relative change in F_2-IsoPs for the Responders was $62.5 \pm 12.6\%$ and $-10.5 \pm 5.9\%$ for the Non-Responders ($P < 0.001$). When assessing the physiological responses to LBNP between the Responders and Non-Responders, there were no

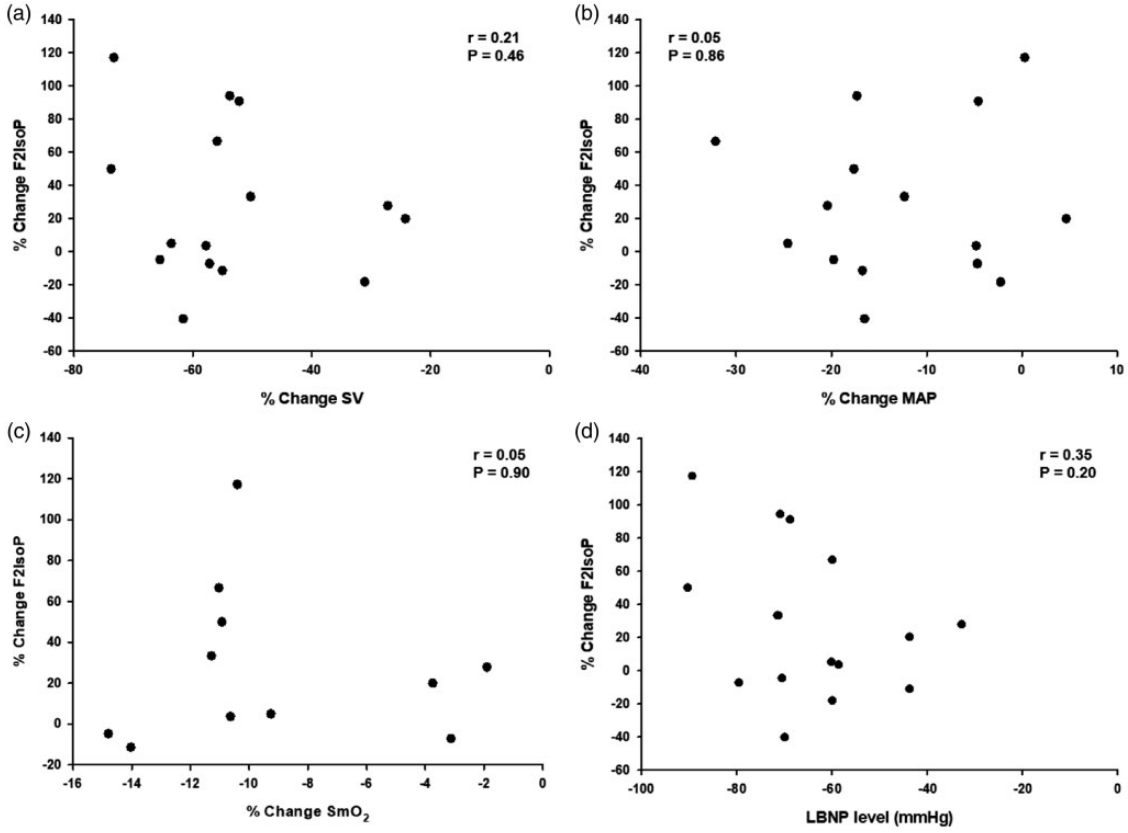

Figure 2. Relationships between percent changes in F_2IsoP concentrations (%Δ from baseline to presyncope) and key hemodynamic responses, and tolerance to lower body negative pressure (LBNP). %Δ [F_2IsoP] was not associated with %Δ SV (Panel a), %Δ MAP (Panel b), %Δ SmO_2 (Panel c), or maximum LBNP level (Panel d). $N = 15$ except for SmO_2 where $N = 11$.

differences in %Δ SV ($-51.3 \pm 6.4\%$ vs. $-56 \pm 4.4\%$, $P = 0.57$), %Δ MAP ($-12.4 \pm 4.3\%$ vs. $-12.8 \pm 3.3\%$, $P = 0.95$), %Δ SmO_2 ($-8.2 \pm 1.7\%$ vs. $-10.4 \pm 2.1\%$, $P = 0.22$), or maximum level of LBNP attained (-65.9 ± 7.1 mmHg vs. -63.2 ± 4.3 mmHg, $P = 0.76$).

Discussion

The objective of this study was to assess whether simulated hemorrhage with maximal LBNP elicits an oxidative stress response. The main finding is that stepwise LBNP to presyncope elicited an increase in F_2-IsoP, an *in vivo* marker of systemic lipid oxidative stress.

Baseline F_2-IsoP concentrations were 23.7 ± 2.4 pg/mL (range: 11–44 pg/mL) in our young healthy human subjects. These concentrations are comparable, albeit slightly lower, than baseline F_2-IsoP concentrations in other studies in young healthy males and females (36 ± 2 pg/mL),[41] young healthy females (~45 pg/mL),[42] and in older healthy males and females (32 ± 15 pg/mL).[43] Pre-syncopal LBNP elicited an average 28.5% increase in F_2-IsoP. Both central hypovolemia (indexed via SV) and relative hypotension were achieved, although neither were directly associated with the change in F_2-IsoP concentrations (Figure 2(a) and (b)).

To our knowledge, only one study has investigated the oxidative stress response to systemic hemorrhage in humans. Corcoran *et al.*[34] showed no change in F_2-IsoP concentration with 20% blood loss, which induced a mild 10% decrease in SV, but no change in SAP following an initial fall from 154 mmHg at baseline to 144 mmHg following 2.5% blood loss. These investigators suggested that the lack of hypotension (although not unexpected due to high baseline arterial pressure and cardiovascular compensation) was a potential limitation in their study, resulting in no change in F_2-IsoP. Indeed, F_2-IsoPs are preferentially generated under low oxygen (hypoxic) conditions,[21] which may have been prevented by maintenance of arterial pressure in the study by Corcoran and colleagues. Accordingly, we postulated that the hypovolemia (53% decrease in SV) and hypotension (12% decrease in MAP) achieved by application of pre-syncopal LBNP, would be directly associated with the increase in circulating plasma F_2-IsoP. While this was the case overall, there were no statistically meaningful associations between the changes in F_2-IsoP concentrations and the magnitude of hypovolemia or hypotension (Figure 2(a) and (b)). This outcome led us to examine the relationship between F_2-IsoP and SmO_2 as a measure of peripheral hypoperfusion. Regional muscle oxygen saturation, assessed by SmO_2 recordings at the forearm, could be an indicator of peripheral vasoconstriction and ischemia in response to the central hypovolemia of LBNP.[32] The NIRS technique does not facilitate differentiation between decreased oxygen supply vs. increased oxygen extraction at the level of the peripheral tissues to definitively interpret the observed reduction in SmO_2. However, as metabolic demand of the peripheral muscle, and thus, oxygen extraction are unlikely to change during LBNP, the reduction in SmO_2 was likely due to peripheral vasoconstriction. While SmO_2 decreased by an average of ~9% from baseline to presyncope, as with the arterial pressure and SV responses, there was no association between the decreases in SmO_2 and the increase in F_2-IsoP (Figure 2(c)).

There are some important methodological considerations that should be discussed in relation to this study. We only assessed one marker of oxidative stress at two discrete time points (baseline and pre-syncope), so it is not clear if increases in F_2-IsoP or other markers of oxidative stress are also occurring at sub-maximal LBNP, just at presyncope, or at time periods well beyond termination of LBNP. For example, lung tissue collected from rats 3 h following a hemorrhage to 35–40 mmHg MAP and shed blood resuscitation exhibited elevated markers of oxidative stress.[44] Similarly, Fatouros *et al.*[45] demonstrated elevated oxidative stress in human subjects up to 48 h following a high-intensity exercise bout. The time course and temporal relationships between LBNP and multiple measures of oxidative stress responses merit further study. Also, our subjects in this study were young and healthy, so the present results may not apply to older subjects or clinical populations who may have different basal oxidative stress or antioxidant capacity, so may respond differently to the LBNP stress. Finally, although we controlled for factors such as exercise, alcohol, caffeine, medications, and dietary supplements for the 24 h before the experiment, there may be other dietary factors, both acute and chronic, that influence F_2-IsoP concentrations in healthy individuals. Studies have shown that acute and chronic diet changes, such as 1 day of dark soy sauce consumption,[46] 17 days of soy protein high in isoflavones,[47] 60 days of vitamin C,[48,49] 63 days of olive oil ingestion,[50] and ≥60 days of vitamin E[48,51] could decrease F_2-IsoP concentrations. Accordingly, more detailed assessment of the subjects' diets may be considered in future studies.

Summary and conclusions

Simulated hemorrhage using LBNP to presyncope elicited an increase in circulating F_2-isoprostanes in young, healthy humans. These findings indicate that presyncopal LBNP could be used as a model to assess interventions to reduce oxidative stress during simulated or actual hemorrhage, such as novel resuscitation fluids. Future studies should include collection and analysis of serial blood samples during and after LBNP to better assess the temporal resolution of peak responses of oxidative stress, and the delayed response following recovery from this model of simulated hemorrhage.

Authors' contributions: All authors participated in elements of the experimental design, collection of the data, analysis and interpretation of the data, and writing and review of the manuscript: FSP, RTM, and CAR designed the study, VLK, JDS and CAR conducted the experiments and collected the data, VLK, CAR, AJR, and FSP analyzed the data, FSP, JDS, AJR, GKA, RTM and CAR interpreted the data and wrote the manuscript, and all authors approved the final version of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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